

Natural halogenated compounds in forest soils

formation, leaching, emissions and spatiotemporal patterns of chloroform and related compounds

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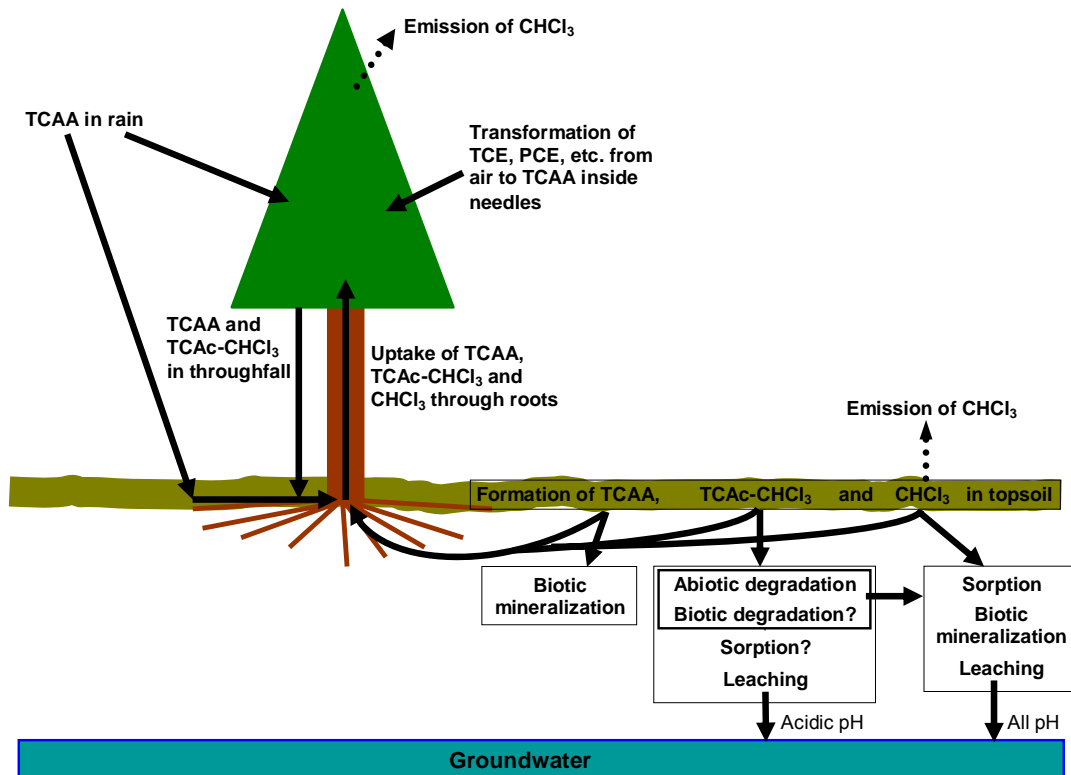
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Natural halogenated compounds in forest soils

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formation, leaching, emissions and spatiotemporal patterns of chloroform and related compounds



Christian Nyrop Albers

February 2010



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&

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Preface

This PhD thesis, entitled “Natural halogenated compounds in forest soils - formation, leaching, emissions and spatiotemporal patterns of chloroform and related compounds” is submitted to meet the requirements for attaining the Danish Ph.D. degree at Roskilde University. The PhD work was initiated March 1st 2007 and the thesis was submitted March 2nd 2010, thus lasting the three years of the Danish Ph.D. Program. Most of the work was carried out at the Geological Survey of Denmark & Greenland (GEUS).

The thesis is divided into two major parts:

1. An introduction to the subject of natural organohalogens with the inclusion of some original work, where this would add to the existing knowledge.
2. The five accompanying papers (listed on page 5) of which two (**I** and **IV**) are published and the other three are submitted to international peer reviewed journals.

Major conclusions and discussions of the Ph.D. work are included in the introduction, but to get the full story, readers will have to consult the individual papers. Throughout the introduction, the five accompanying papers are referred to by their roman numerals (**I** to **V**) written in bold.

Table of contents

Preface.....	3
List of papers	5
Acknowledgments	6
Scope and summary of the thesis	8
Dansk sammendrag (Danish summary).....	11
Abbreviations	13
Introduction.....	15
1. Incorporation of halogens in organic structures in nature	17
1.1 Biotic halogenation	17
1.2 Abiotic halogenation.....	18
1.3 Similarities to the chemical chlorination of drinking water and paper pulp	19
1.3.1 Reaction mechanisms leading to compounds containing a trichloromethyl group	21
2. CHCl ₃ in the terrestrial environment.....	25
2.1 Behaviour and fate of CHCl ₃ in the environment	25
2.1.1 Partitioning between different compartments	25
2.1.2 Abiotic degradation in soil.....	27
2.1.3 Biotic degradation	27
2.2 Anthropogenic sources and fluxes.....	29
2.3 Natural sources and fluxes of CHCl ₃	30
2.3.1 Mode of formation in soil.....	32
2.3.2 Abiotic formation of CHCl ₃ in soil?	32
2.3.3 Natural formation of other trihalomethanes in soil?.....	33
2.4 Guidelines and toxicity thresholds of CHCl ₃ in the environment	33
3. Other naturally occurring trichloromethyl compounds.....	34
3.1 TCAA.....	34
3.1.1 Behaviour and fate in the environment	34
3.1.2 Anthropogenic sources and fluxes	36
3.1.3 Natural sources and fluxes	37
3.1.4 Guidelines and toxicity thresholds of TCAA in the environment	39
3.2 Trichloroacetyl containing compounds	39
4. Organic halogen in the macromolecular structures of SOM (TOX)	41
4.1 Is all TOX chlorine?.....	42
4.2 Formation of TOCl in soil	43
4.2.1 Identified chlorinated structural elements in HS and SOM	43
4.2.2 Main hypotheses concerning the formation of TOCl	45
4.3 Trichloromethyl compounds and TOCl	46
5. On the possible use of ¹³ C stable isotope analysis in natural organohalogen research	48
Concluding remarks and future directions	53
References.....	57
Papers I-V	71

List of papers

This thesis is based on the following original publications, referred to in the text by their roman numerals:

- I. Albers, C. N., Laier, T., Jacobsen, O. S., Vertical and horizontal variation in natural chloroform in two adjacent soil profiles in a coniferous forest, pp. 161-170 in; *Geo-environment and Landscape Evolution III*, Proceedings of the third international Conference on evolution, monitoring, simulation, management and remediation of the geological environment and landscape, WIT Press, Southampton.
- II. Albers, C. N., Jacobsen, O. S., Flores, É. M. M., Pereira, J. S. F., Laier, T., Hot Spots of natural chloroform in coniferous forests – indications of microorganisms being responsible of most of the chloroform produced in temperate coniferous forests, Submitted to *Biogeochemistry* (2009).
- III. Albers, C. N., Laier, T., Jacobsen, O. S. Formation, fate and leaching of natural chloroform in coniferous forest soil, Submitted to *Applied Geochemistry* (2010).
- IV. Albers, C. N., Jacobsen, O. S., Hansen, P. E., Methodological problems in determining TCAA in soils – the discovery of novel natural trichloroacetyl containing compounds and their interference with a common method for determining TCAA in soil and vegetation, *Journal of Environmental Monitoring*, 2010, DOI: 10.1039/b920932h (in press).
- V. Albers, C. N., Hansen, P. E., Jacobsen, O. S., Occurrence and fate of naturally formed trichloromethyl compounds in coniferous forests, Submitted to *Environment International* (2010).

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Christian Nyrop Albers
Copenhagen, February 2010

Scope and summary of the thesis

The scope of the present study is to investigate the occurrence and fate of halogenated organic compounds formed through natural processes in soil. During the last 40 years, halogenated and especially chlorinated compounds have been a major issue in the field of Environmental Chemistry because many environmental problems are caused by human pollution with chlorinated compounds like PCBs, dioxins, chlorinated solvents and many pesticides. Not all halogenated compounds are toxic, however, and furthermore, many halogenated compounds, toxic as well as non-toxic, are naturally occurring in the environment. Halogenated compounds formed by various microorganisms have been known for more than a century and especially during the last twenty years, several studies have investigated the natural occurrence of halogenated compounds in soil and air. Some of these may also reach the groundwater, and then suddenly both a toxicological as well as a regulatory perspective may exist, especially if the compounds are already regulated strictly since they are recognized as pollutants. One such compound is chloroform (CHCl_3), which is a common groundwater pollutant in most industrialized countries, but is now also recognized to be naturally occurring as well.

The **Introduction** reviews the natural, biotic as well as abiotic, processes, which can lead to the formation of halogenated compounds in soil. It is emphasized that one should distinguish between the intended formation of halogenated compounds by different microbial organisms and those apparent unintended side-products, whose formation seems to be mediated by exo-enzymes, excreted for other purposes. This latter unspecific halogenation mechanism is the focus of my thesis. The mechanism seems to be somewhat similar to the unintended formation of chlorinated compounds during disinfection of drinking water with elemental chlorine, where the main by-products are compounds containing a trichloromethyl group (e.g. CHCl_3 and trichloroacetic acid (TCAA)) as well as macromolecular organochlorine (TOCl) with unknown exact structure. The sources, fluxes and fate of these compounds are then reviewed, with special emphasis on what is known about natural concentrations and formation processes. Before the present study, evidence of natural TOCl and CHCl_3 has been provided and it has been made likely that TCAA is also formed naturally in soil. Studies on especially CHCl_3 and TCAA have, however, been concerning only fragments of what is needed in order to understand natural sources, amounts and fates of these compounds. The following five papers address this, and hopefully lead us closer to such an understanding as well as rising a number of new questions.

Paper I discusses the leaching of CHCl_3 formed in the topsoil of coniferous forests to the groundwater, where it is found in concentrations exceeding the Danish quality criteria for abstraction of drinking water. The concentration of CHCl_3 in soil air is measured throughout the unsaturated zone in a coniferous forest, and by calculations on equilibrium between air and water a good correlation to the concentrations in the upper groundwater in the same two profiles is found. Furthermore, a first indication that the spatial variation in CHCl_3 formation is very large is given since within a 10 meters

distance, the concentration of CHCl_3 can differ with more than a factor of 10 throughout the unsaturated zone and a similar difference can be recognized in the upper groundwater. **Paper II** follows up on this spatial variation, and systematically investigates it in the soil air of four coniferous forests. High concentrations of CHCl_3 are found within narrow areas – Hot Spots – varying from ~ 25 to $>400 \text{ m}^2$ in size, with concentrations being typically 20-100 times those in corresponding Low Spots. No visible differences, with regards to e.g. type and density of vegetation or soil texture, exist between Hot Spots and Low Spots, and a number of analyses and experiments are carried out in an attempt to better describe and possibly explain the differences. It is demonstrated that while degradation of CHCl_3 is similar in Hot and Low Spots, both emissions and leaching of CHCl_3 is highest from the Hot Spots, which together with laboratory incubations of soil cores lead to the conclusion that the net formation of CHCl_3 is higher in the Hot Spots. The halogenation degree of the soil (TOCl, TOBr, TOI) is determined and shows no greater difference between a Hot Spot and the corresponding Low Spot, and also the CHCl_3 formation potential seems to be the same between soil organic matter (SOM) from Hot and Low Spots. The origin of the large spatial variation hence remains unknown, but indications point to the fact that domain living fungi may be involved. The consequence of the discovery of CHCl_3 Hot Spots, with regards to the interpretation of previous studies and calculations of global emissions as well as on the design of future studies, is then discussed.

In **Paper III**, CHCl_3 is also the compound of interest, but with a vertical as well as a temporal focus. The variation in CHCl_3 concentration in soil and groundwater is followed during 2-4 years in four coniferous forests, and clear seasonal variations are observed. The CHCl_3 concentration fluctuates somewhat similar to the CO_2 concentration, but with a delay of 3-4 weeks in response to changes in temperature and soil moisture. This delay can also be found when soil is incubated in the laboratory, especially in the fermentation layer of the organic horizon, where most of the formation of CHCl_3 is shown to occur. The delay is a further indication that the formation of CHCl_3 is not of pure abiotic origin, and that the biota involved is relatively slow growing or for other reasons needs time before significant formation of the involved enzymes occur. In laboratory experiments, degradation of CHCl_3 by aerobic microbial activity is shown to occur at all soil depths, but at rather low rates. Furthermore, sorption processes are shown to be important mainly in the upper organic horizons, and in the field, leaching during a rain event is shown to be important for the movement of CHCl_3 , from the production layer to the groundwater.

Paper IV deals with TCAA and a common method for determining TCAA in various environmental compartments. It is shown that especially for soil samples, the TCAA concentrations are by far overestimated due to the presence of interfering compounds containing the trichloroacetyl ($\text{CCl}_3\text{-C=O}$) structural element. This structural element is known from chlorination of drinking water, but has not previously been shown to occur in nature. A critical review on previous reports concerning TCAA in soil is thus given and in perpetuation to this, suggestions on how TCAA and trichloroacetyl containing compounds may be analyzed individually in future studies. The trichloroacetyl containing compounds are further characterized and found to exist both in the macromolecular/solid parts of SOM as well as

in soil water and a number of other compartments of the forests. The trichloroacetyl containing compounds extractable with water and found in groundwater are shown to contain a carboxylic acid group, but no final molecular structure is determined. In **Paper V**, the occurrence of this new group of naturally occurring organochlorines is further studied and its vertical, horizontal and temporal variation is monitored. Similar to CHCl_3 , concentrations of trichloroacetyl containing compounds in soil show great spatial variations, and a clear positive relationship with the CHCl_3 concentration in top soil is found. Trichloroacetyl containing compounds are alkali-labile, but in areas of acidic soil and groundwater, they may leach and reach the groundwater in $\mu\text{g/L}$ -concentrations. They also seem to be taken up into the vegetation through the transpiration system, and then excreted and re-translocated to soil via precipitation, which in open areas contains no such compounds. The trichloroacetyl concentrations seem to be somewhat related to the TCAA concentrations, which are also much higher in throughfall than in rain from open areas. A hypothesis, that also TCAA is formed in soil, taken up by trees, accumulated in needles and then to a certain degree excreted from these, is then proposed. The hypothesis is supported by the observation that the highly varying TCAA-concentrations in needles from different spruce trees are strongly correlated with the concentrations of CHCl_3 and trichloroacetyl containing compounds in the soil at the base of the tree trunk. This suggests a common origin of all the considered trichloromethyl compounds, and then raises the question of why TCAA, as the only compound, cannot be found in neither forest soil nor groundwater. A sorption study shows that sorption of TCAA does occur in the soil, but not to a degree, which will hinder its determination. Mineralization studies, on the other hand, show that the forest soil has a huge potential for mineralizing TCAA in the upper one meter, with half lives well below 24 hours. This mineralization potential provides a suitable explanation of why TCAA may be formed in soil and taken up by vegetation, but does not accumulate to concentrations above the analytical detection limit in the soil and therefore is not of concern in terms of groundwater quality. Sufficient evidence now seems to exist in order to construct a well-founded conceptual model including all important aspects of formation and fate of trichloromethyl compounds in coniferous forests. Such a model is proposed at the end of **Paper V**.

Dansk sammendrag (Danish summary)

Formålet med nærværende afhandling er at undersøge forekomst, dannelse og skæbne af halogenerede organiske stoffer, dannet gennem naturlige processer i jorden. Halogenerede og herunder specielt klorerede stoffer har haft et stærkt fokus inden for miljøkemien i de sidste 40 år, på grund af de mange miljøproblemer der har været relateret til visse af disse stoffer (f.eks. PCB'er, dioxiner, klorerede opløsningsmidler samt visse pesticider). Ikke alle halogenerede stoffer er dog giftige, og mange naturstoffer, giftige såvel som ikke giftige, indeholder halogener. Kloroform er et af disse stoffer, der udover at være et udbredt forureningsstof også har vist sig at dannes naturligt i en række miljøer herunder jord, hvorfra det formodes at nedsive til grundvandsmagasiner i koncentrationer der kan overskride grænseværdien for indvinding af drikkevand.

I introduktionen gennemgås de naturlige processer, der kan føre til dannelsen af halogenerede stoffer i jord. Det understreges, at der bør skelnes skarpt mellem stoffer udskilt fra mikroorganismer med et specifikt formål og uspecifik halogenering induceret af enzymer, udskilt med andet formål. Denne uspecifikke halogenering, der kan sammenlignes med de kloreringsprocesser der foregår under desinfektion af drikkevand, er i fokus i resten af afhandlingen. Som det ses ved drikkevandsklorering, dannes især stoffer med en triklormethyl-gruppe (f.eks. kloroform (CHCl_3) og trikloreddikesyre (TCAA)) samt makromolekylært bundet klor (TOCl) under de uspecifikke halogeneringsprocesser. Kilder, koncentrationer i miljøet samt skæbne af disse stoffer gennemgås, og det kan konkluderes at før denne afhandling, var der rimeligt bevis for eksistensen af naturlig CHCl_3 og TOCl i jord, og endvidere har der været sandsynliggjort at TCAA dannes naturligt. Forudgående studier af især CHCl_3 og TCAA har dog kun givet et fragmentarisk indblik i naturlige kilder, koncentrationer samt skæbne af disse stoffer. Denne afhandling, og specielt de fem inkluderede artikler, vil forhåbentlig lede os nærmere en forståelse af disse aspekter.

I **Artikel I** diskuteres nedsivningen af naturlig CHCl_3 fra overjord i en dansk nåleskov til grundvandet, hvor det findes i koncentrationer der overskrider kvalitetskriteriet for drikkevand. Koncentrationen af CHCl_3 i poreluft følges ned gennem to jordprofiler, og gennem ligevægtsberegninger konkluderes det at der er en god sammenhæng mellem CHCl_3 i den umættede og mættede zone, og at naturlig dannelse i overjord dermed er en meget sandsynlig kilde til "forureningen" med CHCl_3 . Endvidere indikeres en meget stor horisontal variation i koncentrationen af CHCl_3 i poreluft, såvel som i grundvand, hvilket er emnet i **Artikel II**. Her undersøges den horisontale variation i CHCl_3 -dannelsen systematisk i fire danske nåleskove, og alle steder konstateres "Hot Spots" skarpt afgrænset, men ikke synligt adskilt, fra områder med lav dannelse af kloroform. Laboratorieforsøg understøtter forekomsten af disse "Hot Spots", hvorfra betydelige mængder CHCl_3 frigives til atmosfæren og nedsiver til grundvandet. Afgrænsningen af "Hot Spots" udgøres sandsynligvis af domænelevende svampe, men det lykkes ikke at finde den afgørende parameter, der kan give en forklaring.

Konsekvensen af disse "Hot Spots" for tidligere beregninger af globale udslip af naturligt CHCl_3 til atmosfæren, baseret på et spinkelt empirisk grundlag, diskuteres herefter.

Artikel III omhandler også naturlig CHCl_3 , men med fokus på vertikale og tidslige variationer og de følger som dannelsen i overjorden har for grundvandskvaliteten. Monitoring af underjordiske installationer i fire nåleskove igennem 2-4 år, viser klare sammenhænge med den generelle biologiske aktivitet, men med en forsinket respons på 3-4 uger på øget temperatur og jordfugtighed, hvilket indikerer at dannelsen af CHCl_3 er relateret til en relativt langsomt voksende biomasse i jorden. Igen blev feltresultaterne understøttet af laboratorieforsøg med inkubering af intakte jordsøjler. Sorption og nedbrydning af CHCl_3 viste sig at være af betydning igennem den øverste del (sorption) eller alle dele (nedbrydning) af den umættede zone, og forhindrede en simpel beskrivelse af skæbne af det dannede CHCl_3 . Det kunne dog groft estimeres, at ca. 10% af den dannede kloroform nedsiver med regnvand til grundvandet, mens størstedelen udsendes til atmosfæren.

Artikel IV omhandler TCAA og en udbredt analytisk metode til bestemmelse af TCAA, som viser sig, specielt for jord og grundvandsprøver, at give alt for høje koncentrationer på grund af en interfererende gruppe af stoffer. Disse stoffer, der har en trikloracetyl ($\text{CCl}_3\text{-C=O}$) gruppe tilfælles er ikke tidligere fundet i naturen, men viser sig at være udbredt i alle dele af skovsystemet, og med al sandsynlighed at være naturligt dannet. Trikloracetyl-stofferne er ustabile ved pH over ca. 6, hvor de spontant dekomponerer under frigivelse af CHCl_3 , men de kan findes i både jordvand og grundvand, hvor de sandsynligvis er mobile, da de også indeholder en carboxylsyregruppe. Muligheden for fejlfortolkninger af TCAA-koncentrationer i tidligere publikationer diskuteres, og det vises, hvorledes TCAA og trikloracetyl-stofferne vil kunne analyseres hver for sig i fremtidige studier. Dette udnyttes i **Artikel V**, som yderligere undersøger forekomsten af trikloracetyl-stofferne i hele skovøkosystemet, og det vises at i områder hvor pH-værdien i grundvandet er lav, vil trikloracetyl-stofferne kunne findes her sammen med CHCl_3 . Der er en stærk sammenhæng med forekomsten af naturligt CHCl_3 i jord, hvilket tyder på et fælles ophav, mens TCAA ikke kan konstateres i hverken jord eller grundvand. Sorptionen af TCAA til jord viser sig at være for lav til at forklare dette, i fald TCAA som ventet dannes sideløbende med CHCl_3 og trikloracetyl-stoffer. Nedbrydningsforsøg viser derimod at den øverste meter af skovbunden er særdeles veladapteret til mineralisering af TCAA, som foregår med halveringstider på under 24 timer. Det er dermed muligt at TCAA dannes i jorden uden at det ophobes i målbare koncentrationer, hvilket understøttes af forekomsten af TCAA i vegetation, og en positiv sammenhæng mellem denne og koncentrationen af CHCl_3 og trikloracetyl-stoffer i den jord, hvorfra vegetationen opsuger jordvæske.

Tilstrækkelig viden synes nu opbygget til at en velbegrunderet konceptuel model, der inkluderer alle vigtige aspekter af dannelse og skæbne af trikloromethyl-stoffer i nåleskove, kan fremsættes. Dette gøres i slutningen af **Artikel V**.

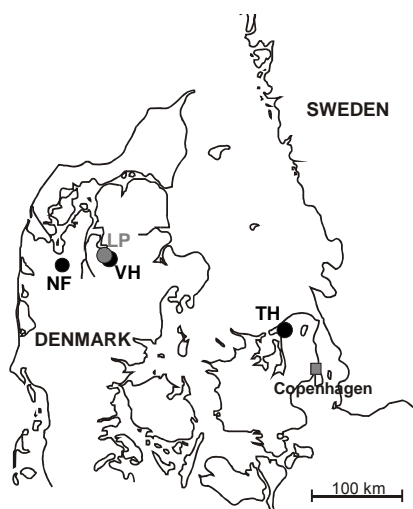
Abbreviations

This list of abbreviation provides not necessarily the “official” definitions but rather presents my use of the various abbreviations.

1,1,1-TCA	1,1,1-trichloroethane
A _h -horizon	Minerogenic top horizon with significant accumulation of organic matter (LOI > 5%) providing it with a black colour
AOX	Adsorbable Organic Halogen (Organic halogen in solution, adsorbable on activated carbon)
B _h -horizon	Minerogenic sub-horizon with accumulation of organic matter (LOI > 1%) providing it with a brownish colour
BPO	Bromoperoxidase
CAM	Chlorinated anisyl metabolite
CPO	Chloroperoxidase (CPO may also be used as the proper noun of the first discovered chloroperoxidase from <i>Caldariomyces fumago</i>)
Da	Dalton (equal to 1 atomic mass unit)
DCAA	Dichloroacetic acid, Cl ₂ CHCOOH
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
dw	Dry weight
F-horizon	The “fermentation-layer” (Partly degraded plant material forming a layer below the L-horizon, in ecosystems with slow degradation of litter. LOI is typically around 90%)
FA	Fulvic acid
fw	Fresh weight
H-horizon	The “humification-layer” (Plant material degraded into fine particles, with the origin often not recognizable. The lowest part of the organic horizon and often somewhat mixed with the underlying soil. LOI > 20% but often somewhat higher)
HA	Humic acid
HS	Humic substances
IPO	Iodoperoxidase
K _d	The partitioning coefficient between soil and water
K _F	The Freundlich coefficient for the partitioning of a compound between soil and water. K _F is equal to K _d , if the sorption to soil is linear
K _{OC}	The partitioning coefficient between organic carbon and water
L-horizon	The “litter-layer”. Dead, but relatively fresh, plant material with no significant degradation
LC ₅₀	The concentration of some compound, where it is lethal to 50% of a population of a specific species
LOQ	Analytical Limit of quantification
LOI	Loss On Ignition (Weight loss when dry soil is heated to 550°C for two hours as surrogate for organic content)
LP	The Liseborg Plantage study site in central Jylland, DK

NF	The Nordre Feldborg study site in Western Jylland, DK
O-horizon	The organic layer on top of the original minerogenic soil. Consists of the L-horizon and, if present, the F- and H-horizons
OM	Organic matter
PCDD/Fs	Polychlorinated dibenzo-p-dioxins / polychlorinated dibenzofurans
PCE	Tetrachloroethene
PNEC	Predicted no effect concentration (The highest concentration not expected to cause harm to a specific organism, population or ecosystem. Typically calculated from toxicological experiments)
ppbv	parts per billion on a volume:volume basis, concentration unit for gases
pptv	parts per trillion on a volume:volume basis, concentration unit for gases
SOC	Soil organic carbon
SOM	Soil organic matter
TCAA	Trichloroacetic acid, Cl_3CCOOH
TCE	Trichloroethene
TH	The Tisvilde Hegn study site in Northern Sjælland, DK
THM	Trihalomethanes
TOBr	Total organic bromide, e.g. in a soil sample
TOCl	Total organic chloride, e.g. in a soil sample
TOI	Total organic iodide, e.g. in a soil sample
TOX	Total organic halogen, e.g. in a soil sample
VH	The Viborg Hedeplantage study site in central Jylland, DK
VOCi	Volatile organic compounds containing chlorine
X	Halogen atom
XPO	Haloperoxidases

Study site abbreviations:



The location of the four study sites, with abbreviations used throughout the thesis.

Introduction

Natural organohalogens in the terrestrial environment, with special emphasis on trichloromethyl compounds formed during unspecific halogenation reactions in soil

1. Incorporation of halogens in organic structures in nature

Due to great scientific and commercial interest in biological halogenation processes, some of these are rather well described. Still, however, large gaps in our knowledge on especially some of the reaction mechanisms exist. In the following section, halogenation processes occurring both within and outside living cells will be described, with an emphasis on the processes that might lead to the formation of chloroform (CHCl_3) and related compounds, of most interest in the present thesis. In addition to the halogenation mediated by enzymes, abiotic halogenation has been shown to occur in soil and this will also be described. Finally, because of the great similarities between some of the natural organohalogenes and compounds formed during chlorination of drinking water, a comparison of substances and reaction mechanisms of natural and artificial chlorination processes will be performed.

1.1 Biotic halogenation

Since the discovery of the first halometabolite in 1896, ~4000 natural halogenated compounds have been identified, of which ~2000 are chlorinated, ~2000 brominated, ~100 iodinated and ~30 fluorinated [for recent reviews, see Winterton, 2000; Gribble, 2004; Wagner *et al.*, 2009]. The first halogenating enzyme to be isolated was CPO from the plant pathogen *Caldariomyces fumago* [Shaw & Hager, 1959], which synthesizes 2,2-dichloro-1,3-cyclopentanediol in large amounts [Clutterbuck *et al.*, 1940]. CPO belongs to a major group among the halogenating enzymes; the haloperoxidases (XPOs). The other major group of halogenating enzymes is FADH_2 -dependent halogenases.

FADH₂-dependent halogenases: These halogenating enzymes are highly substrate specific, requiring FADH_2 , halide and oxygen as (co-)substrates in the halogenation reaction. In the beginning of the reaction, FADH_2 is bound to the enzyme and reacts with O_2 to form FADH-OOH , which reacts further with a halide (X) to form HOX. The HOX is not liberated from the enzyme, but kept by hydrogen bonding in a certain position to react with the desired substrate. In this way, FADH_2 -dependent halogenases are involved in highly specific biosynthesis only [Chen & Van Pée, 2008; Wagner *et al.*, 2009].

Haloperoxidases: XPOs differ from FADH_2 -dependent halogenases since they are far less substrate specific. They are named CPO, BPO etc. from their capability to use different halides as substrates. Differences in redox-potential (E° is 1.36, 1.09 and 0.54 V for Cl^- , Br^- and I^- , respectively) lead to XPOs like lactoperoxidase being capable of oxidizing iodide only, BPOs being capable of oxidizing both bromide and iodide and CPOs utilizing chloride, bromide and iodide as substrates. Beside the halide, XPOs use H_2O_2 as the oxidizing co-substrate and since the redox potential of H_2O_2 is lower than that of fluoride, XPOs cannot oxidize fluoride, and natural fluorine containing compounds are rather rare, requiring the involvement of specific fluorinases [Wagner *et al.*, 2009]. Heme-XPOs, containing an iron atom in the reactive centre, have been shown to halogenate specifically inside the cell, but they can also produce oxidized halogen, e.g. HOCl (Figure 1a), outside the cell that will diffuse until it meets and halogenates a random substrate. Some XPOs contain a vanadate ion (H_2VO_4^-) instead of a heme-group

in their reactive centre, and these Va-XPOs seem to be exclusively exo-enzymes, producing HOX, which will lead to unspecific halogenation (Figure 1b), and so far, no involvement in the formation of halometabolites have been shown for Va-XPOs [Wever *et al.*, 1997; Van Pée & Zehner, 2003].

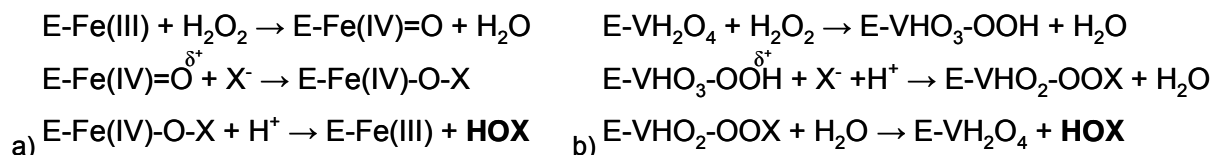


Figure 1. The formation of HOX from H₂O₂ and X⁻ in the reactive centre of a) heme-XPO and b) Va-XPO [Wever *et al.*, 1997; Van Pée & Zehner, 2003; Chen & Van Pée, 2008]. E-Fe is the enzyme-iron complex in heme-containing XPOs. E-VH₂O₄ is the enzyme-vanadate complex in Va-XPOs. X can be either Cl, Br or I.

In the terrestrial environment, XPOs have been isolated from ascomycetes, basidiomycetes, bacteria, lichen, bryophytes, insects and mammals, but they seem to be especially widespread among fungal saprophytes and plant pathogens in the class of hyphomycetes [Neidleman & Geigert, 1986; Vollenbroek *et al.*, 1995]. Previously, fungal XPOs were believed to exist in ascomycetes only, but rather recently; Ullrich *et al.* (2004) demonstrated the presence of a heme-containing CPO in a basidiomycete (*Agrocybe aegerita*). The function of extracellular XPOs, not providing the organism with any useful compound, has been discussed, but no conclusion has been reached. One suggestion is that the reactive oxidized halogen atom may play a role in microbial antagonism in the terrestrial environment [Bengtson *et al.*, 2009], but there seems to be more evidence for the role of XPOs in the degradation of lignin structures. According to Barnett *et al.* (1997) Va-CPO constitutes 60-70% of the total secreted exo-enzymes by the plant pathogen *Curvularia inaequalis*, but the transcription of Va-CPO is suppressed in sugar rich environments, corresponding to a lower need of the enzyme, once the fungal tip has penetrated through the lignocellulose barrier into the plant tissue. CPO-activity has also been detected in forest soil [Asplund *et al.*, 1993], but CPO-exo-enzymes have never been isolated from soil.

In addition to FADH₂-dependent halogenases and haloperoxidases, substrate specific α-ketoglutarate dependent halogenases have been described as being involved in the chlorination of terminal methyl groups of amino acids as a step in the biosynthesis of a number of chlorinated compounds [Chen & Van Pée, 2008; Wagner *et al.*, 2009]. Finally a completely different enzyme system, methyl-halide-transferase, is involved in the formation of the methyl halides CH₃Cl, CH₃Br and CH₃I in bacteria, plants and fungi [Van Pée & Zehner, 2003; Wagner *et al.*, 2009]. Methyl halides are e.g. used as methyl donors during lignin degradation, and are produced in several white rot fungi [Harper, 1997; Anke & Weber, 2006].

1.2 Abiotic halogenation

Most of the natural halogenated compounds identified so far, are without doubt the result of reactions mediated by enzymes. There is, however, evidence for the abiotic formation of certain chlorinated

aliphatics, especially the methyl halides. Keppler *et al.* (2000) showed that the addition of Fe^{3+} led to an increased liberation of CH_3X from soil and using guaiacol as a model organic substrate, an abiotic reaction leading to the formation of CH_3X was shown (Figure 2). Rhew *et al.* (2003) looked at the formation and consumption of CH_3X in forest soil, and while the consumption (degradation) was completely stopped when the soil was steam sterilized, the formation continued. Furthermore, Hamilton *et al.* (2003) demonstrated that Cl^- could be methylated abiotically by pectin, to form CH_3Cl .

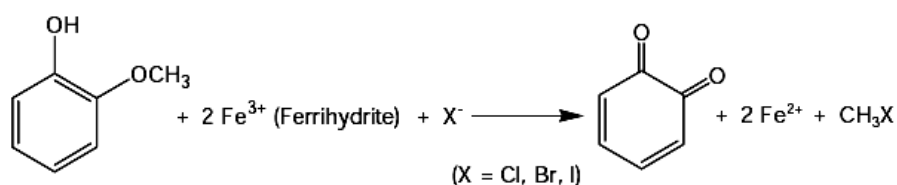


Figure 2. Abiotic formation of CH_3X from guaiacol as the model substrate. Taken from [Keppler *et al.*, 2000].

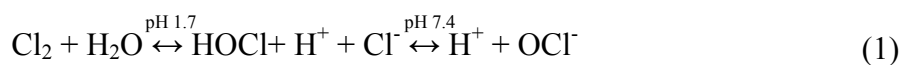
The abiotic formation of dichloroacetic acid (DCAA) and trichloroacetic acid (TCAA) from soil and humic acid in the presence of halide, H_2O_2 and Fe has also been shown [Fahimi *et al.*, 2003]. The proposed reaction pathway was the formation of hydroxyl radicals from Fe^{2+} and H_2O_2 , oxidation of halide to X_2 by the radicals and subsequent unspecific reaction of the oxidized halogen with SOM. The latter part of the reaction would be similar to the unspecific chlorination of SOM caused by extracellular haloperoxidases and also to the artificial chlorination of drinking water (see section 1.3). Apart from DCAA and TCAA, other compounds including CHCl_3 would be expected to be formed, then, and indeed recently, CHCl_3 was demonstrated to be formed in soil, with a 100-fold increase upon addition of $\text{Fe}_2(\text{SO}_4)_3$ or ferrihydrite, H_2O_2 and KCl. CHCl_3 was also formed in model reactions from different polyphenols added Fe^{3+} , H_2O_2 and Cl^- , but the amounts of CHCl_3 formed were very small ($\ll 1\%$ on a carbon:carbon basis).

In conclusion there is evidence for the abiotic formation of organohalogenes, especially the methyl halides. The contribution of abiotic reaction mechanisms compared to enzyme mediated mechanisms is however largely unknown, as it is very difficult and often not important to distinguish between the different reaction mechanisms.

1.3 Similarities to the chemical chlorination of drinking water and paper pulp

As described, oxidized halogen molecules like HOX can be formed in both haloperoxidase mediated reactions and in the Fenton reaction. Since no other reactive halogen species are known in the terrestrial environment, compounds known to be formed in the reaction between HOX and SOM could be expected also to be formed naturally. Because of the unwanted effect on health and environment, numerous studies have been performed on the formation of unwanted by-products during chlorination of freshwater to be used for drinking water and also on the bleaching of paper pulp, which

until the early 1990s was performed mainly with elemental chlorine. When elemental chlorine is added, most of it will be present as HOCl in the pH interval 2-7 (Eq. 1).



Laboratory studies have shown that the chlorination of organic matter with CPO leads to the formation of similar compounds as the ones found during water chlorination, e.g. CHCl_3 , chlorinated acetonitriles, chlorinated acetones, chlorinated acetic acids and chlorinated humic substances (HS) [Asplund *et al.*, 1991; Wannstedt *et al.*, 1990; Hodin *et al.*, 1991; Hoekstra *et al.*, 1995; Haiber *et al.*, 1996]. This would be expected, since CPO is a source of HOCl, when H_2O_2 and Cl^- is present [Wever *et al.*, 1997; Van Pée & Zehner, 2003]. The question is then if the same compounds can be found naturally in the environment, and if the knowledge on compounds formed during chlorination of drinking water can be used to predict the occurrence of a number of natural compounds in soils where CPOs are present. Numerous natural chlorinated compounds have been found in the terrestrial environment. Many of these (e.g. methyl halides and chlorinated anisyl metabolites) have a known biological source and can be denoted as halometabolites, being synthesized with a specific purpose of the synthesizing organism. Some of the natural organohalogenes found in soil and peat do not have a known producer, and are expected to be formed in an unspecific chlorination reaction, however. These are listed in Table 1. As can be seen, all of the organohalogenes listed in the table have also been found as by-products in the chlorination of drinking water and/or paper pulp with HOCl as the chlorinating agent. Even though it must be regarded as an indirect proof, it supports the hypothesis that these natural organohalogenes are produced in an unspecific chlorination reaction, and that the chlorinating agent could be HOCl or another diffusible compound containing oxidized chlorine. This again suggests CPOs to be likely candidates as enzymes responsible for the chlorination in soil, even though HOCl is also suggested to be formed in the Fenton reaction (see Eq. 3, page 38) [Fahimi *et al.*, 2003].

Table 1. Compounds regarded as natural organohalogenes in the terrestrial environment and expected to be formed in an unspecific halogenation reaction and therefore not to be the specific reaction products of biosynthesis. Identification as a by-product during chemical chlorination and suggested main structural precursors are also noted. ? means that this part of the structure is unknown or may vary.

Natural compound	Found in water/pulp chlorination?	Specific condition required during water/pulp chlorination?	Main structural precursor(s)
CHCl_3 , THM ¹	Yes		Numerous
DCAA ²	Yes		Aliphatic acids
TCAA ³	Yes		Numerous
Chlorophenols ⁴	Yes		Phenols
PCDD/Fs ⁵	Yes	Acidic pH	Phenols, HS
Chlorobenzoic acids ⁶	Yes		benzoic acids, HS
$\text{Cl}_3\text{C}-\text{C}=\text{O}-\text{??}-\text{COOH}$ ⁷	Yes	Acidic pH, low HOCl-conc.	Phenolic structures
Chlorinated HS ⁸	Yes		HS

¹[Hoekstra *et al.*, 1998a], ²[Matucha *et al.*, 2007a; Matucha *et al.*, 2007b], ³[Matucha *et al.*, 2007a; Matucha *et al.*, 2007b], ⁴[Silk *et al.*, 1997; Hoekstra *et al.*, 1999b], ⁵[Silk *et al.*, 1997; Hoekstra *et al.*, 1999b], ⁶[Niedan & Schöler, 1997] ⁷[IV], ⁸[Asplund *et al.*, 1989; Grøn, 1991; Grøn & Raben-Lange, 1992; Dahlmann *et al.*, 1993; Bastviken *et al.*, 2007]

1.3.1 Reaction mechanisms leading to compounds containing a trichloromethyl group

This thesis is mainly describing the occurrence and behaviour of natural compounds containing a trichloromethyl group (CHCl_3 , TCAA and trichloroacetyl containing compounds). These are all included in Table 1 and hence regarded as being formed by an unspecific halogenation reaction between HOCl and organic matter. In the presence of HOCl, CHCl_3 has been shown to be formed from both aliphatic substances like citric and acetic acid [Larson and Rockwell 1979, Streicher *et al.*, 1986; Blatchley *et al.* 2003] and aromatic substances like phenols and in particular meta-dihydroxy-benzenes [e.g. Norwood *et al.* 1980; Boyce & Hornig, 1983; Gallard & von Gunten, 2002]. It has been known for more than 30 years that humic substances (HS) are major precursors for the formation of trihalomethanes (THM) and haloacetic acids during water chlorination, but since the structures of HS are highly complicated, and not yet known in detail, the above mentioned acids and phenols have been used as model compounds for HS. Weber *et al.* (2005) found that removal of phenolic and aromatic amino groups from a fulvic acid by enzyme-promoted polymerization led to diminished formation of CHCl_3 and haloacetic acids, supporting that phenolic molecules might be reasonable to use as model compounds. Furthermore, the specific UV-absorbance at 254 nm (A_{254}) of DOM has been shown to be positively correlated with its potential of forming THM and especially TCAA upon chlorination [Hejzlar *et al.*, 1995; Malcolm *et al.*, 1995; Reckhow *et al.*, 2008]. A_{254} is mainly controlled by the aromatic content and the humic acid (HA) fraction, which is the most aromatic fraction of DOM, is the best precursor fraction for the formation of both THM and TCAA [Malcolm *et al.*, 1995; Reckhow *et al.*, 2008].

The formation of CHCl_3 from *m*-dihydroxy-aromatics (e.g. resorcinol) is probably the best described and documented reaction mechanism (Figure 3). The reaction starts with an electrophilic substitution of chlorine at the 2- or 4-position, followed by a sequential substitution leading to a tetrachloro-aromatic ketone or a pentachloro cyclic dione [Boyce and Hornig 1983, De Leer *et al.*, 1985; Rebenne *et al.* 1996]. The high degree of chlorination and oxidation leads to spontaneous hydrolytic ring opening to form keto carboxylic acids, which may be decarboxylated. The enol forms of the ketones are further chlorinated in the presence of HOCl, and a number of trichloroacetyl containing compounds are formed. These compounds are quite stable at acidic pH, but will eventually liberate CHCl_3 , also at low pH, while at alkaline pH the liberation of CHCl_3 is fast and stoichiometric.

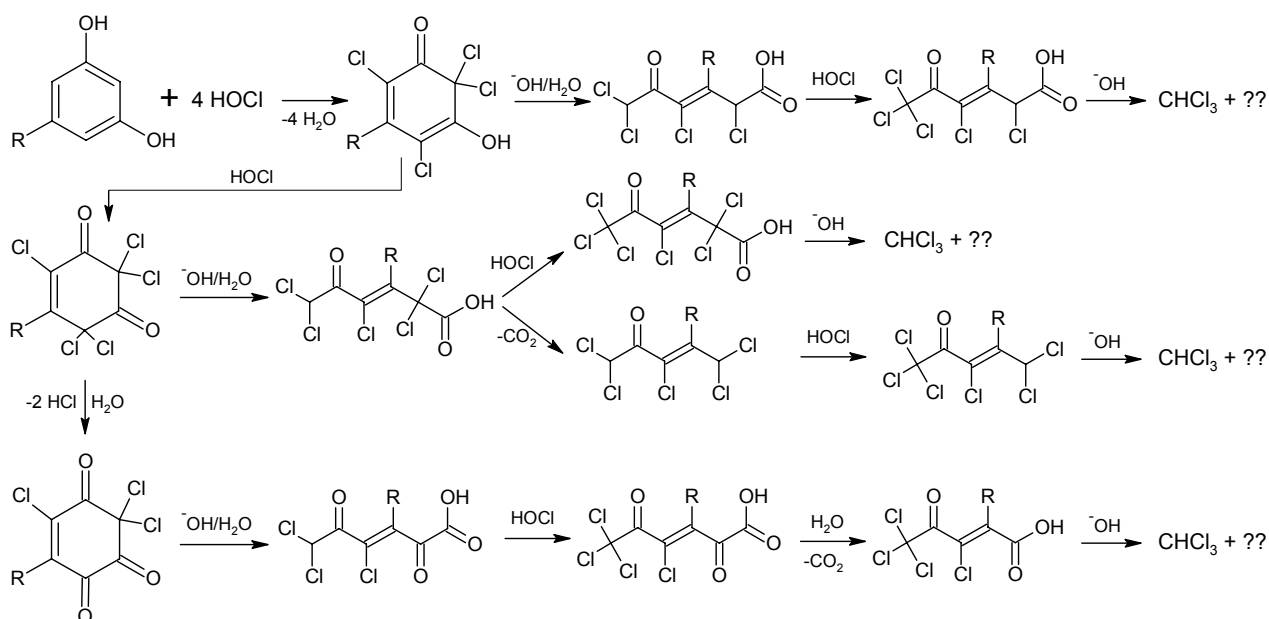


Figure 3. Summary of the proposed reaction mechanism for the formation of CHCl_3 and trichloroacetyl containing compounds from resorcinol and other m-dihydroxy-aromatics [Boyce & Hornig, 1983; De Leer *et al.*, 1985]. R can be e.g. -H, $-\text{CH}_3$ or $-\text{COOH}$.

The focus in the studies investigating and describing the reactions in Figure 3 has been on the formation of CHCl_3 , but TCAA is expected to be another end product, possibly also with trichloroacetyl containing compounds as intermediates. Trichloroacetyl containing compounds have only been reported for chlorination performed at $\text{pH} \leq 7$, and also the formation of TCAA is decreased at alkaline pH [Miller & Uden, 1983; Reckhow *et al.*, 1990; Reckhow *et al.*, 2008]. On the other hand the formation of CHCl_3 from both resorcinol, citric acid (Figure 4) and HS increases with increasing pH [Oliver, 1978; Streicher *et al.*, 1986; Reckhow *et al.*, 1990; Reckhow *et al.*, 2008]. This is partly explained by a preference towards CHCl_3 compared to TCAA at higher pH and by the hydrolysis of trichloroacetyl containing compounds to CHCl_3 at alkaline pH. The first attack of HOCl on resorcinol is also pH-dependent, with increasing rate constant from $\text{pH} \approx 5$ to $\text{pH} \approx 10$, followed by a sharp decrease. This can possibly be explained by a combination of the facts that deprotonated phenols are more activating towards electrophilic substitution and that HOCl is much more reactive than ^-OCl [Boyce & Hornig 1983, Rebenne *et al.* 1996].

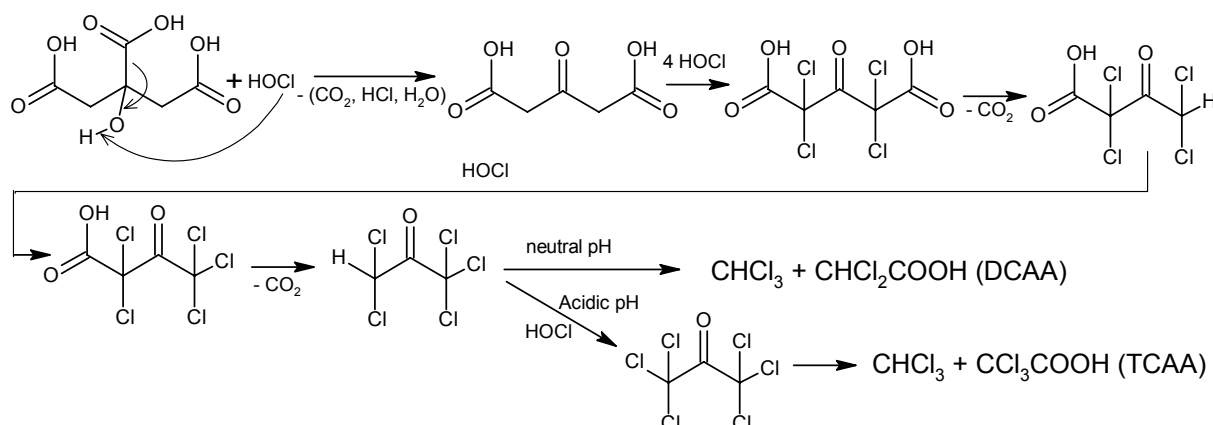


Figure 4. Pathway for the formation of pentachloropropanone (a trichloroacetyl containing compound), CHCl_3 and TCAA from citric acid. Citric acid is oxidized to β -ketoglutaric acid by HOCl, and through a number of enolization processes, the carbonyl-C is subject to chlorination by HOCl and then spontaneous decarboxylations, leading to pentachloro-2-propanone, which will react further according to pH. At neutral pH or above, pentachloropropanone is unstable and spontaneously releases CHCl_3 and DCAA. At acidic pH, pentachloropropanone is stable, but may also be subject to further chlorination to hexachloropropanone, which is unstable and releases CHCl_3 and TCAA. The reaction pathway was originally proposed by Suh *et al.* (1984) and Streicher *et al.* (1986).

Most research on the formation of THM as a by-product in the chlorination of drinking water has focused on meta-dihydroxy-benzenes as THM-precursors. All tested phenols including phenol itself, have, however, shown the potential of releasing CHCl_3 in the reaction with HOCl, although the speed of the reaction and amount of CHCl_3 liberated varies considerably [Norwood *et al.*, 1980; Chaidou *et al.*, 1999; Gallard & von Gunten, 2002; Chang *et al.*, 2006]. Several compounds containing a trichloromethyl group are also formed as major by-products in the chlorination of paper pulp, with structural elements of lignin as the suggested precursors [Lindström & Österberg, 1986]. This suggests, that although the reaction mechanisms sketched in Figure 3 and 4 are possible pathways of formation for trichloromethyl compounds, there are probably several reactions leading to these compounds, which co-occur, when bulk organic matter is exposed to reactive chlorine.

The study by Weber *et al.* (2005) showed, that phenolic moieties in HS has an influence on their potential to form CHCl_3 and TCAA upon chlorination, but non-phenolic aromatics, aliphatic carboxylic acids, carbohydrates and proteins have also been shown to form CHCl_3 and TCAA in the presence of HOCl [Larson and Rockwell, 1979; Streicher *et al.*, 1986; Peters *et al.*, 1992; Chaidou *et al.*, 1999; Navalon *et al.*, 2008]. The study by Blatchley *et al.* (2003) showed that Cu^{2+} catalyses the formation of CHCl_3 from HS and from citric acid, but not from resorcinol and other phenols, indicating, that the reaction mechanism occurring during chlorination of aliphatic acids, might also be important for a part of the liberation of CHCl_3 from HS during water chlorination. Assuming, that the mechanism is the same for the natural unspecific chlorination, this suggests that model compounds of other parts of HS than the aromatic should be considered in studies on the reaction mechanisms leading to natural trichloromethyl compounds.

In paper **V**, it was investigated if CHCl_3 and other trichloromethyl compounds could be formed upon chlorination of bulk forest SOM and HA purified from forest soil. The experiment was carried out at pH 4, which is relevant for forest soil, and it was found that both CHCl_3 , TCAA and trichloroacetyl containing compounds were formed in the reaction (Figure 5). The reactions in Figures 3 and 4 lead to the formation of all these compounds but the formation of CHCl_3 and TCAA has been shown for numerous aromatic and aliphatic precursors, while only very little is known about the formation of trichloroacetyl containing compounds.

The molecular structures of the formed trichloroacetyl containing compounds were not fully revealed, and it is therefore not known if they are similar to the trichloroacetyl containing compounds shown in Figures 3 and 4. In Figure 3, three of the trichloroacetyl containing intermediates are carboxylic acids and one is a neutral compound. The trichloroacetyl containing compounds formed from the forest HA (Figure 5) were found to be a mixture of carboxylic acids, deprotonated at pH 7.5, (28%) and neutral or positively charged compounds (72%) [**V**].

Even though the full structures were not revealed, the experiment does show that the trichloroacetyl structural element ($\text{Cl}_3\text{C}-\text{C}=\text{O}$) is formed from the chemical chlorination of forest SOM, and that compounds containing this structural element could be expected to be formed, if unspecific chlorination occurs naturally in the acidic forest soil. The slightly larger formation of all investigated chlorinated compounds from HA, especially HA from the H-horizon, compared to the original bulk SOM, indicates that HA is probably an important fraction in the context of natural unspecific chlorination in forest soil, but also that it is not the only fraction of SOM functioning as a precursor for the formation of trichloromethyl compounds.

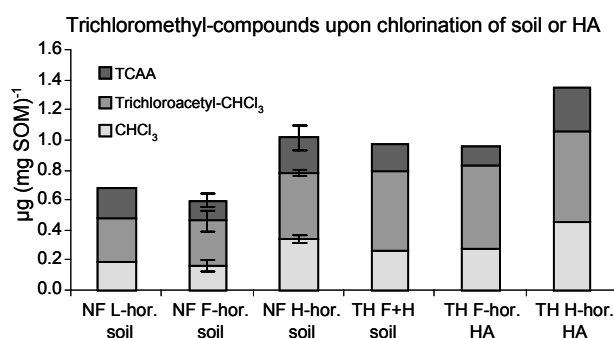
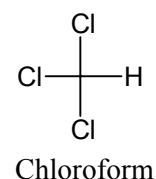


Figure 5. Chlorination of freeze-dried and powdered L, F- and H-horizon from the NF study site, of a mixture of F- and H-horizon from the TH study site and of HA purified from F- and H-horizons in TH. Chlorination conditions: SOM = 1 g L^{-1} . $[\text{NaOCl}] = 0.5 \text{ mM}$. $T = 10^\circ\text{C}$. Reaction time = 24 hours. Taken from [**V**]

The compounds or groups of compounds being investigated in papers **I-V** are mainly trichloromethyl compounds (CHCl₃, TCAA, trichloroacetyl containing compounds) and to a lesser degree halogens bound to macromolecular structures (TOCl, TOBr and TOI). The current knowledge on the occurrence and behaviour of these compounds, in the terrestrial environment, will therefore be reviewed in the following sections, with the inclusion of some data from the five papers as well as a few non-published data obtained during the work leading to this thesis, when these are either supporting existing data or providing new perspectives.

2. CHCl₃ in the terrestrial environment

Chloroform (CHCl₃) is the compound of major interest in this thesis, and the current knowledge on its behaviour and origin in the environment will be reviewed here. This should serve as a background to especially **Papers I-III**, which exclusively deal with the formation and fate of natural CHCl₃, and hopefully put these papers into a greater perspective.



2.1 Behaviour and fate of CHCl₃ in the environment

In environmental and atmospheric chemistry, CHCl₃ has received great interest due to a number of reasons, especially since; 1. It is a major by-product in the chlorination of drinking water. 2. It is one of the most frequent groundwater pollutants in urban as well as rural areas in the United States [Squillace *et al.*, 1999; Squillace *et al.*, 2004; Carter *et al.*, 2008]. 3. It is a major carrier of chlorine to the troposphere and to a lesser degree to the stratosphere, playing a possible role in the degradation of the ozone layer [e.g. Khalil & Rasmussen, 1999; Keene *et al.*, 1999; Aucott *et al.*, 1999; Rhew *et al.*, 2008a].

2.1.1 Partitioning between different compartments

CHCl₃ is a slightly polar organic compound with a water solubility of ~8 g L⁻¹ at 20°C and an octanol-water partition coefficient of ~93 [MacKay *et al.*, 1993]. It is volatile with a boiling point of 61.7°C and a vapour pressure of ~21 kPa at 20°C, and therefore also found in the atmosphere, where it will mainly exist in the gaseous form and to a minor extent in the aqueous phase or associated with solid particles. Estimates of global averages of CHCl₃ concentrations in the atmosphere varies from 10-20 pptv [Harper, 1997; Cox *et al.*, 2003; Gebhardt *et al.*, 2008] with higher concentration in the northern than in the southern hemisphere [Cox *et al.*, 2003]. At 10°C, 15 pptv in the air would correspond to an equilibrium concentration in precipitation of ~1 ng L⁻¹. This is close to the concentrations of CHCl₃ in rain of 0.8-2.3 ng L⁻¹ reported recently for a study in Southern Sweden [Svensson *et al.*, 2007b].

The sorption of CHCl₃ in soil has been shown to be mainly determined by the organic content (Figure 6) [Grathwohl, 1990; III]. This corresponds well with the observation by Wilson *et al.* (1981) that CHCl₃ was only slightly retarded in a soil column with sand low in SOM, compared to the interstitial water. Farrell & Reinhard (1994) found that at SOC-contents less than 0.15%, CHCl₃ was adsorbed to mineral surfaces and Riley *et al.* (2005) found that in sediment with very low SOC-contents ($\leq 0.06\%$), the adsorption was not controlled by SOC but rather by sorption to mineral components. K_d values were estimated to 0.084-0.43 in these low-SOC sediments which was more than 10 times higher than what could be expected from K_{OC} -values. The four soils in Figure 6 represent K_{OC} -values in the range of 22-29 except for the soil with the lowest LOI, which has a calculated K_{OC} of 45. This could indicate that sorption to inorganic material might be relevant at LOI as high as 0.5%, corresponding to an OC content of $\sim 0.25\%$. The K_F -value was still very low (0.27), though, and the leaching of CHCl₃ would probably be only slightly retarded by sorption in such a soil.

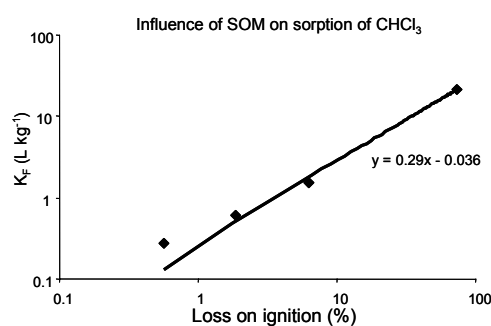


Figure 6. Relationship between SOM-content in soil from two coniferous forests (TH and NF) and the corresponding sorption coefficient, K_F , for CHCl₃. Taken from [III].

The partitioning of CHCl₃ between water and air has been determined in the temperature interval 2-60°C [Görgényi *et al.*, 2002]. At soil temperatures, relevant for temperate soils, the water/air-partitioning coefficient was determined to 21, 15, 13 and 7.9 at 2, 6, 10 and 18°C, respectively. These water/air partitioning ratios can be used to calculate e.g. the concentration of CHCl₃ in soil water from soil air measurements, when the soil temperature is known. This approach was carried out in an attempt to calculate concentrations of CHCl₃ in the uppermost groundwater from CHCl₃ concentrations in soil air throughout two soil profiles in the TH study site [I]. Calculated CHCl₃ concentrations in the soil water just above the groundwater table showed reasonable good agreement with CHCl₃ concentrations actually found in the upper groundwater (Figure 7) and similar agreements were found for four forests monitored during longer time periods [III].

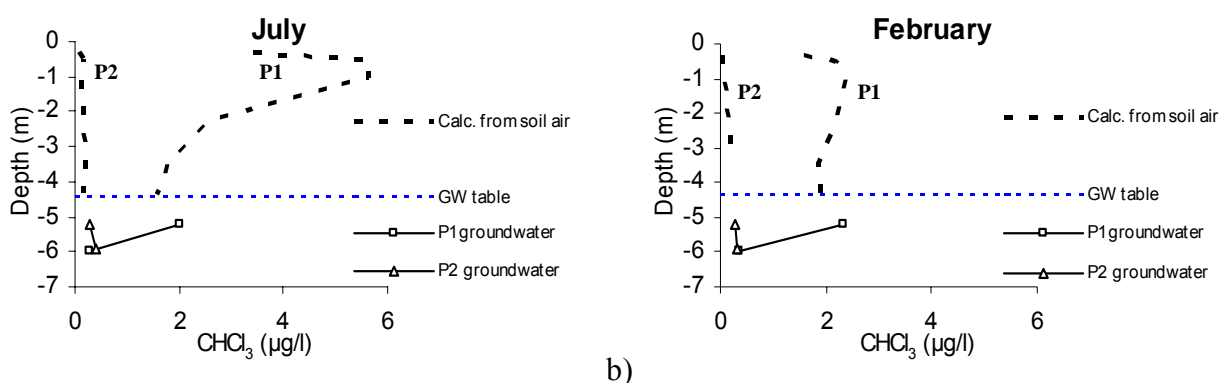


Figure 7. CHCl₃ in groundwater (measured) and in unsaturated soil water (calculated from Henry's law assuming equilibrium between air and water) in the TH study site during a) summer and b) winter. The P1 profile is located in an area with high chloroform production, P2 in an area with low production (see section 2.3 and **Paper II**). Taken from [I].

2.1.2 Abiotic degradation in soil

While CHCl₃ is known to be degraded by OH⁻-radicals in the atmosphere, giving an average atmospheric half-life of ~6 months [Khalil & Rasmussen, 1999; Laturnus *et al.*, 2002], the only reported abiotic degradation mechanism for CHCl₃ in soil is reductive dehalogenation. Pecher *et al.* (2002) found that several polyhalogenated methanes were transformed by Fe(II)/Fe(III)-complexes with a decreasing reaction rate in the order CCl₄>CHBr₃>CHBr₂Cl>CHBrCl₂. The reaction was pH-dependent with faster reaction at lower pH. CHCl₃ was not investigated, but would also be expected to be transformed by such complexes, and Kenneke & Weber (2003) found that Fe(II)/Fe(III)-complexes could dehalogenate CHCl₃ as well as a number of other halogenated methanes. FeS was also found to dehalogenate a number of halogenated methanes, but in iron- and SO₄-reducing sediments, Fe(II)/Fe(III)-complexes seemed to be the most important dehalogenating agents. CHCl₃ is not expected to be degraded abiotically in oxic environments.

2.1.3 Biotic degradation

Based on its chemical structure, CHCl₃ should energetically be equally favorable for reduction under anaerobic conditions and oxidation under aerobic conditions [Vogel *et al.*, 1987]. Most focus has been on anaerobic reductive dechlorination, however, with only a few studies reporting aerobic microbial degradation of CHCl₃. The aerobic degradation of CHCl₃ has in all cases been co-metabolism by different oxygenase expressing bacteria. Strand & Shippert (1986) found that ~3% of the added CHCl₃ was oxidized to CO₂ within five days in soil and similar mineralization rates were found during aerobic degradation of ¹⁴C-CHCl₃ in forest top soil [II; III], while the mineralization was lower in deeper horizons (Figure 8).

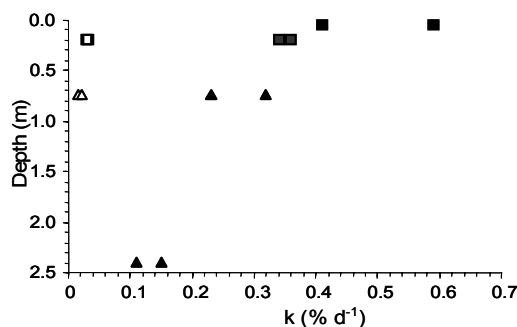
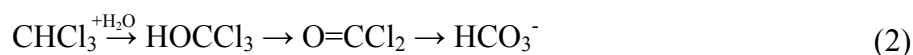


Figure 8. Rate of CHCl₃ mineralization according to the depth of which the soil was sampled. Squares are soil from NF, triangles soil from TH. Open symbols are NaN₃-sterilized soil samples. Taken from [III].

The mineralization reported by Strand & Shippert was 4 times greater in soil acclimated to methane, while acetylene hindered the degradation, indicating that methane-monoxygenase or some other alkane monoxygenase enzyme was involved in the degradation. Methylobacteria are well known to mineralize mono- and di-halomethanes [Trotsenko & Doronina, 2003], while only a few bacteria have been shown to degrade CHCl₃ aerobically [Alvarez-Cohen & Speitel, 2001]. Oldenhuis *et al.* (1989) showed that soil methylotroph *Methylosinus trichosporium* could mineralize CHCl₃ through co-metabolism and Bartnicki & Castro (1994) demonstrated a rapid oxidative mineralization of CHCl₃ by a pure culture of the *M. trichosporium*. The proposed degradation pathway was (Eq.2):



, with the chlorine released as HCl. A few additional studies have shown that methanotrophic strains can mineralize CHCl₃ through co-metabolism [Alvarez-Cohen *et al.*, 1992; Chang & Alvarez-Cohen, 1996], but most studies on the aerobic degradation of chlorinated solvents have been performed with trichloroethene as the model compound. Some bacteria, that utilize aromatic compounds, have non-specific mono- or dioxygenases, which initiate the degradation of the aromatic structures, but also can perform co-metabolism of chlorinated solvents. These bacteria only slowly degrade other chlorinated solvents than chlorinated ethenes, though. Another oxygenase-expressing bacterium, the ammonia-utilizer *N. europaea*, has been shown to co-metabolize CHCl₃ at a rate similar to the degradation by methanotrophic strains [Alvarez-Cohen & Speitel, 2001].

Reductive dehalogenation (halorespiration) is a well known anaerobic degradation pathway for several halogenated solvents, but the bacteria capable of dehalogenating chlorinated ethenes are often not capable of dehalogenating halomethanes [Field & Sierra-Alvarez, 2004]. Gupta *et al.* (1996a, b) showed reductive dehalogenation of CHCl₃ by a mixed sulphate-reducing culture both with and without acetate as primary substrate. In methanogenic environment, CHCl₃ was also shown to be dechlorinated to CH₂Cl₂, with and without acetate as the primary substrate, but at a much lower rate than in the sulphate-reducing culture. Reductive dehalogenation of CHCl₃ leads to the formation of CH₂Cl₂ and not to complete mineralization as the oxidative degradation by methylotrophic microorganisms, but the formed CH₂Cl₂ has been shown to be further degraded [Gupta *et al.*, 1996a, b]. Borch *et al.* (2003)

investigated the degradation of some chlorinated methanes, ethanes and ethenes in a natural soil from the top of an agricultural field, and found no major degradation of CHCl₃ under anoxic (denitrifying) conditions within 40 days when compared with the sterile control, while CCl₄ was completely transformed within 20 days.

2.2 Anthropogenic sources and fluxes

CHCl₃ is produced in huge amounts industrially, with a yearly production exceeding 500 Gg (late 1990s) [McCulloch, 2003]. Most of the industrial CHCl₃ is used for synthesis of other compounds, especially HCFC-22. This refrigerant is to be phased out under the Montreal Protocol during the years 2010-2020, and industrial production of CHCl₃ might hence change significantly in the future. Only ~4% of the industrial CHCl₃ is used as an extractant/solvent in various manufacturing and laboratory processes, and ~half of this is expected to escape to the atmosphere, leaving an estimated pollution with industrial CHCl₃ of ~11 Gg yr⁻¹ (Table 2). The use of oxidized chlorine in a number of bleaching and purification processes leads to a significantly larger anthropogenic contribution of CHCl₃ to the atmosphere of ~54 Gg yr⁻¹ (Table 2). This unintended human-derived CHCl₃ was diminished significantly in the 1990s by the shift towards using ClO₂ instead of HOCl in the bleaching processes of the paper manufacture [AET, 2006], and may decrease further in the future, if alternatives to HOCl become widespread in the water treatment processes. Worton *et al.* (2006) estimated from analyses of CHCl₃ in firn air, that the anthropogenic input to the atmosphere was diminished from 193-226 Gg yr⁻¹ in 1990 to 91-93 Gg yr⁻¹ in 2001, probably mainly due to changes in the paper bleaching procedures during the early 1990s.

Table 2. Emissions of CHCl₃ to the atmosphere from known anthropogenic sources. Data are based on the calculations in Aucott *et al.* (1999).

Source	Estimated release to the atmosphere (Gg yr ⁻¹)	Range including uncertainty (Gg yr ⁻¹)	Year(s) as basis for calculation
Paper manufacture	33	24-42	Late 1990s
Drinking water treatment	9	3-15	Late 1990s
Waste water treatment	3	2-4	Late 1990s
Other water treatment	9	3-15	Early 1990s
Incineration/combustion	2		Late 1990s
Chemical and other industry	11	9-13	Late 1990s
Total anthropogenic	67	43-91	

The anthropogenic emissions estimated by Worton *et al.*, (2006) are somewhat higher than what can be calculated from known sources (Table 2), indicating either unknown sources or even greater uncertainties in the calculations. Total emissions to the atmosphere have been calculated to 350-600 Gg yr⁻¹ [Khalil & Rasmussen, 1999; Keene *et al.*, 1999] based on a global atmospheric lifetime of ~6 months, which was calculated from measured lifetimes in the tropics of ~3 months and in the polar regions of 30-40 months [Khalil & Rasmussen, 1999]. Worton *et al.* (2006) estimated total CHCl₃

emissions of 315-373 Gg yr⁻¹ (year 2001). In summary, this means that anthropogenic emissions are probably currently contributing somewhere in the range of 10-25% of the total emissions to the atmosphere, leaving at least 75% for natural sources.

2.3 Natural sources and fluxes of CHCl₃

No matter what the exact ratio is between anthropogenic and natural contributions to the atmosphere, the major fraction of CHCl₃ emissions seems to originate from natural sources of which several have been identified (Table 3).

Table 3. Identified sources of natural CHCl₃.

Organism or ecosystem	Bibliographic source
Oceans/seas	Khalil & Rasmussen, 1999
Macroalgae	Nightingale <i>et al.</i> , 1995; Laturmus, 2001; Baker <i>et al.</i> , 2001
Phytoplankton	Scaratt & Moore, 1999
Volcanoes	Jordan <i>et al.</i> , 2000
Rice fields	Khalil <i>et al.</i> , 1990a; Khalil <i>et al.</i> , 1998
Termite mounds	Khalil <i>et al.</i> , 1990b
Peatlands	Dimmer <i>et al.</i> , 2001
Tropical forests	Gebhardt <i>et al.</i> , 2008
Temperate forest soil	Hoekstra <i>et al.</i> , 1998a; Haselmann <i>et al.</i> , 2000a; Laturmus <i>et al.</i> , 2000; Hoekstra <i>et al.</i> , 2001; II; III
Terrestrial fungi	Hoekstra <i>et al.</i> , 1998b

While several natural sources of CHCl₃ have been identified, the question on the importance of each source remains largely an open one. The terrestrial environment contributes an important and perhaps even major part of the total release of CHCl₃ to the atmosphere [Keene *et al.*, 1999; O'Doherty *et al.*, 2001; Xiao, 2008]. Since the focus of this thesis is on sources in soil, the concentrations and emissions of CHCl₃ from soil are specified in Table 4. Sources of CHCl₃ exist in several soil environments, and several of the sources seem to contribute significantly to the total emissions of CHCl₃ to the atmosphere (natural + anthropogenic) of 300-600 Gg y⁻¹ [Khalil & Rasmussen, 1999; Keene *et al.*, 1999; Worton *et al.*, 2006]. Much more work is needed however, if one wants to reliably determine the significance of each environmental compartment, which would be important in order to predict the influence of future changes in e.g. climate and hence changes in ecosystem structure and distribution. In that perspective, more knowledge on the responsible organisms and processes might also be needed.

Table 4. Reported emissions and concentrations of natural CHCl₃ in the terrestrial environment. In cases where the authors used their data to extrapolate to emissions from their ecosystem type on a global scale, the overall estimates are also shown. The uncertainty intervals of these calculations are not shown, but are in all cases very large. Only studies where the CHCl₃ was assumed to be natural are included in the table.

Environment	Emissions ($\mu\text{g m}^{-2} \text{h}^{-1}$)	Calc. global emissions (Gg y^{-1})	Bibliographic source
Termite mounds	1.4–13	10–100	Khalil <i>et al.</i> , 1990b
Rice paddies	0.10–4.4	8–50	Khalil <i>et al.</i> , 1990a; Khalil <i>et al.</i> , 1998
Subtrop. shrubland soil	0–0.15	-	Rhew <i>et al.</i> , 2008a
Trop./subtrop. forest soil	0.13–2.2	-	Khalil & Rasmussen, 2000
Temp. peatland	0.01–1.1	4.7 Gg y^{-1}	Dimmer <i>et al.</i> , 2001
Temp. grassland soil	0.01	-	Hoekstra <i>et al.</i> , 2001
Temp. deciduous forest soil	0.02	-	Hoekstra <i>et al.</i> , 2001
Temp. coniferous forest soil	0.01–0.11	-	Hoekstra <i>et al.</i> , 2001
Temp. coniferous forest soil	0.25–17	-	Dimmer <i>et al.</i> , 2001
Temp. coniferous forest soil	0.02–2.7	-	II
Boreal coniferous forest soil	0.10–0.80	48 Gg y^{-1}	Hellén <i>et al.</i> , 2006
Tundra soil	0–1.5	-	Khalil & Rasmussen, 2000
Tundra soil	0.01–1.3	3.9 Gg y^{-1}	Rhew <i>et al.</i> , 2008b
Environment	Soil air conc. (ppbv)	Calc. global emissions (Gg y^{-1})	Bibliographic source
Termite mounds	5.7–358	-	Khalil <i>et al.</i> , 1990b
Temp. grassland soil	0.13	-	Haselmann <i>et al.</i> , 2000a
Temp. deciduous forest soil	0.08	-	Haselmann <i>et al.</i> , 2000a
Temp. coniferous forest soil	0.06–0.4	4.9 Gg y^{-1}	Haselmann <i>et al.</i> , 2000a; Haselmann <i>et al.</i> , 2002
Temp. coniferous forest soil	0.9–7.5	-	Hoekstra <i>et al.</i> , 1998a
Temp. coniferous forest soil	0.3–5.8	-	Laternus <i>et al.</i> , 1995; Laternus <i>et al.</i> , 2000
Temp. coniferous forest soil	0.2–163	-	II; III
Environment	Groundwater conc. ($\mu\text{g L}^{-1}$)	Bibliographic source	
Temp. coniferous forest soil	<0.01–1.6	Laternus <i>et al.</i> , 2000	
Temp. coniferous forest soil	0.3–5.4	I; II; III	

As indicated in Table 4, the variations in CHCl₃ concentrations and emissions from soils are very large, both between different studies, but also within the same studies including samples taken in the same study areas. This phenomenon of high spatial variation was investigated more systematically [II], and it was found that almost the full span in reported CHCl₃ concentrations can be found within distances of a few meters within forest stands with relatively homogeneous vegetation and soil texture (Figure 9). The variation was consistent over a two year period. This large spatial variation makes the calculated values for global emissions even more uncertain, and such calculations should therefore be used with extreme care and with specifications of the great uncertainties possibly associated with the data making the basis of the calculations. In conclusion, much more data are needed in order to determine the importance of various terrestrial systems on the global CHCl₃ budget. The greater the geographical area that can be integrated in these measurements, the more reliable the data will be for further calculations of global emissions from that source, although at the same time, this will probably lead to losses of information regarding another interesting perspective – namely the mechanisms controlling the formation of CHCl₃ in the soil.

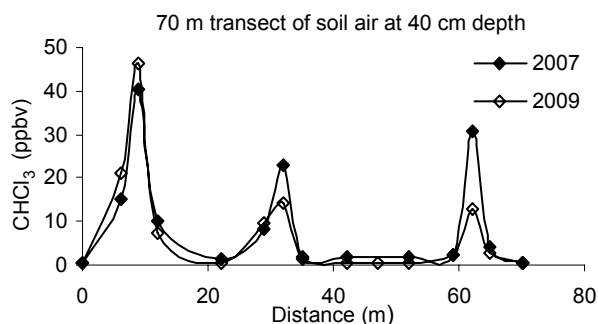


Figure 9. Transect of CHCl₃ concentrations in soil air of a coniferous forest. Soil air was sampled at 40 cm depth in May 2007 and May 2009. Taken from [II].

2.3.1 Mode of formation in soil

In most of the studies on natural CHCl₃ in soil, it has been assumed that the formation is mediated by microbial processes, and several indices exist, which support this hypothesis [Hoekstra *et al.*, 1998a; Hoekstra *et al.*, 1998b; Haselmann *et al.*, 2000b; II; III]. These include the following:

- CHCl₃ is mainly formed in the top soil with high microbial biomass [Hoekstra *et al.*, 1998a; III].
- Certain fungi have been shown to produce CHCl₃ [Hoekstra *et al.*, 1998b].
- Soil with biological activity reduced by electron beam irradiation show only minor releases of CHCl₃ compared to untreated soil [Haselmann *et al.*, 2000b].
- The spatial variation in CHCl₃ formation is huge within areas, which are homogenous regarding various vegetative and abiotic soil parameters, making domain living fungi likely to be participators in the formation [Hoekstra *et al.*, 1998a; II].
- Soil moisture is an important parameter controlling CHCl₃ formation [III].
- Increase in soil moisture and temperature leads to increased CHCl₃ formation but with delays of weeks, indicating growth of responsible organisms before significant increased formation [III].

The current hypothesis on the mechanism of formation is based on the fact that certain microorganisms, mainly fungi, are known to produce halide-oxidizing exo-enzymes, and the resultant oxidized halogen species (e.g. HOCl), will react unspecifically with SOM, as also known from the chlorination of drinking water and paper pulp. This was discussed in a previous section.

2.3.2 Abiotic formation of CHCl₃ in soil?

Very recently, Huber *et al.* (2009) demonstrated the formation of THM from polyphenols, H₂O₂, Fe(III) and X⁻ in laboratory studies. The reaction took place only at very acidic pH (<3.7), and the maximum yield of THM in 32 hours was <<1‰ of the polyphenols on a carbon:carbon basis. The authors did, however, make probable that CHCl₃ could be formed through abiotic processes in a natural soil at pH 4: The soil was freeze dried and rewetted, upon which it liberated small amounts of CHCl₃ at 40°C. This formation of CHCl₃ was increased ~100-fold upon the addition of Fe₂(SO₄)₃ or ferrihydrite, H₂O₂ and

KCl. The speciation of iron influenced the formation of CHCl₃, indicating that the increased formation was not singly caused by HOCl formed by CPO upon the addition of H₂O₂ and KCl. More studies on the possible abiotic formation of CHCl₃ would be interesting in order to support this hypothesis and to try to determine its influence compared to CHCl₃ formed upon microbiological activity.

2.3.3 Natural formation of other trihalomethanes in soil?

Chlorinating enzymes like CPO produce HOCl from H₂O₂ and Cl⁻ and also HOBr and HOI from the corresponding halides. Furthermore, there is a free exchange between halides in HOX that can lead to the formation of HOBr and HOI from HOCl. This is the reason for the formation of brominated trihalomethanes in the chlorination of drinking water [e.g. Nikolaou *et al.*, 2004]. The unspecific chlorination of organic matter leading to the formation of CHCl₃ would therefore be expected to also form small amounts of brominated trihalomethanes, if bromide is present in the soil. Hoekstra *et al.* (1998a) reported the presence of CHBrCl₂ in forest soil air and the concentration profile down to 120 cm depth simulated somewhat that of CHCl₃, but at a concentration of only ~1% of that of CHCl₃ in the same samples (4-42 pptv CHBrCl₂). This concentration was too low to determine an increase in ³⁷Cl in an enrichment experiment. CHBr₂Cl and CHBr₃ could not be measured in the natural soil, but was formed after the addition of a bromide-solution to the soil. Laturnus *et al.* (2000) looked for chlorinated, brominated and iodinated trihalomethanes in forest soil air and groundwater beneath the same forest, but found only CHCl₃ except a very small amount (~22 pptv) of CHBr₃ in the top soil layer. This concentration is, however, within the wide range of reported atmospheric mixing ratios of 0.2-46 pptv [Fogelqvist & Krysell, 1991]. From these two studies it seems likely that other trihalomethanes may be formed in the soil, but only in very small concentrations relative to CHCl₃.

2.4 Guidelines and toxicity thresholds of CHCl₃ in the environment

In high concentrations (ppm range), CHCl₃ acts as a central nervous system depressant in humans, with observed effects down to ~20 ppm (vol:vol in air), and furthermore CHCl₃ is regarded a probable carcinogen [Hathaway *et al.*, 2004; WHO 2004a]. This has led to calculations of acute exposure guideline levels in the range of 29-120 ppm in air for daily exposures of 8 hours to 10 min [USEPA 2010] and a tolerable daily intake of 15 µg kg⁻¹ body weight per day or a tolerable concentration in air of 0.14 mg m⁻³ or ~26 ppbv [WHO, 2004a]. Interestingly, the concentration in soil air may exceed this value (Figure 9 and II).

Except for certain workers in the industry, the most important pathway of CHCl₃ ingestion for humans is through consumption of contaminated or chlorinated drinking water. The WHO has set a quality criterion of 300 µg L⁻¹ for CHCl₃ [WHO, 2007], while the United States Environmental Protection Agency has set a maximum contaminant level of 80 µg L⁻¹ for total trihalomethanes including CHCl₃ and 70 µg L⁻¹ for CHCl₃ as a single compound. Denmark has a much lower maximum concentration of 1 µg L⁻¹ for single VOCl₃, including CHCl₃, in groundwater which is abstracted for the use of drinking

water [Miljøstyrelsen, 2007]. This value has not been set due to toxicological calculations but rather due to the fact that pollution with VOCs is in general recognized as unwanted. If it can be justified that the CHCl_3 in the groundwater is natural, a maximum concentration $10 \mu\text{g L}^{-1}$ is therefore acceptable [Miljøstyrelsen, 2007].

No major ecotoxicological problems have been reported for CHCl_3 in the environment, but while the toxicity of CHCl_3 towards algae, crustaceans and fish is low, CHCl_3 can be rather toxic towards microorganisms and amphibians, with observed effects at 0.1 and LC_{10} at 0.02 mg L^{-1} , respectively [WHO, 2004a]. A PNEC for groundwater biota was estimated to $50 \mu\text{g L}^{-1}$ and for terrestrial wildlife to 183 ppbv in air [WHO, 2004a]. The observed natural concentrations of CHCl_3 in forests (Table 4) hence seem to carry no risk to groundwater biota and only in rare cases approach the PNEC for terrestrial wildlife [II].

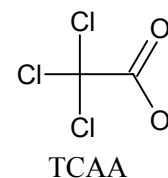
3. Other naturally occurring trichloromethyl compounds

Papers IV and V deal mainly with TCAA and trichloroacetyl containing compounds. This section should serve as a background for these papers as well as putting them into a perspective on the existing knowledge on formation and environmental behaviour of these compounds.

3.1 TCAA

Trichloroacetic acid (TCAA) has been target of a number of investigations, because its presence in conifer needles has been associated with forest decline, especially in central Europe. Furthermore TCAA has been detected in rain, freshwater and soil.

During the last 10-15 years, several indications of its natural occurrence have been reported, but it is not as well accepted as a natural compound as e.g. CHCl_3 . One reason for this may be that a number of very different sources exist for this compound, and it therefore may be difficult to quantify the natural contribution.



3.1.1 Behaviour and fate in the environment

TCAA has a pK_a -value of ~ 0.7 , and is therefore almost exclusively found in its dissociated form in the environment. Deprotonized TCAA is very soluble in water ($\sim 1300 \text{ g L}^{-1}$) and has a low Henry's law constant [Bowden *et al.*, 1998], and the derived preference for aqueous phases is perhaps the reason why only very few studies have been published on the partitioning of TCAA into non-aqueous compartments. Streibig (1980) investigated the sorption of TCAA to various soils and found that it was in general negligible except for a small sorption to two soils with high SOM (19-23%) and clay (19-33%) contents. Haiber *et al.* (1996) reported a low recovery of TCAA from HA-spiked water, but the extraction from the water was carried out at very acidic pH to extract non-dissociated TCAA into

diethyl ether and protonated TCAA may behave very different from its anion. In **V**, the sorption of TCAA to forest soil was investigated, showing a small sorption ($K_F \approx 1$) in the top organic horizon and a negligible sorption in deeper horizons. This indicates that TCAA will leach easily through forest soil, as was also shown for some agricultural soils [Ogle & Warren, 1954].

Abiotic degradation has been speculatively suggested for TCAA in soil [e.g. Frank *et al.*, 1989]. The proposed reaction mechanism is decarboxylation of TCAA \rightarrow $\text{CHCl}_3 + \text{CO}_2$, but such spontaneous decarboxylation has been shown to be very slow at relevant temperatures in pure water [Fairclough, 1938] and lake water [Xiang *et al.*, 2005], while at elevated temperature ($>60^\circ\text{C}$), the decarboxylation is fast.

Under anaerobic conditions, Fe(II)-complexes are known to mediate an abiotic reductive dechlorination of several chlorinated ethanes and ethenes, and though much more interest has been paid towards chlorinated solvents, one study did show the abiotic dechlorination of TCAA by green rust, a mixture of Fe(II) and Fe(III) with an intermediate layer of anions like carbonate or sulphate [Chun *et al.*, 2007]. Abiotic reductive dechlorination might be an important pathway for the degradation of TCAA under anaerobic conditions, but more studies are needed to conclude on this.

Although abiotic degradation of TCAA may be important in soil under anaerobic conditions, microbial degradation is likely to dominate under aerobic conditions and has also been shown to occur without the presence of oxygen (Table 5). TCAA seems to be completely mineralized in soil under aerobic conditions, and metabolites from the degradation is most likely not of environmental concern. Haselmann *et al.* (2000) did show some indications that CHCl_3 could be formed by microbial degradation of TCAA in forest soil, but other authors [Forczek *et al.*, 2001; Matucha *et al.*, 2003a; Matucha *et al.*, 2006] were not able to repeat this observation, and in general TCAA seems to be completely mineralized in aerobic soils.

Table 5. Overview of reported microbial degradation pathways for TCAA. Data gathered from the reviews by Slater *et al.* (1997), Schöler *et al.* (2003) and Field & Sierra-Alvarez (2004).

	Isolation of strain(s)?	Degradation products
Aerobic processes		
Unspecified degradation with growth	Yes	CO_2 , Cl^-
Co-metabolic degradation	Yes	CO_2 , Cl^-
Anaerobic processes		
Halorespiration	Yes	DCAA
Unspecific anaerobic degradation	Yes	Acetate, CH_4 , CO_2 , Cl^-

Two major classes of research on microbial degradation of TCAA, separated in time and in focus, exist: From ~1955-1985 (the period in which TCAA was used as an herbicide) most degradation studies were carried out with agricultural soils. From ~1990 until present, focus has been on TCAA in forests (as a secondary air pollutant affecting forest health and in the last 10 years also as a natural product of the forest soil) and most degradation studies in this period were carried out with forest soils. Very

different rates of TCAA degradation are achieved in soils from forests and agricultural fields, but in all soils investigated, there has been a significant degradation within the tested time interval (typically much less than one year). In agricultural soils, there is a lag phase of 2-3 weeks followed by relatively fast degradation [Jensen, 1957; Smith, 1974; McGrath, 1976]. With a second application, no lag phase is observed, and TCAA is quickly mineralized [McGrath, 1976]. This adaptation to fast degradation of TCAA was retained in the soil for almost three years. In forest top soil, no lag phase exists and TCAA is degraded with a half-life of typically less than 24 hours [Forczek *et al.*, 2001; Schröder *et al.*, 2003; Matucha *et al.*, 2003a, 2003b, 2006; V]. In deeper parts (below 1 m) of forest soils, there is also a degradation potential, but only after a lag phase [V]. One could then speculate that TCAA is not naturally present in significant amounts to sustain a degrading population in agricultural soils and in forest sub soils, while in forest top soil, there is a clear microbial tolerance, indicating a regular input of TCAA.

3.1.2 Anthropogenic sources and fluxes

There are at least six identified potential anthropogenic sources of TCAA in the environment;

- By application as an herbicide
- As a metabolite in the degradation of pesticides
- From abiotic oxidation of VOCl in the atmosphere
- From biotic oxidation of VOCl in biota
- As a disinfection by-product in the chlorination of drinking water
- As a by-product in the bleaching of paper pulp

TCAA as a pesticide. From ~1950 to the late 1980's TCAA as its sodium salt was intensively used as an herbicide in several countries in Europe, in USA and elsewhere. In Denmark, TCAA was one of the most intensively used active ingredients, but ~1990 it was banned in the European Union due to its indiscriminative mode of action.

TCAA as a degradation product. TCAA is a known degradation product of the two organophosphate insecticides trichlorfon (O,O-diethyl (RS)-O-(1,2,2,2-tetrachloroethyl) phosphorothioate) and chlorethoxyfos (dimethyl (RS)-2,2,2-trichloro-1-hydroxyethylphosphonate). None of these insecticides are currently in use in the EU, but trichlorofon was in use from the 1950s to the early 1990s.

Abiotic oxidation of VOCl. The two chlorinated solvents 1,1,1-trichloroethane (1,1,1-TCA) and tetrachloroethene (PCE) are known precursors of TCAA in the atmosphere [Peters, 2003; Cape *et al.*, 2006]. The atmospheric degradation of 1,1,1-TCA involves trichloroacetaldehyde (chloral) as an intermediate, and a small part of the formed chloral will after uptake into cloud water be further oxidized to TCAA [McCulloch, 2002]. The yield of TCAA from atmospheric 1,1,1-TCA has been calculated to be between ~1% [McCulloch, 2002] and 14% [Jordan & Frank, 1999]. The atmospheric degradation of PCE proceeds mainly through reaction with OH-radicals, leading to no formation of

TCAA. 10-15% of atmospheric PCE will however react with Cl-radicals leading mainly to the formation of the pentachloroethoxy radical ($\text{CCl}_3\text{CCl}_2\text{O}^\cdot$) of which 40-50% will end up as TCAA [Hoekstra & Juuti, 1999; McCulloch, 2002]. These somewhat uncertain calculations have led to estimates of TCAA-concentrations in European precipitation originating from 1,1,1-TCA and PCE in the interval of $0.11 \mu\text{g L}^{-1}$ [McCulloch, 2002] to $0.30 \mu\text{g L}^{-1}$ [Jordan & Frank, 1999]. The atmospheric concentration of 1,1,1-TCA has, however, decreased drastically since the 1990's due to its regulation through the Montreal Protocol [AGAGE, 2010]. An average of $0.24 \mu\text{g L}^{-1}$ was found in rain and snow samples in Switzerland in 1997 [Berg *et al.*, 2000], which is slightly higher but not too different from the average of $0.11 \mu\text{g L}^{-1}$ found during regular sampling at the TH study site [V]. TCAA concentrations $\leq 0.10 \mu\text{g L}^{-1}$ have, however, also been detected in old firn and glacier ice deposited before anthropogenic emissions of 1,1,1-TCA and PCE occurred [Haiber *et al.*, 1996; Von Sydow *et al.*, 1999].

Biotic oxidation of VOCl. C_2 -chlorocarbons like 1,1,1-TCA, PCE and trichloroethene (TCE) are transformed by living plant cells, probably in the chloroplasts, into TCAA. This has been shown for both coniferous [Plümacher & Schröder, 1994; Weissflog *et al.*, 2007; Strycharz & Newman, 2009] and deciduous [e.g. Newman *et al.*, 1997; Strycharz & Newman, 2009] tree species and is probably a general phenomenon. Also in mammals, C_2 -chlorocarbons are metabolized mainly into TCAA, which is then excreted through urine, and TCAA in urine is even used as a biomarker for TCE exposure [Forkert *et al.*, 2003].

TCAA as a by-product from disinfection and pulp bleaching. As previously discussed, TCAA is, together with CHCl_3 , among the major by-products during chlorination of natural waters and HS and especially the aromatic part of HS have been shown to be good precursors for TCAA. The concentration of TCAA in chlorinated drinking water can reach hundreds of $\mu\text{g L}^{-1}$, but the importance of this formation pathway for TCAA is currently decreasing because of the introduction of alternatives to Cl_2 , such as ozonation.

To remove the last 5-10% lignin in pulp, each tonne of pulp is traditionally bleached with 60-70 kg Cl_2 leading to ~ 4 kg organic Cl [Kringstad & Lindström, 1984]. A major part of the extractable chlorinated by-products is TCAA, as much as 53% by weight [Lindström & Österberg, 1986]. Since ~ 1990 , the use of Cl_2 in the paper industry has been decreasing, [AET, 2006], and this industry may therefore not be such an important source of TCAA today.

3.1.3 Natural sources and fluxes

Hoekstra & De Leer (1993), Hoekstra *et al.* (1995) and Haiber *et al.* (1996) were the first to suggest that a significant part of the TCAA in the environment could be natural. This viewpoint is still somewhat controversial, though. The widespread occurrence of TCAA in especially vegetation [Frank *et al.*, 1990; Juuti *et al.*, 1996; Weissflog *et al.*, 2003; Stidson *et al.*, 2004; V] may be an indication that TCAA is naturally occurring, but it is not easy to distinguish this TCAA, from the TCAA that may be formed through oxidation of VOCl. Hoekstra *et al.* (1999a), found a weak positive relationship between TCAA

in samples taken from soil and peat and CHCl_3 in soil or peat air (Figure 10a), while there was no relationship with any other chlorinated solvent. Since CHCl_3 is known to be formed naturally in soil and TCAA might be formed in reactions also leading to formation of CHCl_3 , this may indicate that the TCAA was natural. The concentration of TCAA was rather low (up to $0.3 \mu\text{g} (\text{kg dw})^{-1}$ in soil and up to $4.6 \mu\text{g} (\text{kg dw})^{-1}$ in peat), but in studies where a compound specific analysis of TCAA is used [Hoekstra & De Leer, 1993; Hoekstra *et al.*, 1999a; Peters, 2003; Scott *et al.*, 2005], concentrations of TCAA in non-polluted soils are always low and often below the detection limit. In **Paper V**, an apparent positive relationship between TCAA in spruce needles and CHCl_3 in soil next to the tree trunk was found (Figure 10b), supporting the possible relationship in soil and peat, reported by Hoekstra *et al.* (1999a).

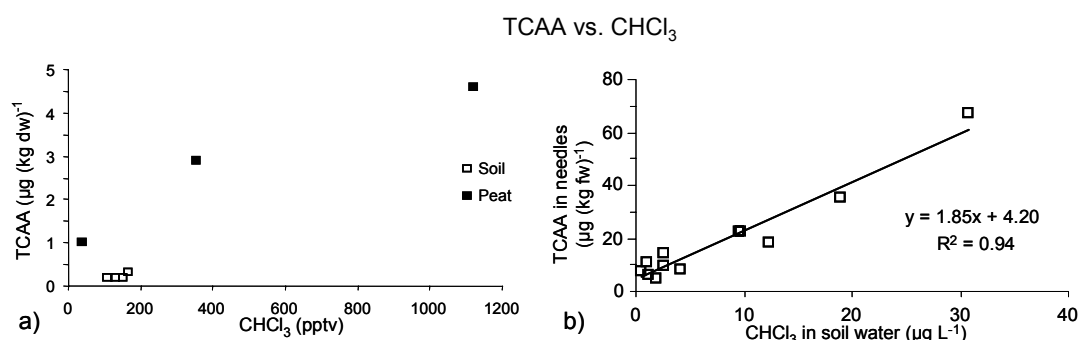


Figure 10. a) Possible relationship between TCAA in soil and peat and CHCl_3 in soil air at the same sampling site. After Hoekstra *et al.* (1999a). b) TCAA in spruce needles vs. water-extractable CHCl_3 in soil (~15 cm depth) at the base of the trunk of the tree from which the needles were sampled. Taken from [V].

In vivo formation of TCAA in soil has never been reported, but recently Matucha *et al.* (2007a & b) showed the formation of ^{36}Cl -TCAA (and ^{36}Cl -DCAA) in forest soil by adding $^{36}\text{Cl}^-$ to both an organic forest soil and a mineral forest soil rich in organic matter in a laboratory experiment. The formation corresponded to less than 0.1% of the added ^{36}Cl but was never the less the first and as yet only direct evidence of the natural formation of TCAA in soil. The formation was ascribed to biological activity, but this was not proved in the experiments.

Abiotic formation of TCAA has also been shown to occur from coniferous forest soil, commercial HA and from phenols with the addition of Fe^{3+} or Fe^{2+} , Cl^- and H_2O_2 [Fahimi *et al.*, 2003]. The mechanism is suggested to be mediated by the Fenton reaction (eq. 3):



$\cdot\text{OH}$ may then oxidize Cl^- to reactive Cl that can then react with organic matter. The authors suggest that TCAA is formed abiotically in soil in addition to its enzyme-induced formation. Further experiments would be needed, though, to reveal if this abiotic mechanism is significant in natural systems.

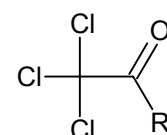
3.1.4 Guidelines and toxicity thresholds of TCAA in the environment

TCAA has been used extensively as an herbicide, and is a known phytotoxic substance that is taken up by the transpiratory system with effects below $1 \text{ mg (kg soil)}^{-1}$. According to Forczek *et al.* (2001), TCAA damages the photosynthetic apparatus of coniferous trees at needle concentrations above $\sim 60 \text{ } \mu\text{g kg}^{-1}$ and Ahlers *et al.* (2003) calculated a predicted no effect concentration in soil ($\text{PNEC}_{\text{soil}}$) for pine and spruce of $2.4 \text{ } \mu\text{g (kg dw)}^{-1}$. The lowest LC_{50} reported for the aquatic environment is $300 \text{ } \mu\text{g L}^{-1}$ for a species of green algae [Lewis *et al.*, 2004]

TCAA is not very toxic to mammals or other animals and the WHO guideline for TCAA in drinking water is $200 \text{ } \mu\text{g L}^{-1}$ [WHO, 2004b]. For aquatic animals, the lowest acute LC_{50} reported is $1200 \text{ } \mu\text{g L}^{-1}$ for a shrimp and for the terrestrial environment, an LC_{50} of $0.1 \text{ } \mu\text{g cm}^{-2}$ ($\sim 125 \text{ } \mu\text{g kg}^{-1}$) for an earthworm has been reported [Lewis *et al.*, 2004]. This acute toxicity was converted to a $\text{PNEC}_{\text{soil}}$ of $2.5 \text{ } \mu\text{g kg}^{-1}$, very similar to the $\text{PNEC}_{\text{soil}}$ of $2.4 \text{ } \mu\text{g kg}^{-1}$ calculated for coniferous trees.

3.2 Trichloroacetyl containing compounds

When chemical chlorination of organic matter is performed at low pH and low chlorine dose, various compounds containing a trichloroacetyl group are formed along with CHCl_3 and TCAA (Figure 3 and 4) [Boyce & Hornig, 1983; De Leer *et al.*, 1985; Streicher *et al.*, 1986; Reckhow *et al.*, 1990; V]. Hoekstra *et al.* (1998a)



Trichloroacetyl group. R = C, H

proposed that such compounds might also be formed in the processes leading to the formation of CHCl_3 in forest soil, but this was not further investigated and not until the work leading to the present thesis [IV; V], such compounds were discovered in natural environments. As shown in these two papers, trichloroacetyl containing compounds were present in all investigated forest compartments (spruce xylem, spruce needles, throughfall, soil, soil water and groundwater). Especially in top soil, trichloroacetyl containing compounds were by far the dominating trichloromethyl compounds, but both in the organic horizon and in the shallow minerogenic horizons, it is well correlated with CHCl_3 (Figure 11), indicating a possible relationship between the formation of the two compounds.

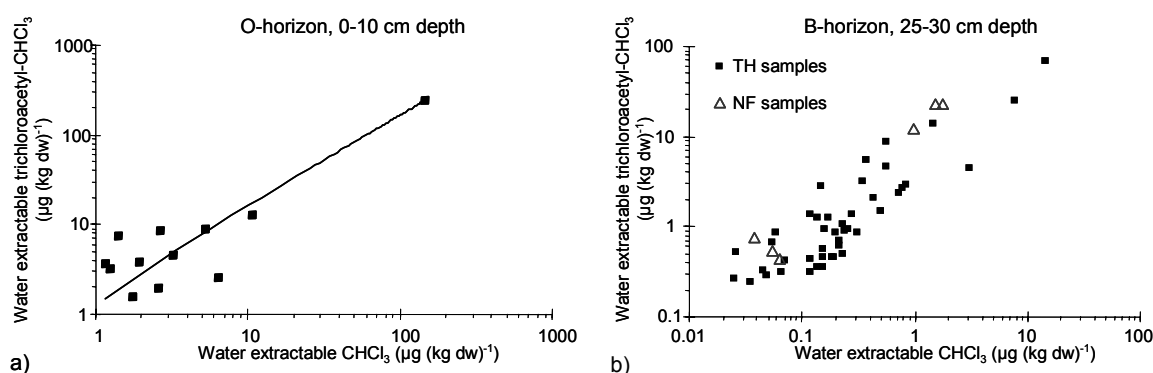


Figure 11. Relationship between water extractable trichloroacetyl- CHCl_3 and CHCl_3 in a) 13 samples of O-horizon sampled in TH [unpublished results] and b) soil from 25–30 cm depth in TH (44 samples) and NF (6 samples) [V].

Furthermore, the work presented in [IV and V] shows that the major fraction of trichloroacetyl-CHCl₃ in top soil is present in non extractable compounds or in compounds only extractable with NaOH (Figure 12a), and also that there is a positive relationship between the two fractions of trichloroacetyl containing compounds, although the ratio between them differs between sampling sites (Figure 12b).

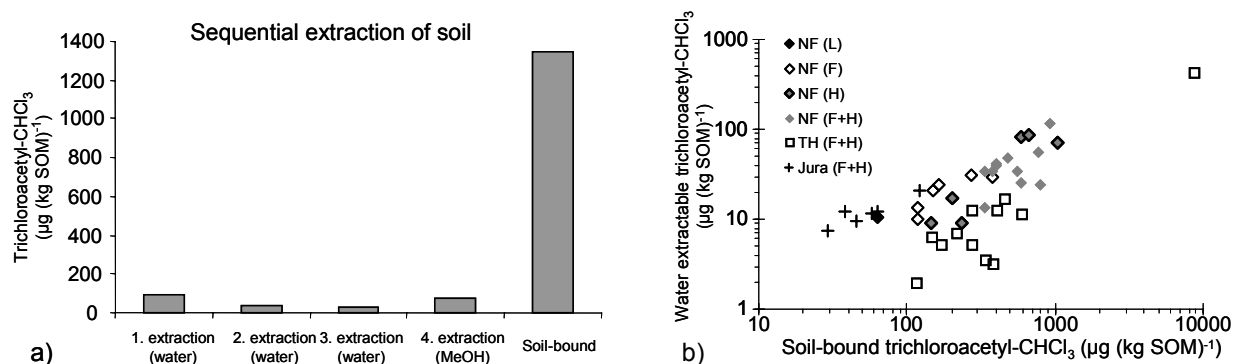


Figure 12. a) Trichloroacetyl-CHCl₃ in sequential extracts of soil from the organic H-horizon in NF. Solvent was either pure water or 80% MeOH. Soil bound is the trichloroacetyl-CHCl₃ liberated from the soil when 0.1M NaOH is added after the sequential extraction. Taken from [IV]. b) Relationship between water extractable and soil bound trichloroacetyl-CHCl₃ in two Danish (NF and TH) and two Swiss (both Jura) coniferous forests. Taken from [V].

Even though the majority of trichloroacetyl groups in soil are present in macromolecular structures, it was made probable in **Paper IV** that most of the trichloroacetyl-CHCl₃ in soil pore water and in groundwater consists of easily leachable substances containing both a trichloroacetyl and a carboxylic acid group. In two cases, compounds with both these structural elements have been identified as products from the chemical chlorination of resorcinol and HA in the laboratory (Figure 13). Whether or not it actually is these or similar compounds that are formed naturally in top soil and then subsequently transported to the groundwater remains to be shown in future studies, but based on existing knowledge they are the most likely suggestions.

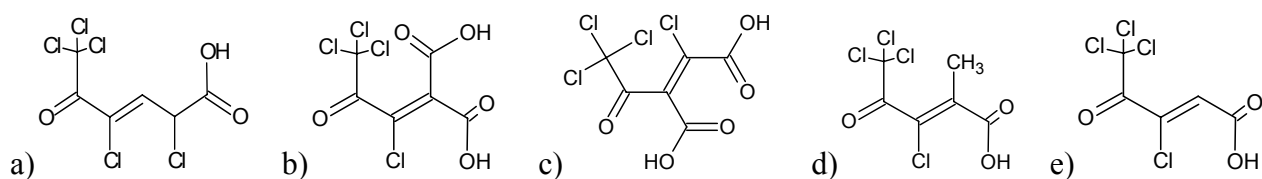


Figure 13. The six previously published structures fulfilling the structural elements of trichloroacetyl containing compounds identified in soil and groundwater samples in IV. a) from Boyce & Hornig (1983), b)-e) from De Leer *et al.* (1985). The compounds were formed during artificial chlorination with low chlorine dose and only at pH-values below neutral. See Figure 3 for the proposed reaction pathway leading to a), b), d) and e).

Trichloroacetyl containing compounds without a carboxylic acid group were also present in the forest ecosystem, especially in needles and throughfall [IV]. Such compounds have been found during chemical chlorination at low pH of both resorcinol [Boyce & Hornig, 1983] and citric acid [Streicher *et al.*, 1986] (Figure 4).

4. Organic halogen in the macromolecular structures of SOM (TOX)

More than 20 years ago, organochlorine compounds in the environment were thought to be mainly of anthropogenic origin, and special equipment and procedures were developed, by which the total amount of organic chlorine could be determined, either as total organic halogen (TOX, used for soil and sediment samples) or as total adsorbable organic halogen (AOX – used for aqueous samples). The analysis is usually done by combustion of a sample after removal of inorganic halogen and subsequent determination of the formed halides by titration with silver ions. The method cannot distinguish between halides, but TOX and AOX is calculated as if all halogen atoms were chlorine, and the group parameter is taken as a measure of total pollution with halogen containing compounds in either solid or liquid samples. The method is still in use today, but ~20 years ago, it was shown that TOX and AOX were often not describing pollution in an environmental sample, but rather the natural background of halogen containing organic compounds. TOX and AOX were shown to be present in surface water, soil, peat and groundwater from around the world [Müller & Schmitz, 1985; Asplund *et al.*, 1989; Grøn, 1991; Asplund & Grimvall, 1991]. Most of the samples were from non-industrial areas, and some of the groundwater samples were several thousand years old and therefore had not been exposed to human pollution with organochlorines. AOX in surface water was found to be correlated with the colour and hence HS-concentration [Asplund & Grimvall, 1991], and halogens were found to be a natural element in both HAs and FAs (Table 6) [Asplund *et al.*, 1989; Grøn, 1991; Grøn & Raben-Lange, 1992]. Since the discovery of natural TOX in the terrestrial environment, a large number of studies have investigated the concentrations as well as processes leading to changes in concentrations of TOX, especially in forest soil. The reported concentrations are summarized in Table 6, and some general trends can be extracted:

- In soil, the fraction TOX/SOM increases with depth [Hjelm *et al.*, 1995; Öberg & Grøn, 1998; Öberg, 1998].
- Soil leachates have higher TOX/SOM fractions than the bulk SOM [Öberg & Grøn, 1998; Öberg & Sandén, 2005].
- The major Cl-pool in soil is organic, but in soil leachates, the major Cl-flux is that of chloride [Rødstedth *et al.*, 2003; Öberg & Sandén, 2005; Svensson *et al.*, 2007a].

Also, a number of trends on the processes leading to formation and degradation of TOX in soil have been found:

- The TOX/SOM fraction increases during the degradation of litter [Hjelm *et al.*, 1995; Öberg *et al.*, 1996b; Öberg *et al.*, 1997].
- Fresh plant material contains only small amounts of organic chlorine, but after partial degradation, the Cl is mainly organic [Myneni, 2002; Leri *et al.*, 2008].
- Low pH promotes the formation of TOX relative to SOM in soil [Öberg *et al.*, 1996a; Johansson *et al.*, 2003a].

- Nitrogen fertilizer decreases the formation of TOX during degradation of litter and/or increases mineralization of TOX [Öberg *et al.*, 1996; Johansson *et al.*, 2001].
- TOX is positively correlated with the chloride concentration in soil [Johansson *et al.*, 2001; Johansson *et al.*, 2003b; Öberg & Sandén, 2005].
- Anaerobic conditions hinder the formation of TOX [Thomsen, 2006; Bastviken *et al.*, 2009].

Table 6. Organic chlorine contents of various terrestrial compartments. Only studies, where the organochlorine was suggested to be natural and where the SOM or SOC contents of the soils were also given, are included. If only SOC was given, a SOM carbon-content of 50% (by weight) was used to set TOCl-data relative to SOM. In most studies only the group parameter TOX was measured, and concentrations were given as if all X was Cl. In Keppler *et al.*, 2000, Keppler & Biester (2003), Putschew *et al.*, 2003, Biester *et al.* (2004), **II** and **V** the halides were quantified individually. ? means data not available.

Compartment	TOCl (range)	Vegetation
Throughfall (mg (g DOM) ⁻¹)	1.1-5.0 ¹	Spruce
Fresh plant material (mg (g OM) ⁻¹)	0.003-0.1 ²	Various
Litter (mg (g SOM) ⁻¹)	0.05-0.4 ³	Spruce, Pine
Organic horizon, forest (mg (g SOM) ⁻¹)	0.1-1.2 ⁴	Spruce, Pine, Beech
Minerogenic horizon, forest (mg (g SOM) ⁻¹)	0.4-2.6 ⁵	Spruce, Pine, Beech
Minerogenic horizon, non-forest (mg (g SOM) ⁻¹)	0.3-1.4 ⁶	Various
Purified HS (mg (g SOM) ⁻¹)	0.2-2.0 ⁷	?
Forest soil leachate (mg (g DOM) ⁻¹)	0.3-18 ⁸	Spruce, pine
Groundwater (mg (g DOM) ⁻¹)	0.5-4 ⁹	Various
Peat (mg g ⁻¹)	0.2-2.0 ¹⁰	Sphagnum, cushion plants

¹[Öberg *et al.*, 1998], ²[Asplund, 1995; Öberg *et al.*, 1997; Flodin *et al.*, 1997b] ³[Hjelm *et al.*, 1995; Öberg & Grøn, 1998; **V**], ⁴[Asplund *et al.*, 1989; Asplund & Grimvall, 1991; Asplund *et al.*, 1994; Hjelm *et al.*, 1995; Hjelm *et al.*, 1999; Johansson *et al.*, 2001; **II**], ⁵[Asplund *et al.*, 1989; Asplund *et al.*, 1994; Hjelm *et al.*, 1995; Öberg *et al.*, 1996a; Öberg & Grøn, 1998], ⁶[Asplund *et al.*, 1989; Asplund & Grimvall, 1991; Asplund *et al.*, 1994; Keppler *et al.*, 2000] ⁷[Asplund *et al.*, 1989; Grøn, 1991; Dahlmann *et al.*, 1993; CN Albers *et al.*, unpublished data], ⁸[Öberg & Grøn, 1998; Rodstedth *et al.*, 2003; Öberg & Sandén, 2005], ⁹[Grøn, 1991], ¹⁰[Asplund *et al.*, 1989; Asplund & Grimvall, 1991; Keppler & Biester, 2003; Putschew *et al.*, 2003; Biester *et al.*, 2004]

4.1 Is all TOX chlorine?

Most of the data in Table 6 were measured as the group parameter TOX, with the moles but not the speciation of X given by the TOX-apparatus. TOX on a weight basis is then calculated as if all X was Cl. This has the following implications, if Br and/or I are present in significant quantities:

- The stated weight-based TOX is too low, since Br and I have higher atomic masses.
- TOCl is smaller than the stated TOX.

In three studies, other detectors than the colorimetric were used after combustion of soil samples, making it possible to quantify TOI, TOBr and in two cases also TOCl individually. Maw & Kempton (1982) did not determine TOCl, but found TOBr concentrations of 0.2-0.3 mg (g SOM)⁻¹ and TOI concentrations of 0.03-0.06 mg (g SOM)⁻¹. Keppler *et al.*, (2000) reported that in German grassland and forest soil, TOBr and TOI together constituted less than 10% of TOX, but in two Hawaiian forest soils,

TOBr and TOI concentrations were higher, constituting up to 18% and 41% of TOX, respectively. In **Paper II**, TOBr concentrations of $0.06\text{--}0.12\text{ mg (g SOM)}^{-1}$ and TOI concentrations of $0.01\text{--}0.03\text{ mg (g SOM)}^{-1}$ in organic soil from a coniferous forest were found. This constituted 15-19 % of the total TOX in those soils. In HA purified from the H-horizon in TH, TOCl was 0.89 mg g^{-1} , TOBr was 0.13 mg g^{-1} and TOI was 0.03 mg g^{-1} [Unpublished data].

For peat samples, differentiated TOX-concentrations have been reported also, with TOCl ranging from $0.3\text{--}1.5\text{ mg g}^{-1}$, TOBr ranging from $0.02\text{--}0.3\text{ mg g}^{-1}$ and TOI values ranging from $0.004\text{--}0.04\text{ mg g}^{-1}$ [Putschew *et al.*, 2003; Biester *et al.*, 2004].

All in all, the determination of TOX from unspecific titration with silver ions, seems to lead to only minor miscalculations of TOCl in most cases, but organic Br and I seems to always constitute a part of TOX, and element specific determination of TOX should certainly be preferred in future studies.

4.2 Formation of TOCl in soil

4.2.1 Identified chlorinated structural elements in HS and SOM

Total organic chlorine (TOCl) in a soil sample is a measure of all non-volatile organic chlorine present in that soil. A small fraction of TOCl (typically 0.5-3% in forest O-horizon and peat and <10% in minerogenic horizons) is extractable with water [Asplund *et al.*, 1994; Hjelm *et al.*, 1995; Silk *et al.*, 1997; Johansson *et al.*, 2001]. The water extractable fraction has a higher chlorination degree than bulk SOM but is correlated with DOC in the extract. It therefore probably consists of a combination of humic substances with TOCl-concentrations similar to or slightly higher than in the bulk SOM plus some smaller water soluble molecules like TCAA and trichloroacetyl containing carboxylic acids [IV] and possibly additional unknown chlorinated compounds. Another fraction of TOCl (0.05-15% in forest O-horizon) has been found to be extractable with organic solvents [Hjelm *et al.*, 1999]. This fraction has been found mainly to consist of chlorinated anisyl metabolites (CAMs) (Figure 14), which are metabolites of a large number of basidiomycetous fungi [De Jong *et al.*, 1994; Field *et al.*, 1995; Verhagen *et al.*, 1998; Hjelm *et al.*, 1999], where they probably take part in lignin degradation as well as function as chemical defence agents [Anke & Weber, 2006].

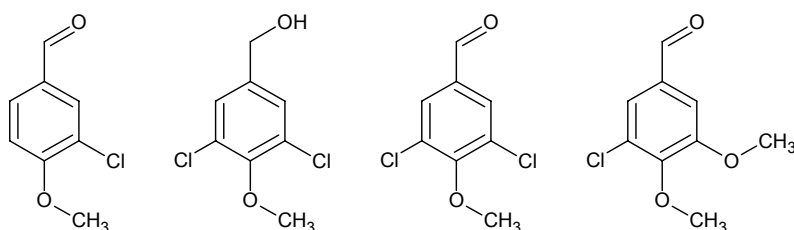


Figure 14. Examples of chlorinated anisyl metabolites (CAMs) found in rotting wood and forest soil [De Jong *et al.*, 1994; Hjelm *et al.*, 1996; Hjelm *et al.*, 1999].

The major part of TOCl is neither extractable with water nor organic solvents, however. It is expected to be a part of the bulk SOM, partly in HS, which are known to contain chlorine (Table 6) and partly in

other macromolecular parts, e.g. as chlorinated lignin structures. The base-leachable fraction of SOM, which mainly consists of HS, has been found to contain ~20% of total TOCl (determined as TOX) as well as ~20% of SOM [Hjelm & Asplund *et al.*, 1995]. Within the base-leachable fraction up to 50% of TOCl was found in a fraction with estimated molecular weight >10 kDa, and only ~10% in the fraction with apparent molecular weight <1 kDa [Hjelm & Asplund, 1995].

Myneni (2002) used x-ray absorption spectroscopy to show that organic chlorine in HA and FA isolated from soil, mainly was bound to aromatic structures, while aliphatic chlorine constituted less than 30% of TOCl. Using the same analytical methods, it has also been shown, that TOCl in weathering leaves shifts from mainly aliphatic to mainly aromatic during degradation. This was found both in natural samples and during laboratory experiments with an ascomycetous fungus as the degrading organism [Myneni 2002; Leri *et al.* 2007]. The aromatic chlorine seems mainly to be part of phenolic structures [Leri *et al.*, 2007].

Oxidative degradation of HS and bulk SOM using KMnO_4 and H_2O_2 [Dahlmann *et al.*, 1993; Dahlmann *et al.*, 1994; Johansson *et al.*, 1995; Flodin *et al.*, 1997b] as well as pyrolysis-GC/AED and pyrolysis-GC/MS [Flodin *et al.*, 1997a] have been used in order to identify structural elements in TOCl (Figure 15). In all cases, identified structures could account for only a few percent of the total TOCl, and the identified structural elements are therefore either not the dominating structures of TOCl or alternatively the applied methods have not been very quantitative. While evidence for the quantitiveness of the applied chemical degradation method lacks, there is good evidence that the identified chlorinated products were not formed during the degradation procedure and hence are true structural elements of TOCl [Dahlmann *et al.*, 1993; Flodin *et al.*, 1997b].

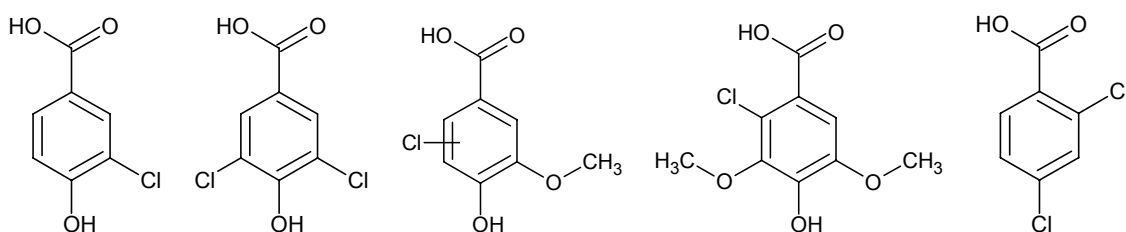


Figure 15. Some of the aromatic chlorinated structures identified after oxidative degradation of HS and bulk SOM [Dahlmann *et al.*, 1993; Dahlmann *et al.*, 1994; Hjelm & Asplund, 1995; Johansson *et al.*, 1995; Niedan *et al.*, 2000]. Phenols and carboxylic acids were identified as their ethyl- and methyl esters respectively, because of the necessity to protect these groups during the oxidative degradation. Pyrolysis of FA revealed similar structural elements [Flodin *et al.*, 1997a], and so did oxidative degradation of wood colonized by the Va-CPO producing fungus *Curvularia inaequalis* [Ortiz-Bermúdez *et al.*, 2007].

As part of the work leading to this thesis, the trichloroacetyl structural element ($\text{CCl}_3\text{-C=O}$) was detected in forest SOM (further discussed in other sections). This is the first aliphatic chlorinated structural element, which has been identified in macromolecular SOM.

4.2.2 Main hypotheses concerning the formation of TOCl

There are two hypotheses concerning the formation of macromolecular organohalogens in soil. The main hypothesis is that TOCl is formed during an unspecific chlorination of SOM with HOCl derived either from extracellular chloroperoxidases [Asplund *et al.*, 1993; Johansson *et al.*, 2003b], from abiotic reactions involving H₂O₂ and iron [Fahimi *et al.*, 2003] or from a combination of biotic and abiotic processes [Bastviken *et al.*, 2009]. The alternative hypothesis suggests that macromolecular TOCl is formed by the incorporation of chlorinated metabolites excreted by microorganisms, e.g. CAMs from basidiomycetous fungi [Field *et al.*, 1995; Hjelm, 1996; Hjelm *et al.*, 1996].

The unspecific chlorination of paper pulp leads to some of the same structural elements found in the oxidative degradation and pyrolysis of HS and SOM [Dahlmann *et al.*, 1993; Dahlmann *et al.*, 1994; Flodin *et al.*, 1997a]. Furthermore, Ortiz-Bermúdez *et al.* (2007) showed the formation of similar structural elements in lignin, isolated from partly degraded aspen and spruce wood, inoculated with the Va-CPO producing ascomycete *Curvularia inaequalis*. These are strong indications that part of the formation of TOCl in forest soil could take place as an unspecific chlorination of lignin or lignin-derived structures in SOM. The trichloroacetyl structural element (CCl₃-C=O), reported in this thesis as a structural element in SOM, gives CHCl₃ upon treatment with alkali and is a well known disinfection by-product in the chemical chlorination of dissolved HS during drinking water treatment [De Leer *et al.*, 1985; Reckhow *et al.*, 1990]. This is a further proof, that at least part of the TOCl in forest soil is formed during unspecific chlorination.

Looking at Figures 14 and 15, there is, however, a striking similarity between some of the CAM metabolites and the structural elements identified during oxidative degradation of HS and SOM. If the CAMs were demethylated to form phenols and then incorporated into SOM, the structures in Figure 15 could actually be derived from CAMs. The demethylation of the methoxy group in CAMs by anaerobic bacteria have been demonstrated [Milliken *et al.*, 2004], and it has been suggested that such demethylation reactions would occur readily in the environment under various conditions [De Jong *et al.*, 1994]. If the CAMs are demethylated, the formed chlorophenols will possibly be bound covalently to humic structures by mediation of commonly occurring soil enzymes like laccase and peroxidases. This reaction has been demonstrated in model studies using various other chlorophenols [Hatcher *et al.*, 1993; Bollag & Dec, 1995].

In conclusion it seems highly likely that at least part of the TOCl is formed by unspecific chlorination reactions in the soil and furthermore that at least a part of this unspecific chlorination is caused by biotic activity. The possibility that incorporation of fungal metabolites into HS and other macromolecular parts of SOM plays an important role can however not be excluded, and one possible hypothesis could be that two or several modes of formation co-exist in the soil. This was also previously suggested [Öberg *et al.*, 1997].

4.3 Trichloromethyl compounds and TOCl

CHCl₃ and TCAA have been found in presumably natural concentrations up to 146 (Figure 11a) and 1.3 [Hoekstra *et al.*, 1993] $\mu\text{g kg}^{-1}$, respectively in the organic top soils of coniferous forests, but in general, the concentration of especially CHCl₃ is much lower in soil. In comparison, concentrations of organic chlorine (TOCl) in coniferous forest top soil have been reported to vary from 0.1-1 g (kg SOM)⁻¹ [Hjelm *et al.*, 1995; Öberg & Grøn, 1998; II]. Even though no measurements of both CHCl₃ or TCAA and TOCl in a single soil sample have been reported, this points to the fact that single compounds like CHCl₃ and TCAA usually constitute much less than 1‰ of the total pool of organic chlorine in such soils. The concentration of soil bound trichloroacetyl-CHCl₃ in the organic horizon of forest soil is much higher than that of CHCl₃ and TCAA, usually between 0.1-1 mg (kg SOM)⁻¹, and in one extreme case (the same sample containing 146 $\mu\text{g kg}^{-1}$ CHCl₃) 8 mg (kg SOM)⁻¹ [IV, V]. On a weight basis, this structural element generally still constitutes only ~1‰ of the total organic chlorine in macromolecular structures of SOM, however [V].

In deeper horizons of forest soil, CHCl₃ might constitute a greater part of the total pool of organic chlorine than in top soil. In **Paper V**, soil samples from 30 cm depth in TH were reported to contain water extractable CHCl₃ up to a concentration of ~15 $\mu\text{g (kg soil)}^{-1}$ or ~1 mg (kg SOM)⁻¹ and water extractable trichloroacetyl-CHCl₃ up to a concentration of ~68 $\mu\text{g (kg soil)}^{-1}$ or ~5 mg (kg SOM)⁻¹. Since [TOCl] in minerogenic forest soil is typically ~1 g (kg SOM)⁻¹ (Table 6), CHCl₃ might be expected to constitute close to 1‰ of TOCl in that sample and water extractable trichloroacetyl-CHCl₃ an additional ~5‰. Since TCAA was absent or present in very low concentrations in soil samples taken from 30 cm depth in TH and since soil bound trichloroacetyl-CHCl₃ is expected to constitute ~1‰ of TOCl [V], the trichloromethyl structural element seems to constitute less than 1% of TOCl, even in minerogenic horizons. Despite the uncertainties in the above calculations, there is no doubt that CHCl₃ and related compounds constitute a minor portion of the total pool of organic chlorine in forest soil.

A weak, but statistically significant, positive relationship was found between TOCl and soil bound trichloroacetyl-CHCl₃ in forest soil [V]. If CHCl₃, TCAA and trichloroacetyl containing compounds are formed in an unspecific chlorination reaction, the formation of these compounds should also lead to an increased chlorination degree of SOM in general. If unspecific chlorination is only one of two or more co-occurring pathways of formation of TOCl, as proposed above, this might explain the weakness of the relationship between trichloromethyl compounds and TOCl or in other words; the formation of trichloromethyl containing compounds will increase TOCl concentration, but the formation of TOCl will, depending on formation pathway, not necessarily lead to the formation of trichloromethyl compounds.

The major pool of organic Cl in soil seems to be HS and other macromolecular structures, of which the majority of chlorine-containing structural elements are currently unknown. In the context of biogeochemical cycling of Cl in the terrestrial environment, CHCl₃ might however be an important compound because of its tendency to quickly escape the area of formation either by evaporation to the atmosphere or leaching to deeper soil layers. The turnover time of macromolecular chlorine is

probably much longer. The pool of macromolecular organochlorine in top soil of a coniferous forest in Sweden was recently estimated to 13 g Cl m^{-2} , while the leaching from top soil and net mineralization of macromolecular chlorine in the same area each were estimated to be $\sim 0.2 \text{ g Cl m}^{-2} \text{ y}^{-1}$ [Öberg *et al.*, 2005]. The study area in TH has been estimated to emit CHCl_3 to the atmosphere in a range of $0.05 \text{ } \mu\text{g m}^{-2} \text{ h}^{-1}$ during the winter time to $0.6 \text{ } \mu\text{g m}^{-2} \text{ h}^{-1}$ during the summer time [II]. Taking the average of these two values as the estimated yearly average, this gives an average CHCl_3 emission to the atmosphere of $\sim 0.003 \text{ g m}^{-2} \text{ y}^{-1}$ in the total study area of TH. Looking at CHCl_3 Hot Spots only, similar calculations based on data from [II] gives a CHCl_3 emission to the atmosphere of $\sim 0.010 \text{ g m}^{-2} \text{ y}^{-1}$. In addition, $\sim 0.001 \text{ g CHCl}_3 \text{ m}^{-2} \text{ y}^{-1}$ was reported to leach to the groundwater from a CHCl_3 Hot Spot in TH [III], giving a total flux of CHCl_3 from the soil in TH Hot Spots of $\sim 0.01 \text{ g Cl m}^{-2} \text{ y}^{-1}$ or $\sim 5\%$ of the $0.2 \text{ g Cl m}^{-2} \text{ y}^{-1}$ estimated for the flux (leaching from top soil) of TOCl [Öberg *et al.*, 2005]. In comparison, Dimmer *et al.* (2001), reported emissions of CHCl_3 from two coniferous forests in Ireland in September corresponding to 0.002 and $0.15 \text{ g m}^{-2} \text{ y}^{-1}$, respectively. The reported values of CHCl_3 fluxes from forest soil thus suggest, that even though the pool of CHCl_3 in top soil is very low compared to TOCl ($\ll 1\%$), the flux of CHCl_3 and hence its influence on the biogeochemical cycling of chlorine is most likely considerable.

5. On the possible use of ¹³C stable isotope analysis in natural organohalogen research

Carbon is a mixture of two stable isotopes, ¹²C (~99%) and ¹³C (~1%). The ratio of ¹³C/¹²C (R) varies between different organic and inorganic compounds, and the isotope ratios are typically set relative to the international belemnite standard (VPDB) and expressed as in eq. 4:

$$\delta^{13}\text{C} (\text{‰}) = (R_{\text{sample}} / R_{\text{standard}} - 1) \times 1000 \quad (4)$$

Atmospheric CO₂, the origin of most terrestrial organic carbon, has a δ¹³C-value of ~ -8‰, and plant material is typically showing some depletion of ¹³C relative to this value, depending on the mode of photosynthesis (Figure 16).

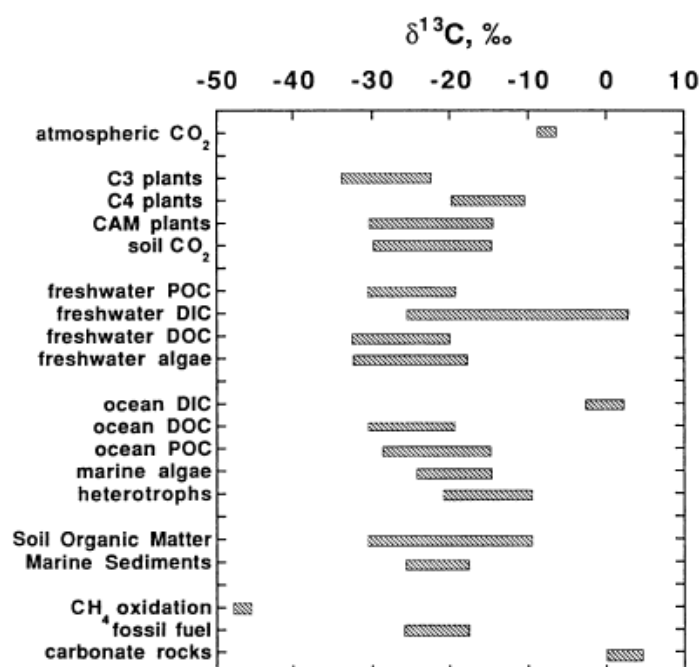


Figure 16. Typical average δ¹³C-values in various major organic and inorganic carbon pools and compounds. Taken from [Trumbore & Druffel, 2002].

Industrial CHCl₃ is produced through the chlorination of methane from natural gas. Methane is particularly depleted in ¹³C (Figure 16), and so is CHCl₃ formed from methane. Various compound specific isotope analyses of industrial CHCl₃ have revealed δ¹³C-values from -43 to -63‰ [Holt *et al.*, 1997; Jendrzewski *et al.*, 2001; Laier *et al.*, 2005]. Natural CHCl₃ is expected to be formed from the aromatic and possibly also aliphatic part of the bulk SOM and HS and could therefore be expected to show a somewhat heavier isotope signature. Laier *et al.* (2005) found δ¹³C-values from -13 to -27‰ for presumably natural CHCl₃ in groundwater beneath coniferous forests and similar values were confirmed in the four study sites of this thesis (Table 7).

Table 7. $\delta^{13}\text{C}$ for natural CHCl_3 in the upper groundwater abstracted from four coniferous forests. [Unpublished data].

Location	TH	VH	LP	NF
$\delta^{13}\text{C}$ (‰)	-20.1	-19.0	-16.7	-29.7

These values were supplemented with various analyses of CHCl_3 in forest soil ($\delta^{13}\text{C}$ -values from -24 to -30‰) and CHCl_3 emitted from forest soil to the atmosphere (-24 to -27‰) [Albers, C.N. & Breider, F., unpublished data].

Natural trichloroacetyl- CHCl_3 apparently shows a somewhat heavier isotope signature (Table 8).

Table 8. $\delta^{13}\text{C}$ -values for natural trichloroacetyl- CHCl_3 . [Unpublished data].

Forest	Soil horizon	$\delta^{13}\text{C}$ (‰)
TH	F+H	-9.6
TH	F+H	-10.1
NF	H	-10.2
LP	Groundwater	-13.2

An experiment was set up, in order to investigate the $\delta^{13}\text{C}$ -values for the trichloromethyl (CCl_3) structural element of both CHCl_3 , trichloroacetyl containing compounds and TCAA, formed upon chemical chlorination of forest SOM:

A number of different samples were chlorinated (different horizons from the NF site, F+H-layer sample from the TH site, a purified HA from the TH site and finally a commercial soil HA). The samples were chlorinated with small amounts of HOCl at pH=4 (phosphate buffer). Triplicates of each sample type were then analyzed as they were ($\delta^{13}\text{C}$ of CHCl_3 formed directly upon chlorination). Another set of triplicates were purged with N_2 to remove CHCl_3 and afterwards added NaOH to hydrolyse trichloroacetyl containing compounds ($\delta^{13}\text{C}$ of the CCl_3 -group in trichloroacetyl containing compounds). Finally a third set of triplicates were added NaOH, purged with N_2 to remove both CHCl_3 and trichloroacetyl- CHCl_3 and then heated (after adjustment of pH to below neutral) to convert TCAA into CHCl_3 . The results are summarized in Figure 17.

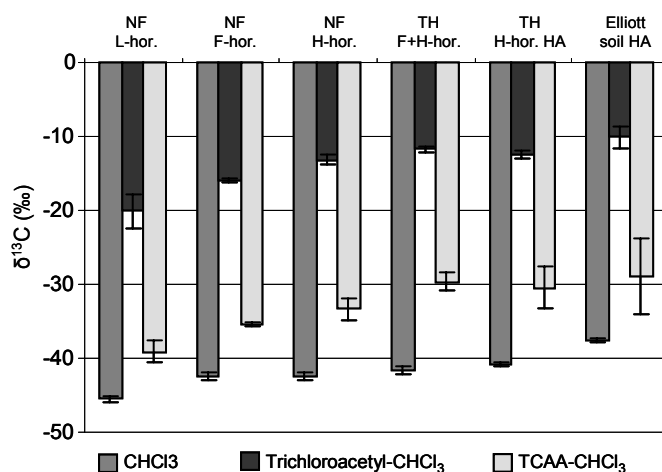


Figure 17. $\delta^{13}\text{C}$ -values of CHCl_3 formed directly upon chlorination or by conversion of either trichloroacetyl containing compounds or TCAA, also formed during the chlorination. Error bars are standard deviation of three replicates. Chlorination conditions and most of the SOM-fractions were similar to those in Figure 5. [Unpublished data].

There are several interesting features in this experiment. First of all it is interesting that the CHCl_3 formed directly upon the chlorination shows a $\delta^{13}\text{C}$ between -45 and -38‰. These values are far from the values found in natural CHCl_3 . The data have a trend with the $\delta^{13}\text{C}$ showing the lightest signature in the L-horizon and a somewhat heavier in the F- and H-horizons, probably caused by the general enrichment in ^{13}C during degradation of SOM [Gleixner *et al.*, 2002]. The CHCl_3 derived from TCAA shows similar tendencies as CHCl_3 but with 5-10‰ heavier $\delta^{13}\text{C}$ signature. For CHCl_3 liberated from trichloroacetyl containing compounds, similar trends between samples and depths were seen as for the other compounds, but the $\delta^{13}\text{C}$ -values were much higher.

Humic substances from different environmental compartments have $\delta^{13}\text{C}$ -values between -22 and -28‰ [Gleixner *et al.*, 2002]. The Elliott HA, is a commercial HA with a $\delta^{13}\text{C}$ -value of -22.6‰ [IHSS, 2010]. This relatively ^{13}C -enriched HA produced in all cases the heaviest trichloromethyl compounds. If the $\delta^{13}\text{C}$ -values of the parent material on average were around -25‰, the experiment shows that CHCl_3 and TCAA formed upon chlorination is depleted in ^{13}C and trichloroacetyl- CHCl_3 is enriched in ^{13}C . This could then either be due to different discriminations of carbon isotopes in the reactions leading to the formation of the compounds or due to different precursors with very different $\delta^{13}\text{C}$ -values within the individual SOM or HA fractions. The latter seems somewhat unlikely, but it is not possible to conclude finally from this single experiment.

These results leave four main options for the interpretation of the $\delta^{13}\text{C}$ -values (-13 to -30 ‰) found for natural CHCl_3 in relation to what is found in the laboratory experiment:

1. The CHCl_3 collected in various soils and groundwater is formed in a reaction very different from the chemical chlorination with HOCl , leading to a very different isotope signature.

2. The CHCl₃ is partly degraded after formation in the soil, leading to a heavier isotope signature, as is well known for various degradation mechanisms [Meckenstock *et al.*, 2004; Elsner *et al.*, 2005].
3. The CHCl₃ collected in various soils and groundwater is a mixture of CHCl₃ formed directly upon chlorination and CHCl₃ formed by hydrolysis of trichloroacetyl containing compounds.
4. Mainly trichloroacetyl containing compounds are formed at first, and later in the reaction, a fraction of these are liberating CHCl₃. This reaction must then be discriminating with regard to carbon isotopes, liberating ¹³C-depleted CHCl₃ and leaving the remaining trichloroacetyl containing compounds enriched in ¹³C.

The latter two options would also explain the wide range of $\delta^{13}\text{C}$ -values, which have been found for natural CHCl₃, since either the ratio between CHCl₃ formed directly and CHCl₃ formed from trichloroacetyl containing compounds or the fraction of trichloroacetyl containing compounds liberating CHCl₃, would not be constant.

The above experiments are early and non-conclusive experiments, but they show that analyses of $\delta^{13}\text{C}$ -values in CHCl₃ and related compounds may not only help to determine if their origin is natural, but may also help to reveal the mechanisms leading to their natural formation.

Concluding remarks and future directions

Based on the Introduction as well as the enclosed manuscripts

From the literature survey it is clear, although this is still not common knowledge among all environmental scientists and other professionals, that substantial evidence exists for the widespread formation and occurrence of natural halogenated organic compounds throughout the terrestrial environment. Microbial as well as abiotic processes occurring in soil may lead to a variety of iodinated, brominated and especially chlorinated compounds, which may be found in a wide range of concentrations. The main focus of my thesis has been the unspecific chlorination that takes place in especially forest soil. Unspecific chlorination may lead to a number of chlorinated compounds, with different behaviour and fate in the environment. Before the work leading to the present thesis, CHCl_3 was rather well established as a natural compound, but data on almost all aspects concerning formation and fate were scarce and at the same time inconsistent, e.g. regarding environmental concentrations and fluxes. With the great spatial variation in chloroform concentration and possibly production, shown in **Paper II**, it is now easier to interpret the very different results obtained in different papers, although it also makes the various calculations on global concentrations and emissions presented in these plus various review papers even more uncertain. In combination with the results on leaching of CHCl_3 presented in **Paper I** and **III**, it is hopefully also clearer now, that groundwater may be naturally “polluted” with CHCl_3 although the natural background concentrations may vary markedly due to the spatial variation in production as well as a number of other possible factors, especially degradation by microbes.

Steps toward a better understanding of the mechanism behind the formation of CHCl_3 , was also taken with the discovery of trichloroacetyl containing compounds in **Paper IV** and its relationship with CHCl_3 and possibly TCAA in **Paper V**. This discovery was a further proof that CHCl_3 and TCAA are formed during unspecific halogenation reactions in soil, and the various sorption and degradation experiments performed with these two compounds provide evidence why they may not share environmental fate, despite a common origin in the soil. The concentration of trichloroacetyl containing compounds in the topsoil was found to be huge compared to both CHCl_3 and TCAA, but still to be a minor fraction of total organic chlorine in the soil.

A number of research questions remain to be solved in future studies and new ones have emerged as well, on the basis of this thesis. The more important questions among these include:

What are the halogenating enzymes and/or other biotic as well as abiotic factors controlling unspecific halogenation in soil?

Since most research has been done in the forest environment, what about fields, grassland, tundra etc.? Do these areas contain the same chlorination mechanisms and compounds?

What is the full molecular structure of water extractable trichloroacetyl containing compounds and are some of these of toxicological concern, e.g. in areas where they occur in groundwater abstracted for drinking water use?

What is the fate of trichloroacetyl containing compounds in soil – especially the great pool in the top soil? What is e.g. the risk of this massive CCl_3 pool to be liberated as CHCl_3 , upon a forest clearfelling and the subsequent rise in pH value?

How can we distinguish between pollution and natural background concentrations of halogenated (and other) compounds? Is the use of stable isotopes the solution, at least for some compounds like e.g. CHCl_3 ?

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Papers I-V

I

Vertical and horizontal variation in natural chloroform in two adjacent soil profiles in a coniferous forest

Christian N. Albers, Troels Laier, Ole S. Jacobsen
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Christian N. Albers • Troels Laier • Ole S. Jacobsen

Christian Nyrop Albers has contributed to the project as described below:

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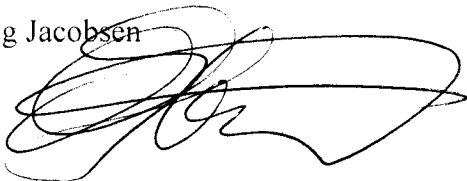
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| c) Implementation of the project / empirical work | 2 |
| d) Analysis, interpretation and discussions | 2 |

Date: 23-02-2010


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Vertical and horizontal variation in natural chloroform in two adjacent soil profiles in a coniferous forest

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Abstract

Naturally produced chloroform is occasionally detected in soil and groundwater of non-urban areas in concentrations that can exceed the regulatory levels. In this study, we present the distribution of naturally produced chloroform in a temperate coniferous forest from top soil to upper groundwater. Chloroform is most likely produced by the activity of fungi, and the production varies therefore with the domains of certain unidentified fungi. We show that the differences in concentration within 10 metres can approach two orders of magnitude in the top soil. The difference in chloroform concentrations in upper groundwater within the same distance is almost one order of magnitude. The concentration of chloroform in the top soil varies significantly with season, while season affect deeper levels much less.

Keywords: chloroform, fungi, groundwater, unsaturated zone, natural organohalogens.

1 Introduction

Halogenated organic compounds in the environment are usually viewed as a result of human activity. Even though this is often true, more than 3800 naturally produced halogenated organic compounds have been identified so far [1]. In non-urban environments, chloroform (CHCl_3) is occasionally detected in the groundwater where no obvious anthropogenic source is present [2]. Furthermore, global estimations of chloroform production have shown that less than 10% is of anthropogenic origin whereas marine and terrestrial production must make up more than 85% [3,4].



In Denmark, the concentration of chloroform was not allowed to exceed 1 µg/L in groundwater abstracted for drinking water. However, as a result of recent investigations on natural chloroform [5], this value has now been extended to 10 µg/L, provided a natural source is likely to exist in the recharge area and the waterworks can verify, that the chloroform has a natural origin. Industrial and natural chloroform may be distinguished using stable carbon isotopic analysis with industrial chloroform having a $\delta^{13}\text{C}$ range from -46 to -63 ‰ and naturally produced chloroform having $\delta^{13}\text{C}$ from -13 to -27‰ [6].

One laboratory study has shown a net production of chloroform in the organic horizon of a temperate spruce forest soil [7]. This production was most likely the result of microbial activity. Several studies have shown that the soil air of coniferous forests in general have chloroform concentrations significantly elevated to the background atmospheric concentration of ~20 pptv [8]. The concentrations range from 2–10 times atmospheric concentration in a temperate coniferous forest on clayey soil [9] to more than 300 times the atmospheric background in a temperate Douglas fir forest [10]. Based on the limited number of studies, forest soils with a well developed organic top layer seems to be the prerequisite for high chloroform net production and emission compared to grasslands and forests devoid of such organic horizon, as was also concluded by Hoekstra *et al.* [11].

Various studies indicate that fungi might be responsible for the production of chloroform in forest soils. Haselmann *et al.* [7] found that both air drying and sterilization of the soil diminished the net production of chloroform, Hoekstra *et al.* [10] suggested that fungal enzymes like chloroperoxidases could be involved in chloroform production and three fungal strains were shown to produce chloroform when grown on sterilized forest O-horizon [12].

Naturally produced chloroform has been detected in soil air to a depth of 7.5 m below the surface [13]. Furthermore, chloroform was detected in nearby shallow groundwater wells. However, the concentrations of chloroform in groundwater, up to 1.6 µg/L, were much higher than would have been anticipated from the measured soil air concentrations using Henry's Law. The authors could not explain this paradox. In the present paper we show that concentration of chloroform within an area may be so variable that narrow sampling of both soil gas and groundwater is necessary to be able to establish a relation between chloroform concentrations in gas phase and water phase. This is even the case for areas with the same type of vegetation. We also found that although high natural chloroform concentrations are mostly found in coniferous forests, it is not straightforward to judge the potential chloroform production from vegetation type and general soil parameters alone.

2 Materials and methods

2.1 Location

The study area is a small part of a forest, far from industrial activity, at Tisvilde Hegn, Northern Sjælland, Denmark. The area is close to a monitoring well showing high chloroform content in the shallow groundwater. Regular analyses by the Danish National Groundwater Quality Monitoring Program during the last



17 years have shown chloroform concentrations up to $6 \mu\text{g/L}$ [14]. The subsurface of the area consists of diluvial sand covered by aeolian sand in the top 0.5 m. The area was forested in the 19th century to prevent further soil erosion. This forestation of a soil very low in nutrients has resulted in a well-developed O-horizon, 5-20 cm thick. The vegetation is mainly old Scots Pine (*Pinus sylvestris*) and young Common Spruce (*Picea abies*). Vegetation below the trees of the dense forest is sparse grass and moss.

2.2 Sampling

Preliminary investigation revealed high spatial heterogeneity in chloroform concentration and two permanent profiles for monitoring chloroform in soil air and in the top groundwater were established: one profile (P1) was established where chloroform concentration in the top soil air was high, and the other profile (P2) was established 10 metres away, where chloroform concentration in the top soil air was ~ 100 times lower than in P1. Each profile was established by drilling by hand (diameter ~ 10 cm) to 6 m depth. Eight 5 cm brass filters were then placed from the bottom and up at specific intervals, with two filters below the groundwater level (~ 4.5 m below surface) and six filters in the unsaturated zone (Figure 1). Each filter was connected to a 6 mm nylon tube that was later used for sampling. The original sand was refilled and between each filter, a layer of bentonite was placed to avoid shortcut between filters.

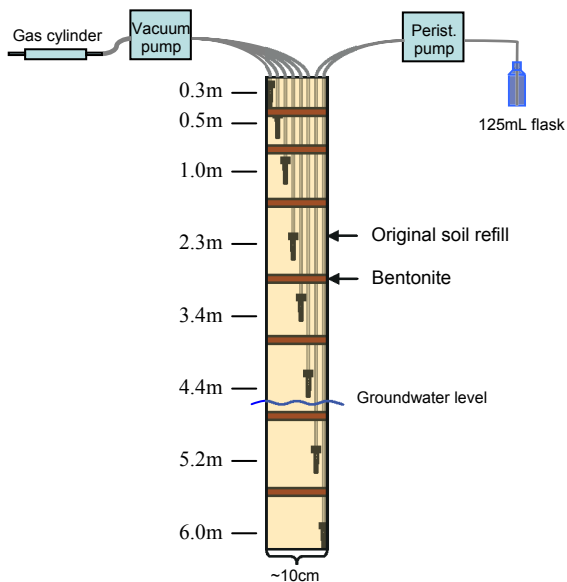


Figure 1: Sketch of the installation placed in each of the profiles 1 and 2 and the sampling of soil air from the top six filters and groundwater from the lowest two filters.

Soil samples were taken from P1 at eight intervals down to 480 cm. pH was determined in a 1:1 water/soil slurry. Water content was determined by drying for 24h at 105°C. The content of soil organic matter (SOM) was determined as loss on ignition (2h, 550°C). Soil texture was determined by sieving with weighing of the fractions >2mm (gravel), 0.6-2mm (coarse sand), 0.2-0.6mm (medium sand), 0.063-0.2mm (fine sand) and <0.063mm (silt and clay).

Groundwater was drawn from the filters below the groundwater table in both profiles with a peristaltic pump. A minimum of 1 L was pumped to waste before filling 125 mL glass flasks and closing with screw caps containing an aluminium liner. Gas bubbles in the flasks were avoided by the sampling procedure. Soil air was sampled from the top six filters by vacuum pumping 3-6 L air through a steel cylinder that was then closed in one end and pressurized to ~1.5 bar.

2.3 Chemical analyses

Chloroform was analyzed on a gas chromatograph equipped with an ECD detector. The analytical procedure was similar to that described by Busenberg and Plummer [15] for chlorofluorocarbons (CFC) in age-dating of young groundwater. Chloroform and other halocarbons were trapped on a pre-column at -30°C, which was then heated to 95°C. Separation of gas constituents was done on a 1.7 m packed column, Poracil-C, at 70°C, pre-column back-flush technique was used to complete the analysis of each sample within 11 min. For gas samples normally 15 mL was used for analysis, and for water samples 30 mL was used. Detection limit for chloroform in gas samples was 10 pptv and for water samples 0.0002 µg/L.

By using Henry's law, which is essentially the equilibrium partitioning between gas and water phase for a given gaseous substance based on its partial pressure at a certain temperature, one can calculate the concentration in water from the measured concentration in air, assuming equilibrium. Calculation of equilibrium concentrations in soil water was performed using the Henry's law constants determined in [16]. Temperature has a marked influence on the partitioning coefficient, and for chloroform, the equilibrium concentration in water is twice as high at 6°C as at 18°C for the same concentration in the gas phase [16]. The temperature in the unsaturated zone will differ during the different seasons, especially in the upper part of the unsaturated zone and based on temperature measurements at 0.2 and 5 m depth we did our calculations with a temperature range from 16–12°C (from highest to deepest filter) in July and a range of 3–8°C (from highest to deepest filter) in February.

3 Results

Stable carbon isotope analysis of chloroform in groundwater from the monitoring well mentioned above indicated that chloroform was most likely of a natural origin (results not shown), and a nearby area upstream this well was chosen for further investigation. Preliminary investigation of soil air chloroform concentration at 30 cm depth in a 100×140 m grid gave the impression of large



differences in chloroform concentration at locations close to each other and with no visible differences above ground. Three small areas of 20-40 m² had particularly high chloroform concentrations and two permanent sampling profiles (Figure 1), 10 m apart, were established within and outside one such area, where chloroform concentrations in soil air differed significantly; ~27000 pptv at P1 and ~250 pptv at P2.

3.1 Soil data

The subsurface lithology of the two locations P1 and P2 appeared to be similar from visual inspection of samples collected during fieldwork. Samples from P1 were chosen for further examination of the lithology of the unsaturated zone and uppermost aquifer (Table 1). The top 10 cm of the profile was mainly organic (O-horizon) with three visible organic layers: At the top was a ~3 cm thick litter layer (L-layer) consisting of slightly degraded plant material. Below the L-layer, a fermentation layer (F-layer) of ~4 cm thickness was recognizable. This layer consisted of partly degraded needles and branches with visible fungal hyphae. Below the F-layer a humic layer (H-layer) of ~3 cm thickness was clearly visible. In this layer, the original plant structure was hardly recognizable. Below the O-horizon, greyish sand appeared, indicating a partial washout of iron hydroxides and organic material. However, no Bh horizon was found below. No dark A-horizon existed and yellowish fine to medium sand dominated throughout the profile (Table 1). The clay and silt content (<63µm) was below 10% at all depths but showed some variation being low especially in the old aeolian sand just below the organic layer and in the very homogeneous sandy layer from 2-4 m depth.

Table 1: Soil data from P1. pH was determined in a 1:1 water/soil slurry. SOM was determined as loss on ignition at 550°C. The textural data were determined by sieving.

Depth (cm)	pH	H ₂ O (%)	SOM (%)	>2mm (%)	0.6-2mm	0.2-0.6mm	0.063-0.2mm	<63µm (%)
0-10	4.1	64.6	74	-	-	-	-	-
10-30	3.9	7.7	2.0	3.6	1.4	66	26	2.9
30-50	4.2	7.6	1.8	1.4	3.4	65	24	6.5
50-100	4.4	8.7	1.8	14	7.0	31	39	8.9
100-180	5.2	7.2	0.57	13	6.8	45	26	9.6
180-300	6.3	4.4	0.32	0.5	11.8	72	13	2.2
300-400	7.3	3.4	0.24	0.4	6.0	79	13	1.3
400-480	7.6	15.5	0.38	0.4	2.5	38	52	7.7

The profile showed a gradual increase in soil pH with depth from ~4 at the top to almost 8 at 4.5 m depth. Analysis of groundwater showed a pH-value of around 7.8. Soil sample appearance indicated that aerobic conditions prevailed all through the unsaturated zone and uppermost aquifer. Groundwater from filters below the water table contained ~10 mg/L O₂.



3.2 Chloroform in soil air

The samples taken from the two profiles confirmed the initial findings of a very large difference in chloroform concentrations in the top soil of P1 (~100000 pptv) and P2 (~2500 pptv), Figure 2(a). In July, the concentration profiles approach one another with depth (Figure 2(a)), but a difference of approximately one order of magnitude is still seen just above the groundwater at 4.4 m depth.

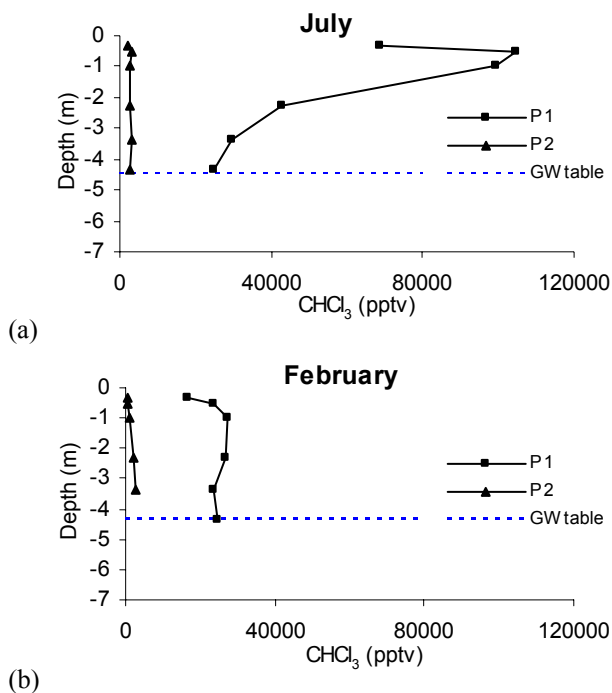


Figure 2: Chloroform in soil air at the two profiles during (a) summer and (b) winter.

Chloroform in the soil air was determined in both summer and winter time and showed large differences between these two seasons as expected for a biologically derived process (Figure 2). This difference was especially clear in the top three filters (0.3–1 m depth) with a ~4 times decrease from July to February in both P1 and P2. In the lower two filters for air sampling (3.4 and 4.4 m), the chloroform concentration was approximately the same in July and February. During winter, rising groundwater made sampling of air from the lowest filter in P2 impossible.

3.3 Chloroform in groundwater

Groundwater was sampled from the two filters below the groundwater table. Dates for sampling of soil air and groundwater were identical. The change in

chloroform concentration in groundwater from July to February was small for all four filters, Figure 3. Furthermore, chloroform concentrations were almost identical ($\sim 0.3 \mu\text{g/L}$) 1.5 m below the groundwater table at both profiles. However, 0.7 m below the groundwater table the chloroform concentrations differed by almost one order of magnitude. Thus, the difference in chloroform concentration between P1 and P2 just above and below the groundwater table appears to be of the same order of magnitude, but the difference has disappeared moving an additional 80 cm down.

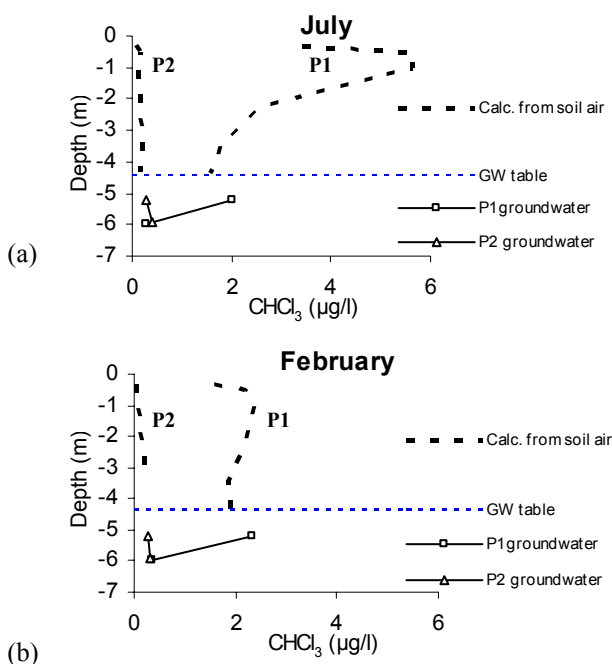


Figure 3: Chloroform in groundwater (measured) and in unsaturated soil water (calculated from Henry's law assuming equilibrium between air and water) during (a) summer and (b) winter.

Even though the exact concentration in the soil water is hard to calculate we wanted to get an impression of whether there was any connection between the concentrations we measured in the unsaturated zone with what we measured in the groundwater. We therefore calculated the corresponding concentration of chloroform in the soil water from the measured concentrations in the soil air (Figure 3) as described in section 2.3. The calculated soil water concentrations in P1 in July range from almost $6 \mu\text{g/L}$ in 0.5-1 m depth to $\sim 1.6 \mu\text{g/L}$ just above the groundwater table. This fits well with the measured concentration of $2.0 \mu\text{g/L}$ in the groundwater at 5.20 m depth. In February, the calculated concentrations were $\sim 1.9 \mu\text{g/L}$ for the lowest air filter, and this still fits well with the measured chloroform concentration at 5.20 m depth of $2.3 \mu\text{g/L}$. In the upper part of P2,

the calculated concentration of chloroform in the soil water varies from $\sim 0.2 \mu\text{g/L}$ in July to $0.06 \mu\text{g/L}$ in February. In the deepest part of the unsaturated zone, the calculated concentration is $\sim 0.16 \mu\text{g/L}$ in July. Unfortunately there was no sample from P2 at 4.4 m depth in February due to a rise in groundwater level, but based on the concentration at 3.4 m depth, a somewhat similar value of $\sim 0.2 \mu\text{g/L}$ could be expected in the top groundwater at this time of sampling. As seen in Figure 3, the concentration in the top groundwater in P2 was $\sim 0.3 \mu\text{g/L}$ in both July and February, which is 50-90% higher than what could be expected from the concentrations in the soil air in February and July respectively.

4 Discussion

The large difference in chloroform concentration in the soil air of almost two orders of magnitude between two locations only 10 m apart and with no visible differences above or below ground is considerable. Hoekstra *et al.* [10] found spatial variation in the concentration of chloroform in soil air within a 60×180 m grid but with a more moderate variation of 920-7400 pptv. A large horizontal variation in net chloroform production fits well with the hypothesis that chloroform is formed by the activity of domain-living organisms like many fungal species. Considering the decrease in chloroform concentration in P2 from July to February (from ~ 2500 pptv to ~ 600 pptv), there is no doubt however, that there is also a small production of chloroform at this location, but the increase down the profile is probably caused by sideways diffusion from places of larger production, such as P1.

A clear increase in chloroform concentration is seen from 0.30 to 0.50 m depth at both P1 and P2, Figure 2a. Laturmus *et al.* [13] found a similar increase in chloroform concentration in soil air until ~ 0.75 m, followed by a slow decrease towards the groundwater table. Hoekstra *et al.* [10] also found an increase in chloroform in soil air down to ~ 0.5 m. The reason for this increase is not immediately intelligible if the production of chloroform takes place in the O-horizon where the most fungal activity exists. Assuming that the production of chloroform occurs either in the fungi or by exo-enzymes excreted into the soil water, chloroform will be dissolved in the soil water after formation. One explanation of the increasing concentration during the first 0.5-1 m could then simply be an indicator of the time it takes for equilibrium between the water and gas phase to be established. Another explanation could be that part of the chloroform is formed from precursor molecules at deeper soil layers. The unspecific chloroperoxidase-mediated reaction suggested for the formation of chloroform also leads to the formation of potential chloroform precursors like trichloroacetic acid [10], which might later be converted to chloroform either abiotically or by microbial degradation. Hoekstra *et al.* [10] could not find any formation of chloroform from ^{37}Cl below 20 cm depth despite the increase in chloroform concentration down to ~ 0.5 m, and this fits well with the hypothesis, that some of the chloroform is formed below the domains of the fungi, but with high dependence of the fungi above. This question needs further studies to be fully explained.



One interesting feature of Figure 2 is that the large differences in chloroform concentrations in the top soil of the two profiles is still of approximately one order of magnitude at 4 m depth. This is somewhat surprising considering the small distance between the two profiles. That diffusion does not eliminate the differences between the two profiles could indicate that the movement of chloroform in the unsaturated zone is mainly due to movement in the soil water. This hypothesis is quite logic in the sense that according to Henry's law, the concentration of chloroform is ~13 times higher in the soil water than in the soil air at 10°C [16], but it is in some conflict with the previously suggested hypotheses that chloroform enters the groundwater mainly through diffusion [13].

We calculated the soil water chloroform concentrations from the measurements in the soil air, in an attempt to see a connection between chloroform concentration in the unsaturated and in the saturated zone below. The calculated soil water concentrations can probably be viewed as minimum concentrations, since chloroform is formed in water, and if equilibrium is not established, it can then be assumed, that it will be in favour of a larger soil water concentration than expected. A larger concentration in the soil water could still fit quite well with the measured chloroform concentrations in the top groundwater of P1, since some dilution might be expected even here. Also in P2, where chloroform concentration in the groundwater is at least 50% higher in the groundwater, than what was expected from the soil air measurements, the water filtrating through this soil profile must be either mixed with water of higher chloroform content when reaching the aquifer or equilibrium has not been fully established between water and air.

In conclusion, we have shown that naturally produced chloroform can enter the groundwater. Concentrations in an aquifer will however be extremely hard to predict, especially from single soil air measurement but also from single measurements of the shallow groundwater of a test well.

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II

**Hot Spots of natural chloroform in forests – indications of
microorganisms being responsible of most of the chloroform produced
in temperate coniferous forests**

Christian N. Albers, Ole S. Jacobsen, Érico M. M. Flores, Juliana S. F. Pereira, Troels Laier
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(Partly revised according to requested revisions from *Biogeochemistry*)

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Hot Spots of natural chloroform in coniferous forests – indications of microorganisms being responsible of most of the chloroform produced in temperate coniferous forests

Authors:

Christian N. Albers • Ole S. Jacobsen • Érico M. M. Flores • Juliana S. F. Pereira • Troels Laier

Christian Nyrop Albers has contributed to the project as described below:

0 = No contribution

1 = Minor

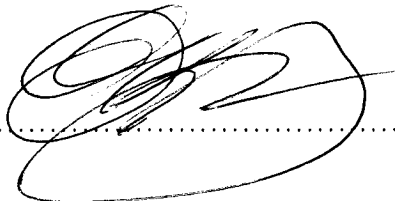
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| b) Strategy for the project / choice and development of methods | 2 |
| c) Implementation of the project / empirical work | 2 |
| d) Analysis, interpretation and discussions | 2 |

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Hot Spots of natural chloroform in coniferous forests

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indications of microorganisms being responsible of most of the chloroform produced in temperate coniferous forests

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Abstract

15

Natural chloroform in soil gas below four coniferous forest sites was studied. High concentrations were found within narrow areas – Hot Spots – varying from ~25 to >400 m² in size, with chloroform concentrations being typically 20-100 times those in corresponding Low Spots. Attempts to localize Hot Spots by visual inspection with regard to type and density of vegetation failed. Possible differences between Hot and Low Spots could be emission, leaching or degradation of chloroform. However, emissions of chloroform from Hot Spots were ~10 times higher than from

20 Low Spots and similarly the chloroform concentration in groundwater below a Hot Spot was ~10 times higher than below the corresponding Low Spot. No differences in chloroform mineralization rates were observed between sites and incubation of soil cores confirmed a larger net formation of chloroform in the Hot Spots.

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Various soil parameters were measured in order to compare the soil sampled from Hot and Low Spots. The halogenation degree of organic soil samples was in the same range, although slightly higher in the humification layer of the Hot Spot. The chloroform formation potential of the soil organic matter showed differences between soil horizons but not between sites.

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The high levels of chloroform in the Hot Spots are probably best explained by differences in chlorinating activity caused by an uneven distribution of yet unidentified microorganisms, since differences in soil organic matter quality and in emission, leaching and degradation of chloroform as well as a number of additional soil parameters could be completely ruled out.

Keywords

35 Flux • Forest • Natural chloroform • Natural organohalogens • Soil • Spatial variation

Introduction

Chloroform (CHCl₃) is a volatile chlorinated aliphatic compound. It is a common groundwater pollutant in urban areas as well as in rural areas (Squillace et al. 1999; Squillace et al. 2004). In
40 Denmark, chloroform has repeatedly been found as the single pollutant in groundwater beneath coniferous forests in concentrations often exceeding the local groundwater quality criteria of 1 µg/L (Laternus *et al.*, 2000; Jacobsen *et al.*, 2007). The occurrence of chloroform in groundwater of otherwise fine quality indicates a natural origin and it is well established that chloroform is a natural
45 substance, with only 10-25% of the annual flux of 0.3-1.0 Tg/yr to the atmosphere originating from human activity (Laternus et al. 2002; McCulloch 2003; Worton et al. 2006). Atmospheric chloroform is present at concentrations of 10-15 pptv in background locations of the northern hemisphere (NOAA 2009) and based on atmospheric monitoring in Mace Head, Ireland, chloroform emissions in the western and central part of Europe seem to be highest from terrestrial regions in
50 Ireland, Scotland and Scandinavia, while the emissions are much smaller from the dense populated areas (Ryall et al. 2001).

The presence of natural chloroform in temperate coniferous forests has been observed for more than 10 years (e.g. Hoekstra et al. 1998a; Hoekstra et al. 2001; Dimmer et al. 2001; Haselmann et al. 2002; Svensson et al. 2007) but even though the concentrations were always higher in the soil air than in the ambient air, highly varying concentrations and fluxes have been measured both within
55 and between forests. Before the presence of natural chloroform in forest soil was known, Frank et al. (1989) reported chloroform concentrations as high as 14000 pptv at 30 cm depth, which was ~400 times the atmospheric background at the same location, but with variations of more than one order of magnitude between and within three different forested areas. Similar concentrations in soil air of coniferous forests have been reported by Frank & Frank (1990) and Hoekstra et al. (1998a),
60 also with variations as high as a factor of 10 within single forests. Haselmann et al. (2002) found smaller concentrations in a temperate spruce forest, with only 2-10 times the atmospheric background in air samples taken at 0-10 cm depth. Hoekstra et al. (2001) and Dimmer et al. (2001)

both investigated the flux of chloroform from forest soil and found differences of more than one order of magnitude between different vegetations.

65 The highly elevated concentrations in coniferous forest soils, and positive fluxes from these, are strong indications that a natural formation takes place in such soils. A more direct proof of chloroform formation in forest soil is the observed increase in the isotope fraction of ^{37}Cl in soil air chloroform, when Na^{37}Cl was added to forest top soil (Hoekstra et al. 1998a).

70 The formation of chloroform in forest soil has been ascribed unspecific chlorination facilitated by extracellular enzymes excreted by fungi (Hoekstra et al. 1998a), although some fungi seem to produce chloroform intracellularly (Hoekstra et al. 1998b). The hypothesis of the unspecific chlorination is partly adopted from research on macromolecular organochlorine, which is present in forest soils in concentrations of $\sim 1 \text{ mg g}^{-1}$ of soil organic matter (SOM) (Asplund et al. 1989; 75 Asplund & Grimvall 1991; Hjelm et al. 1995; Öberg & Grøn 1998). This macromolecular organochlorine is supposed to be formed when chlorinating enzymes oxidize chloride to reactive halogen species like HOCl that react unspecifically with SOM. A pool of chlorinating enzymes with similarities to chloroperoxidase (CPO) known from the plant pathogen *Caldariomyces fumago* has been shown to exist in forest soil (Asplund et al. 1993), and the formation of chloroform was then 80 suggested to be the result of a biotic reaction (1) followed by an abiotic reaction (2) (Hoekstra et al. 1998a):



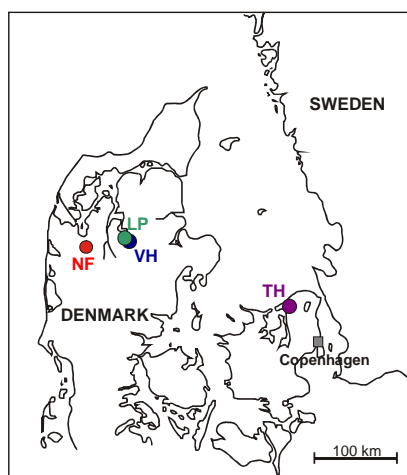
85 The last reaction is expected to be similar to what has been shown to occur, when chloroform is formed as a major by-product in the chlorination of drinking water (Rook 1977; Boyce & Hornig 1983; Deborde & von Gunten 2008). In addition to the haloperoxidase-catalyzed reaction, Huber et al. (2009) recently demonstrated the abiotic formation of trihalomethanes from polyphenols added 90 Fe(III), H_2O_2 and the increased formation of chloroform in soil when Fe(III) was added. The reaction seemed to take place mainly at very acidic pH (< 3.7).

In conclusion, it seems well established that chloroform is formed in temperate coniferous forests. The reported concentrations in soil are highly varying, however, and a significant part of this

95 variation has been reported within single forests. This makes it hard to calculate local and global
fluxes of chloroform to both the atmosphere and the groundwater. One purpose of our study was
therefore to more systematically investigate spatial variation of soil chloroform between and within
temperate coniferous forests. The second purpose was to see if the spatial variation could be
explained by variation in formation or removal of chloroform and if this could help to reveal the
100 mechanism of formation which is still on a very hypothetical level.

Materials & Methods

Study sites



105 Figure 1. Map of the location of the four study sites in Denmark. NF is Nordre Feldborg, LP is Liseborg Plantage, VH is Viborg Hedeplantage and TH is Tisvilde Hegn. See text for GPS-coordinates.

Two forests were chosen as study sites because monitoring wells with $\mu\text{g/L}$ -concentrations of presumably natural chloroform were present in the vicinity (Figure 1). In Nordre Feldborg (NF), the
110 study site ($8^{\circ}57'01''$ E, $56^{\circ}22'51''$ N) is a stand of 40 year old Norway Spruce (*Picea abies*) as the dominating vegetation. No underwood is present, but the forest floor is partly covered with moss. The soil is sandy, with both diluvial and aeolian sand in the area, which has been forested since approximately 180 years. In Tisvilde Hegn (TH), the study site ($12^{\circ}03'40''$ E, $56^{\circ}02'22''$ N) is a mixed stand dominated by older Scots Pine (*Pinus sylvestris*) and younger Norway Spruce with also
115 a few Birch trees (*Betula pendula*) present. No underwood or moss is present. The soil is 200 to 400 years old aeolian sand, which has been forested for at least 150 years. The top sand is partly washed out, and the area is in the initial phase of a podzolization. Two additional forests near the town of

Viborg, Viborg Hedeplantage (VH) (9°22'14" E, 56°25'37" N) and Liseborg Plantage (LP) (9°21'33" E, 56°25'42" N), were included to confirm a general pattern of spatial variation. These forests were chosen because of problems with presumably natural chloroform in concentrations above the quality criteria in the nearby water works (Jacobsen *et al.*, 2007). The two additional study sites are mixed stands with Norway Spruce as the dominating vegetation and apart from mosses no underwood is present. The soil is diluvial sand with well developed A-horizons. All four forests are temperate hemiboreal coniferous plantations on sandy soils and at all four locations, a 5-25 cm thick organic horizon has developed on top of the sand. The organic horizon typically consists of a litter (L-) horizon at the top followed by a fermentation (F-) horizon. Below the F-horizon, a humification (H-) horizon partly mixed with sand can be recognized.

Field studies

Soil air sampling. A stainless steel soil air lance was used to obtain soil air samples (Figure 2a). The lance was forced into the soil and air was drawn through the screen of the lance at 40 cm depth using a membrane pump (Rietschle Thomas, Schopfheim, Germany) at a speed of $\sim 2 \text{ L min}^{-1}$. The soil air was led through a 200 mL stainless steel gas cylinder with two terminal valves. After 3 minutes the exit-valve was closed and the cylinder was pressurized to $\sim 1.5 \text{ bar}$ by continued pumping before closing the inlet valve. The gas cylinders were brought to the laboratory and analyzed within 48 h. Reproducibility of the method was tested by analysis of consecutive gas samples, which showed a standard deviation $< 3\%$ of the average value of three samples. Furthermore, possible errors due to chloroform interacting with our standard steel cylinders during storage was checked by comparing with samples collected in electropolished steel cylinders (Swagelok Company, Solon, OH) and in 30 mL glass cylinders. No errors were found. Prolonged storage up to two weeks resulted in only minor changes in chloroform and CO_2 content. Sorption of chloroform to the PE-tubing used to connect the pump with the air lance and the steel cylinder was undetectable during laboratory tests using ^{14}C -chloroform and the tubing did not release chloroform or other volatile halogenated compounds in detectable amounts.

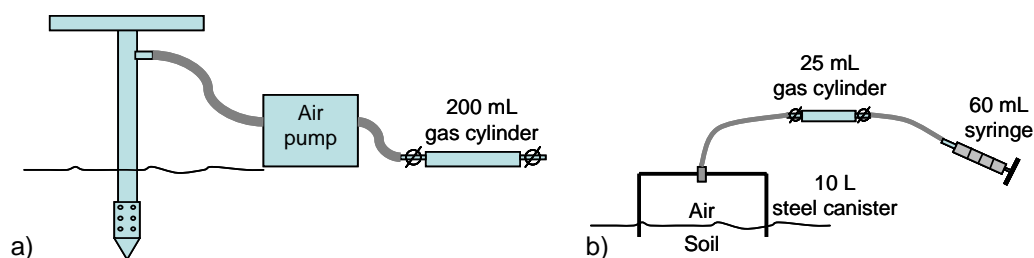


Figure 2. a) Setup for obtaining soil air samples from 40 cm depth. b) Setup for measuring fluxes of gases from soil.

Flux measurements. The fluxes of chloroform and methyl chloride from the forest floor were investigated in NF and TH using a simple static chamber approach (Figure 2b). Ten litre stainless steel canisters were equipped with valves and used as flux chambers. A 25 mL glass gas cylinder with two terminal valves was connected to a chamber and a 60 mL syringe. After pressing the edge of the canister ~ 1 cm into the soil, it was left closed for two hours and a gas cylinder was then connected to the valve that was then opened. The syringe was drawn forth and back 20 times, in order to flush the gas cylinder and to mix the air in the flux chamber. The inlet valve of the gas cylinder was then closed and the gas sample pressurized to ~ 2 bars with the syringe piston. Samples were taken in duplets, with typical variation of 5-10% between replicates. The gas samples were brought to the laboratory and analyzed within 48 hours. The flux from soil to the atmosphere was calculated from the increase in chloroform concentration during two hours. Preliminary tests revealed that the increase during the two hours was not completely linear (see supplementary material Figure S1). The first derivative of the fitting function at $t=0$ was compared with the slope of the first order equation calculated from a single point measurement and the flux at $t=0$ could then be estimated from the flux calculated from the single measurements at $t \approx 2$ h. The soil temperature and moisture was monitored on an hourly basis in TH, as described elsewhere (Albers et al. Submitted).

Chemical analyses, gases. Pure CHCl_3 (>99.5%) for analytical standards was purchased from Sigma-Aldrich (Steinheim, Germany).

Air samples were analyzed for chloroform, CFCs and other C_1 -organohalogenes on a gas chromatograph equipped with an ECD detector (GC-8A, Shimadzu, Kyoto, JP). The analytical procedure was similar to that described by Busenberg & Plummer (1992) for chlorofluorocarbons (CFC) in age-dating of young groundwater. Briefly, the halocarbons were trapped at -30°C on a pre-column, which was then heated to 95°C . Separation of gas constituents was done on a 1.7 m

packed column, Poracil-C, at 70°C. Pre-column back-flush technique was used to complete the
175 analysis of each sample within 11 min. Fifteen mL of gas sample was used for analysis. The limit of
quantification (LOQ) for chloroform was ~10 pptv and for methyl chloride ~7 ppbv.
Analysis of O₂ and CO₂ in gas samples was done on a Mikrolab GC82 gas chromatograph equipped
with a thermal conductivity detector (TCD) (Mikrolab, Aarhus, DK). Separation of the gas
constituents was carried out at 60°C on 2 columns packed with molecular sieve 5A and Porapak C
180 using helium (60 mL/min) as the carrier gas. The LOQ for CO₂ was 0.04%.

Chemical analyses, soils. pH was measured in a 2:1 water:soil slurry. Chloride and water
extractable iron were determined in subsoil as follows: 5 g soil was shaken for 24 hours with 5 mL
MilliQ-H₂O, and the slurry was then centrifuged (3000g, 10 min.). Chloride in the supernatant was
185 determined by ion chromatography (Dionex DX500 IC). Total iron in the supernatant was
determined spectrophotometrically at 520 nm after complexation of the reduced iron with 2,2'-
bipyridine (Moss & Mellon 1942). The background absorption in the supernatant, constituting 10-
20% of the total absorption at 520 nm, was subtracted from the total absorption.
Soil for the determination of total organic halogens was sampled with steel cylinders (H: 18 cm and
190 Ø: 6 cm) in November 2008. The soil columns were stored cold and closed until arrival in the
laboratory and the A_h-horizons from just below the H-horizons were then extracted with water for
chloroform-determination as a quick test, to see differences in chloroform concentrations between
soil columns. The different layers of the organic horizons were freeze dried and grinded to fine
powder in a coffee mill followed by further grinding in an agate mortar. The powdered soil was
195 then divided in two. One portion was taken for further analysis as it was (TX, total soil
chlorine/bromine/iodine) and one portion was thoroughly washed three times with HNO₃/KNO₃
(0.02M/0.2M) solution (liquid:soil ratio of ~30) and then once with Milli-Q water to remove all
inorganic halogen present. After freeze drying, this portion of the soil sample was analyzed
similarly for halogens (TOX, total organic chlorine/bromine/iodine). The loss of organic chlorine in
200 such a washing procedure has previously been shown to be less than 1% for organic horizons
(Asplund et al. 1994; Silk et al. 1997). Total inorganic halogen (TIX) was calculated as (TX –
TOX), which with the accuracy of the analytical procedure gave LOQ for TIX of ~2 µg/g. A total of
24 soil samples (triplicate F- and H-horizons, NF Hot and Low Spot, TX and TOX) were then
digested for the determination of total organic halogens using microwave-induced combustion
205 (MIC), which was recently proposed to digest organic samples with complex matrices (Flores et al.

2007; Moraes et al. 2007). The conditions during the MIC-procedure were as follows: Triplicate soil samples (about 200 mg) were pressed as pellets using a hydraulic press set at 3 ton for 1 min for further digestion by MIC. Conditions were chosen according to previous work (Flores et al. 2008) using 6 mL of 50mM (NH₄)₂CO₃ as absorbing solution. The heating program was 1400 W for 10
210 min (reflux step) and 0 W for 20 min for cooling. After combustion, the resultant solution was transferred to a polypropylene vessel and diluted with water to 30 mL. Accuracy was evaluated using certified reference material of coal (NIST 1632c) and spiked samples were used to evaluate the recovery of halogens. After MIC, Cl was determined by ion chromatography and Br and I were determined by inductively coupled plasma mass spectrometry (ICP-MS). Operational conditions of
215 halogens determination by IC and ICP-MS were selected according to literature (Flores et al. 2008).

Laboratory experiments

Net formation of chloroform. Eight soil cores were sampled in steel cylinders (H: 10 cm and Ø: 6 cm) in TH in May 2007. The soil cores were incubated immediately at 10°C in the dark in 1 L
220 closed glass jars with a sodium bicarbonate based plastisol-lined lid equipped with a luer lock port. This valve was connected directly to the injection port of the GC/ECD, for injection into the air-evacuated 15 mL sample loop. Two x 0.2 mL air was sampled for CO₂ and O₂ analysis, respectively. The incubated jars were analyzed once a week for 5 weeks. Due to fairly high consumption of oxygen, we found it necessary to add 40 mL pure O₂ to each jar at the end of each
225 analysis. The net formation was calculated from the average increase in chloroform concentration from day 6-34. Pilot studies with injection of chloroform to similar soil cores showed that equilibrium between soil cores and the surrounding air was established within 30-50 hours.

Chlorination of soil samples. Freeze dried and powdered top soil samples were chlorinated
230 chemically in order to detect any differences in chloroform formation potential between soil sites and horizons. Soil corresponding to 15.5 mg SOM was weighed into a 16 mL glass vial, 15 mL 0.1M phosphate buffer (pH = 4.0) was added followed by 50mM NaOCl-solution to a final OCl⁻ conc. of 0.5mM. The low HOCl/SOM ratio, as compared to those used in the chlorination of drinking water, was chosen because the natural concentration of reactive chlorine in soil, most
235 likely is very low compared to that in a water purification system. The vials were then placed in the dark at 10°C, partly to mimic natural soil conditions, partly to minimize losses of chloroform to the

small headspace and to the air, when taking subsamples for analysis. After 24 hours, the reaction was quenched with 100 μL 1M $\text{Na}_2\text{S}_2\text{O}_3$ and after centrifugation (1000g, 15 min.), 100 μL of the supernatant was diluted to 125 mL with chloroform-free N_2 -purged MilliQ-water and 30 mL of the solution was analyzed on purge & trap GC/ECD.

Mineralization of chloroform. 30 g O-horizon, homogenized by hand and with roots and other greater particles removed, or 30 g 2-mm-sieved A-horizon were added to 600 mL glass jars equipped with a sodium bicarbonate based plastisol-lined lid with a 9 mm silicone-septum. All jars were equipped with a small glass container for CO_2 -absorbing liquid (1M NaOH) and 3 mL aqueous $^{14}\text{C-CHCl}_3$ (ARC Inc., St. Louis, MO, radiochemical purity >99%) solution was then added to the soil in each jar (170000 DPM/mL, corresponding to a total of ~ 350 $\mu\text{g/kg}$ soil wet weight, which corresponds to some of the highest concentrations found in natural soils). The lid was closed immediately after the addition of $^{14}\text{C-CHCl}_3$ and a stainless steel needle with a 3-way valve was pierced through the septum and into the small glass container. After having added 3 mL of CO_2 -absorbing liquid (1M NaOH) to the container, the 3-way valve was closed. Because of its high volatility, some of the $^{14}\text{C-CHCl}_3$ is likely to move from the soil into the CO_2 -trap. It was therefore necessary to develop a method to distinguish between $^{14}\text{CO}_2$ and $^{14}\text{C-CHCl}_3$ in the absorber fluid. The NaOH was changed every 3-10 days and the $^{14}\text{CO}_2$ originating from the mineralization of $^{14}\text{C-CHCl}_3$ was separated from dissolved $^{14}\text{C-CHCl}_3$ as follows: After thorough mixing, 1 mL of the absorber was added to each of two 2 mL centrifuge tubes (Sarstedt, Nümbrecht, Germany) containing either 1 mL H_2O or 1 mL 1M BaCl_2 . BaCO_3 was allowed to precipitate for 10 minutes at 5°C and all tubes were centrifuged (10000g, 2 min.). 1 mL from each tube was then counted on LSC, and the total $^{14}\text{CO}_2$ released from the soil was calculated from the difference between the subsample with water added (containing both $^{14}\text{CO}_2$ and $^{14}\text{C-CHCl}_3$) minus the subsample with BaCl_2 added (containing only $^{14}\text{C-CHCl}_3$). Preliminary experiments showed that BaCl_2 had no influence on the dissolved $^{14}\text{C-CHCl}_3$ and that the method had high accuracy and reproducibility.

Statistical software. Spatial interpolation of chloroform concentrations in soil was performed with Surfer 8.02 (Golden Software Inc., Golden, CO). The Ordinary Linear Kriging method was used to perform interpolation between sample points.

Simple and multiple regressions on chloroform concentration (dependent variable) and CO_2 concentration or soil characteristics (independent variables) were performed using KyPlot Version

2.0 (KyensLab Inc.). Since data on neither chloroform concentrations in soil nor chloroform fluxes
270 from soil were normally distributed, the non-parametric Spearman's rank correlation coefficient
was used to determine if correlations were statistically significant.

Results

Preliminary investigations in the study areas revealed spatial variations in chloroform concentration
275 in the soil air of up to two orders of magnitude between as well as within single stands. These
surveys were done with tens to hundreds of meters between sampling points. Denser sampling with
1.5 to 10 m between sampling points revealed that the variation was not completely random, with
points of high chloroform concentrations located close to each other (Figure 3). These areas with
high concentrations will from now on be termed "Hot Spots" whereas all other areas with
280 chloroform concentrations lower than ~5 ppbv (but typically lower than 1 ppbv) in the soil air will
be termed "Low Spots". In NF the Hot Spot was rather large (at least 400 m²) and only one Hot
Spot was located within the 30 x 72 m area we investigated. In TH, the Hot Spots were much
smaller (25-50 m²) and three Hot Spots were located within a 20 x 70 m area (Figure 3).

285 In order to investigate whether the Hot Spot pattern was a general phenomenon in coniferous
forests, two additional forests near Viborg, DK, were investigated for chloroform concentrations at
40 cm depth, and again an immense variation was found both between and within stands.
Additional sampling around two sampling points having high chloroform concentration confirmed
the presence of Hot Spots, having very narrow areas of distribution, similar to those in TH (Figure
290 3).

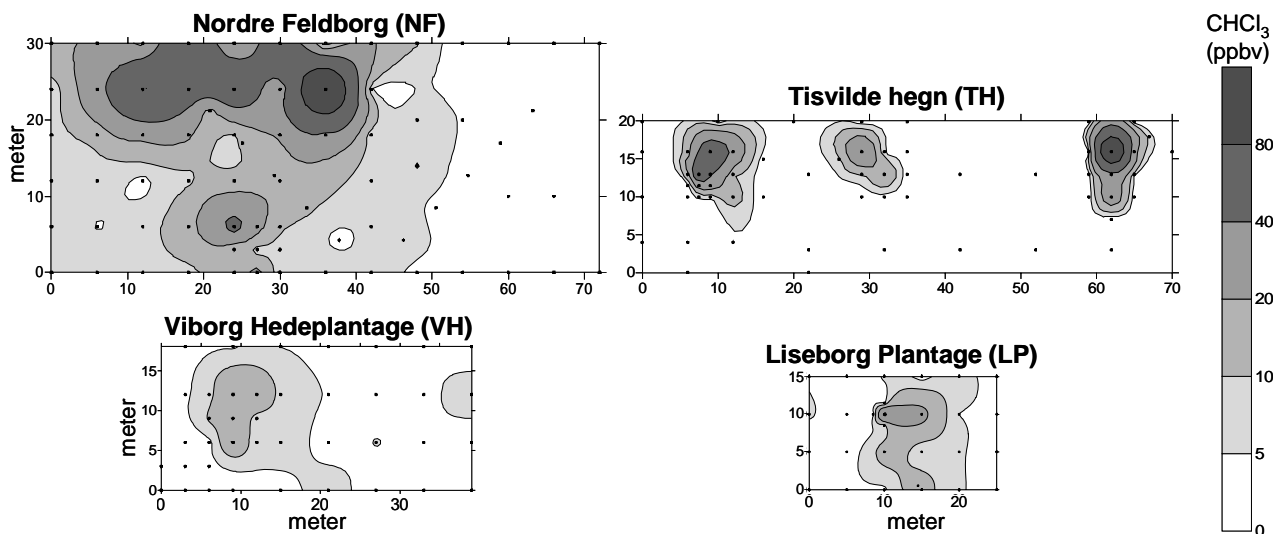


Figure 3. Contour maps of Ordinary Kriging on chloroform concentrations (ppbv) measured in soil air at 40 cm depth. Shaded colours show Hot Spots of chloroform with concentrations typically 100-1000 times the atmospheric background concentration. The white areas show Low Spots with typically ~10 times the atmospheric background concentration. The black dots show the location of the below ground air samples. The samples in TH were collected in spring 2007. The samples in NF were collected in autumn 2007 and spring 2008 at times during the seasonal cycles where chloroform soil air concentrations in NF were similar (Albers et al. Submitted). Samples in VH were collected in February and November 2008 and in LP in November 2009. Atmospheric concentrations of chloroform at the date(s) of sampling (20 cm above soil surface) varied from 0.034-0.18 ppbv.

A 70 m transect through the three Hot Spots in TH illustrates the huge variation within distances of just a few metres (Figure 4a). Forty four of the 60 sampling points in TH were marked during the first sampling in 2007 and these were re-sampled two years later showing very similar chloroform concentrations to what was found in 2007 (Figure 4a and b).

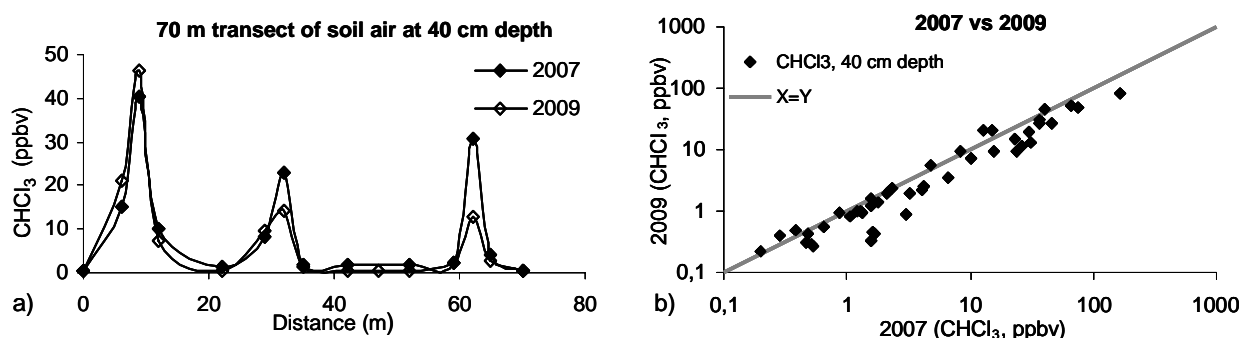


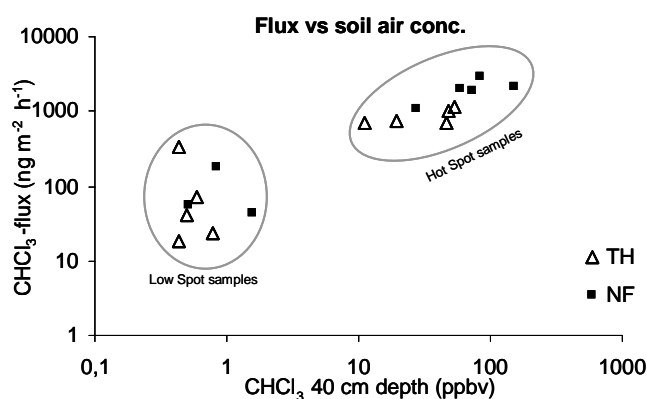
Figure 4. a) Transect in TH traversing the three Hot Spots. Soil air was sampled at 40 cm depth in May 2007 and May 2009. b) Comparison of the 44 sampling points in TH that were sampled in both years. The 1:1 line is shown. Note the logarithmic scale on both axes.

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At 32 sampling points in the TH study site, several soil parameters in addition to chloroform (CO₂ concentration in soil air, thickness of the organic layer (varying from 3.5-20 cm) and furthermore pH (varying from 3.8-4.8), chloride (varying from 1.8-10.9 mg kg⁻¹ soil) and total iron (varying from 0.12-3.5 mg kg⁻¹ soil) in aqueous extracts of soil from 25 cm depth) were measured to try to explain the spatial variation in chloroform concentrations. As will be discussed later, these parameters could all be expected to influence the formation of chloroform. The correlations were very poor for all measured parameters, except for CO₂, where the Spearman's rank correlation coefficient was significant (p = 0.005) although the R² of a simple regression was only 0.26. CO₂ was as the only independent variable measured on additionally 9 air samples and chloroform versus CO₂ for all 41 samples is shown in Figure 6a. Performing multiple regressions with the different measured parameters as independent variables, did not result in any additional significant relationships, than what could be explained by the slightly positive relationship between chloroform and CO₂ concentrations.

325 **Chloroform fluxes from soil**

The higher concentrations of chloroform in the Hot Spots, in principle could be due to a relatively lower loss of chloroform caused by emission from the soil. In order to test this working hypothesis, flux measurements of chloroform were performed in both Hot and Low Spot areas in NF and TH. If the structural soil conditions are truly similar in Hot and Low Spots, some positive relationship would be expected between chloroform concentration in the upper soil and chloroform emission to the atmosphere, and this was indeed found to be the case at both sites (Figure 5).



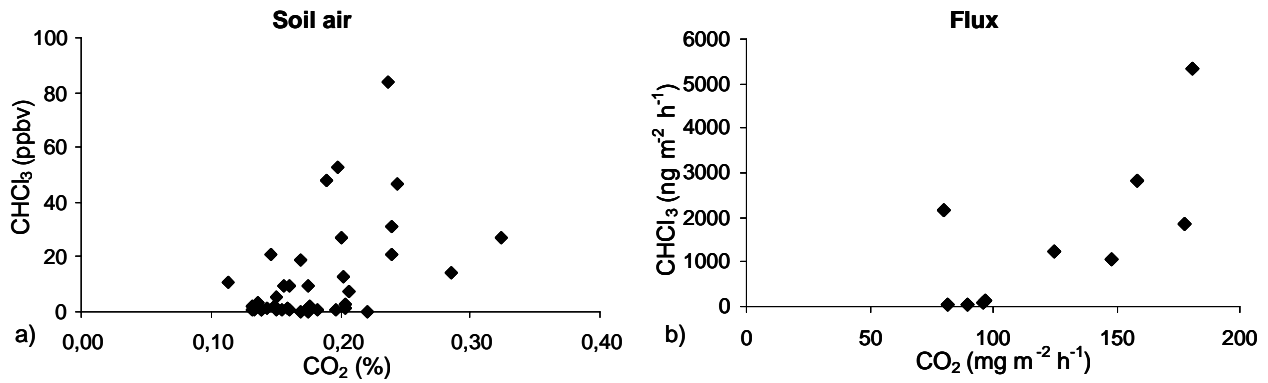
335 Figure 5. Relationship between chloroform concentrations in the upper soil (40 cm depth) and chloroform fluxes from the exact same sample point in NF (n=8) and TH (n=10), respectively. Both sites were sampled in June 2009. Note the logarithmic scale on both axes.

340 The flux-measurements at the two sites showed somewhat similar results (Table 1), although the differences in emissions between Hot and Low Spot were slightly smaller in TH, especially in the winter measurement.

345 Table 1. Average (std. dev.) chloroform-fluxes in NF and TH in March, June and August (TH only) 2009 and calculated emissions from the investigated forest areas, where the distribution between Hot and Low Spots are known (estimated 40% Hot and 60% Low Spot in NF and 15% Hot and 85% Low Spot in TH). All values are in $\text{ng m}^{-2} \text{h}^{-1}$. n = 5. Values for each chamber in June and the exact location of the chambers can be seen in Supplementary Figure S2 & S3.

	NF		TH	
	Low Spot	Hot Spot	Low Spot	Hot Spot
March 2009	16 (15)	214 (148)	40 (36)	86 (30)
Average forest soil emission	95		47	
June 2009	84 (59)	1998 (93)	98 (135)	864 (216)
Average forest soil emission	850		213	
August 2009			276 (437)	2676 (1584)
Average forest soil emission			636	

350 The air samples collected during the flux-measurements in June (NF) or August (TH) were analyzed with respect to CO_2 and CH_3Cl in addition to chloroform. In NF, the chloroform flux showed no relationship to the flux of either CO_2 (Flux range = $39\text{-}150 \text{ mg m}^{-2} \text{h}^{-1}$) or CH_3Cl (Flux range = $104\text{-}4012 \text{ ng m}^{-2} \text{h}^{-1}$), with $p = 0.79$ and 0.99 for Spearman's rank correlation, respectively. In TH the chloroform flux was slightly positively related to CO_2 (Flux range = $80\text{-}177 \text{ mg m}^{-2} \text{h}^{-1}$) with $p = 0.07$ (Figure 6b), while the CH_3Cl flux was too low to be determined.

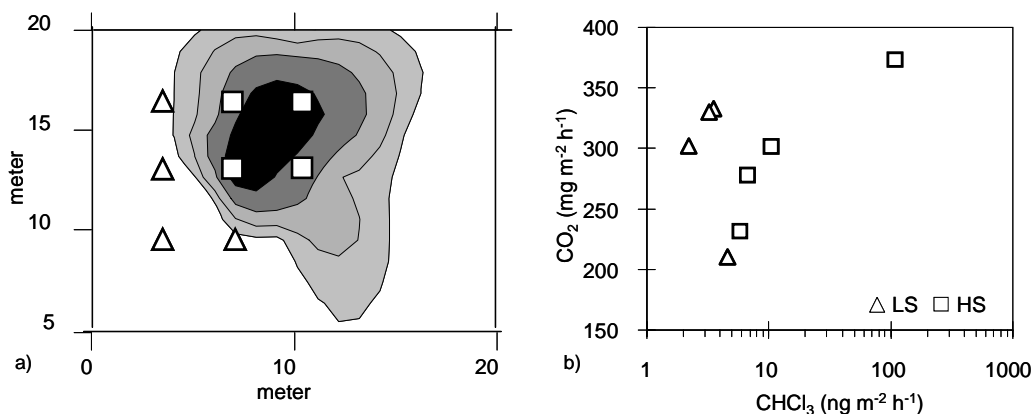


355 Figure 6. Possible relationships between chloroform and CO₂ in TH in a) soil air samples from May 2009, b) flux measurements from August 2009.

Soil incubation study

360 Four Hot Spot and four Low Spot soil cores (0-10 cm depth sampled in TH (Figure 7a)) were incubated for 5 weeks in the dark at 10°C to confirm that the elevated concentrations and emissions found in the Hot Spot soil in vivo were due to a higher rate of chloroform formation in the soil itself and not caused by e.g. input from vegetation. Differences in the net formation of more than one order of magnitude were found both within and between the Hot and Low Spot samples, but the net formation in the Hot Spot samples were in all four cases larger than in the Low Spot samples (Figure 7b, first axis). No significant difference in CO₂-formation was observed between the Hot and Low Spot samples (Figure 7b, second axis).

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370 Figure 7. a) Distribution of the eight 10 cm soil cores from TH used in the incubation study, four from the Low Spot (triangles) and four from one of the Hot Spot (squares) areas (Figure 3). b) Results of the laboratory incubation study, presented as the average chloroform net production (ng m⁻² h⁻¹) from day 6-34 (first axis). CO₂-production is also shown (second axis) from day 6-34. Note the semi-logarithmic scale.

Degradation of chloroform

The higher concentrations, emissions and net formations of chloroform in the Hot Spots, points to a
375 higher gross formation of chloroform in the Hot Spot soil. Degradation of chloroform in the soil
cannot, however, be excluded to influence the distribution of Hot Spots and Low Spots and a few
laboratory experiments using ¹⁴C-labeled chloroform were performed to check if differences in
aerobic degradation rates could be found. The results of these experiments did not show a larger
mineralization of chloroform in the Low Spot, actually the opposite was seen with soil from the O-
380 horizons and no difference was observable in the A-horizons (Table 2).

Table 2. Mineralization of added ¹⁴C-chloroform in soils from NF Hot and Low Spot to ¹⁴C-CO₂. The degradation rates are given as average % per day (min. - max.) during the first three days of the experiment. n=2.

Soil sample	O-horizon		A-horizon	
	Low Spot	Hot Spot	Low Spot	Hot Spot
Degradation rate, day 0-3	0.68 (0.67-0.70)	0.93 (0.92-0.93)	0.35 (0.32-0.37)	0.32 (0.31-0.32)

385 Total organic halogens

Assuming chloroform to be formed during unspecific natural halogenation processes in the soil, one
could expect that a higher chloroform concentration would somehow be related to a higher degree
of chlorination of the soil organic matter (SOM) in general. In order to check if such a relationship
exists, the total organic and inorganic chlorine, bromine and iodine contents were determined in
390 different fractions of the soil. The F- and H-horizons of three soil columns from the Hot Spot and
three soil columns from the Low Spot in NF were analyzed for this purpose (Table 3). There
seemed to be some trends in the halogen contents especially between soil layers, but differences
between sites were minor.

The soil columns, from which the F- and H-horizons were isolated for total halogen analysis, also
395 contained the top few cm of the A_h-horizon. This soil was analyzed for water-extractable
chloroform, as described in the Materials and Methods section, in order to test, whether there was
indeed a significant difference in chloroform concentration between the Hot and Low Spot soil
samples, which were subsequently analyzed for total organic halogen. This test showed water-
extractable chloroform concentrations from 0.01-0.03 µg/kg in the Low Spot samples and 1.0 – 2.5
400 µg/kg in the Hot Spot samples, indicating that such a difference did exist.

Table 3. Results of the determination of total organic and inorganic halogens in samples from NF. Three replicate soil samples were sampled for each soil type and each soil sample was analyzed in triplicates. Numbers in parentheses are the range of the halogen content of the three independent soil samples. TO(X) = Total organically bound (chlorine/bromine/iodine). TI(X) = Total inorganic (chlorine/bromine/iodine). All values are in $\mu\text{g/g}$ either on soil organic matter (SOM)-basis (TO(X)) or on soil basis (TO(X) and TI(X)). BQL means below the quantification limit of $\sim 2 \mu\text{g/g}$.

	Hot Spot F-hor.	Low Spot F-hor.	Hot Spot H-hor.	Low Spot H-hor.
TOCl (SOM-basis)	580 (414-691)	560 (531-607)	623 (400-812)	533 (486-561)
TOCl (soil-basis)	469 (383-631)	515 (475-569)	347 (179-591)	176 (128-261)
TICl (soil-basis)	161 (50-217)	257 (242-285)	88 (37-185)	30 (4-61)
TOBr (SOM-basis)	78 (60-109)	63 (55-77)	95 (70-121)	93 (85-106)
TOBr (soil-basis)	61 (55-67)	57 (52-69)	48 (41-62)	29 (24-40)
TIBr (soil-basis)	BQL	BQL	BQL	BQL
TOI (SOM-basis)	23 (19-32)	16 (14-19)	24 (22-25)	25 (22-27)
TOI (soil-basis)	18 (17-20)	15 (13-17)	13 (8-22)	8 (6-11)
TII (soil-basis)	BQL	BQL	BQL	BQL

Chlorination experiments

Another factor that may influence the formation of chloroform could be the quality of the SOM regarding its chloroform formation potential. Attempts to characterize the organic matter from NF Hot and Low Spot were carried out, but elemental analyses of C, H and N-contents and solid state ^{13}C -NMR spectra showed no differences between Hot and Low Spot samples (results not shown). To detect differences in chloroform precursors, a chlorination study with the same 12 soil samples, which were used for total halogen determination, was performed. The chlorination of both F- and H-horizons showed no difference between Hot and Low Spot but some variation between the soil horizons (Figure 8).

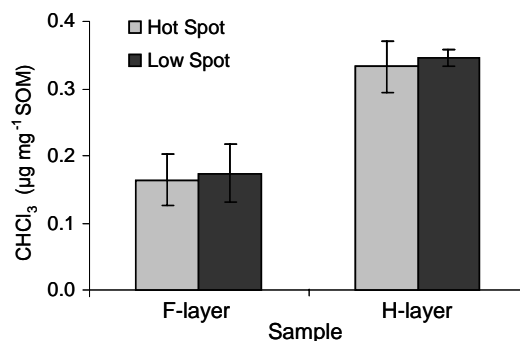


Figure 8. Results from chlorination study with freeze dried and powdered F- and H-horizon soil samples from NF Hot and Low Spots. Error bars are the standard deviation of three replicates. Chlorination conditions: Soil = 1 g/L. $[\text{OCl}^-] = 0.5\text{mM}$. $T = 10^\circ\text{C}$. $\text{pH} = 4.0$. Reaction time = 24 h.

Discussion

Chloroform in soil air

425 Our first surveys were done with tens to hundreds of meters between sampling points in order to see
if the chloroform concentrations were different between areas with visible differences in vegetation
type, density of vegetation, age of stands, light penetration through the canopy and moss coverage
on the forest floor. Variations in chloroform concentrations in the soil air of almost two orders of
430 magnitude were found in these surveys, but surprisingly enough, similar variations were found
within single stands with no visible differences. More detailed investigations revealed that the
distribution of sampling points showing high concentrations of chloroform was not completely
random, and the forest floor seemed to be divided into Hot Spots and Low Spots regarding the
concentration of chloroform. When we recognized this pattern, systematic samplings with distances
between sampling points as low as 1.5 m were performed, to demarcate the Hot Spots. The sizes of
435 the Hot Spots varied depending on study site, but in TH and probably also in VH and LP, the Hot
Spots seemed quite small, with areas from 25-50 m². The size and distribution of these Hot Spots
were unchanged during a 2-year period. In NF, the Hot Spot was too large to be completely
demarcated in this study, but had an area of at least 400 m². Careful visual inspections were
performed to note any differences in vegetation, understory, moss coverage etc., but the Hot Spots
440 and Low Spots were indistinguishable by eye.

Chloroform concentrations in the soils of temperate coniferous forest have previously been
investigated, and Hoekstra et al. (1998a) did a somewhat dense sampling campaign, with 16
sampling points within a 60 x 180 m area in a Dutch Douglas Fir forest. The campaign revealed
spatial variation in chloroform concentration of ~one order of magnitude, with almost the full range
445 of variation observed within 20 m, which was the distance separating the closest located sampling
points. This variation observed in a Dutch coniferous forest could indicate that chloroform Hot
Spots are also present in that forest. Similarly, the great variations in chloroform concentrations and
fluxes reported by various authors are easier to interpret, if the occurrences of Hot Spots and Low
Spots are widespread in forests in general.

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Fluxes of chloroform

Emissions to the atmosphere

The emissions of chloroform from the forest floor in Hot and Low Spots in NF and TH were measured in March, June and August (TH only). The fluxes of chloroform from the soil to the atmosphere were much higher during summer than during winter and in TH, the flux was much higher after a wet period of the summer (August; soil temperature $\sim 14^{\circ}\text{C}$, soil moisture ~ 0.041 vol/vol both measured at 15 cm depth) than after a long dry period of the summer (June: soil temperature $\sim 11^{\circ}\text{C}$, soil moisture ~ 0.010 vol/vol both measured at 15 cm depth). This apparent positive influence of both temperature and soil moisture supports the hypothesis that microbial/fungal activity is involved in the formation of chloroform. For all dates measured, the fluxes of chloroform were much larger from the Hot Spots, as would be expected when the soil concentrations are much larger. More important here though, this rules out the possibility that the increased concentrations in the Hot Spots are caused by differences in emissions of chloroform. Literature data on natural chloroform emissions from soil are rather scarce but in three previous studies, such emissions have been measured in the field. Hoekstra et al. (2001) reported average chloroform emissions of $10\text{-}110\text{ ng m}^{-2}\text{ h}^{-1}$ from Dutch temperate forest soils with various vegetations (sampled in April), Hellén et al. (2006) reported average chloroform emissions of $100\text{-}800\text{ ng m}^{-2}\text{ h}^{-1}$ during 6 measurements in a Finnish boreal pine forest (sampled from April to June) and finally Dimmer et al. (2001) reported average chloroform emissions of $251\text{ ng m}^{-2}\text{ h}^{-1}$ in a temperate Irish pine forest and $\sim 16650\text{ ng m}^{-2}\text{ h}^{-1}$ in a temperate Irish spruce forest (both sampled in September). Except for the exceptionally high chloroform emission from the Irish spruce forest, our results seem comparable with the data presented in the literature. In all three papers, a rather large spatial variation was found. In this paper, we show the spatial variation to be huge but somewhat systematic, which together with dependence on temperature and moisture points to the influence of yet unidentified microorganisms with well demarcated distributions in the soil. Such a difference in microbial activity was also mentioned by Hoekstra et al. (2001) as a possible explanation of the spatial variation in chloroform emission that they reported and patchiness in the distribution of litter degrading fungi is well known in forest soil (e.g. Osono 2007; Snajdr et al. 2008).

Chloroform and methyl chloride emissions showed no relationship. This was expected since the presumed modes of formation are very different, with methyl halides being formed either by direct bio-synthesis in fungi (Harper et al. 1988; Field et al. 1997) or by abiotic nucleophilic substitution

of methoxy groups in pectin or lignin (Hamilton et al. 2003; Keppler et al. 2005). The lack of relationship between emissions of these two chlorinated compounds has been reported earlier for forest and shrubland soil (Dimmer et al. 2001; Rhew et al. 2008), while a positive relationship was
485 found in a peat bog (Dimmer et al. 2001).

As indicated by the large standard deviations in the flux experiment (Table 1), the spatial variations in chloroform emissions from soil to the atmosphere within both Hot and Low Spots are huge, and one should therefore be careful to calculate average forest emissions based on only 5 Hot and 5 Low Spot samples. The calculated average forest soil emissions in Table 1 nevertheless can give
490 some idea of the average chloroform emission from the forest soil, which is of great interest, when estimating the influence of this environmental compartment on the global atmospheric chloroform budget.

Leaching to the groundwater

495 The higher chloroform concentration in the Hot Spot could in principle be due to a lower leaching and/or diffusion of the chloroform towards the groundwater. As indicated earlier (Albers et al. 2008), the chloroform concentration is ~10 times higher in the upper groundwater below one of the Hot Spots in TH than below the adjacent Low Spot (location of abstraction wells is shown in Supplementary Figure S3). Monitoring of the groundwater just below the groundwater table
500 throughout 2 years showed that the concentration below the Hot Spot varied from 2.0 – 5.3 µg/L (Albers et al. Submitted) and below the Low Spot from 0.2-0.4 µg/L. This strongly indicates that transport of chloroform towards the groundwater is higher from the Hot Spot. Furthermore, it shows that also in the groundwater, large spatial variations in chloroform concentrations will exist. This has been reported previously (Albers et al. 2008; Albers et al. Submitted), and the existence and
505 distributions of chloroform Hot Spots in soil, that we present in this paper, may help to explain why great variations in natural chloroform in the groundwater will exist.

Formation and mineralization of chloroform

The soil incubation study with soil from TH confirmed that there was a higher net formation of
510 chloroform in the Hot Spot than in the Low Spot, but with huge variations between samples. Except for one sample, the Hot Spot samples showed much lower net formation than what would be expected from the flux measurements performed in the field. It is possible though, that even within

a Hot Spot the variation is great when sampling only 28 cm² of soil as we did. Preliminary investigations actually showed that the chloroform concentration in the top organic horizon could vary by a factor of more than 10 within a distance of just a few decimetres (results not shown).

We performed a mineralization experiment with ¹⁴C-labeled chloroform to discover any differences in aerobic degradation rates between soil from a Hot and a Low Spot. The study sites are all characterized by well oxygenated sandy soils (Albers et al. Submitted), and even though anaerobic niches might theoretically occur in the organic top soil after heavy rain events, any degradation that could cause the permanent difference between Hot and Low Spots would be expected to be aerobic. Abiotic degradation of chloroform is known in anoxic sediments (Kenneke & Weber 2003), but no abiotic aerobic pathway for the degradation of chloroform is known and the only known aerobic degradation pathway for chloroform is co-metabolic degradation by various oxygenase-expressing bacteria (Bartnicki & Castro 1994; Alvarez-Cohen et al. 1992; Chang & Alvarez-Cohen 1996). This degradation pathway leads to complete mineralization of the chloroform molecule and we therefore performed a mineralization experiment. The experiment revealed that a small mineralization of chloroform takes place in the forest soil, but more important here, the mineralization was not higher in the Low Spot than in the corresponding Hot Spot (Table 2). Mineralization can therefore not explain the differences in chloroform concentrations and since other (partial) degradation apart from mineralization is unlikely, higher degradation in the Low Spots seems not to explain the presence of the Hot Spots. In the literature, not much can be found on aerobic degradation of chloroform in natural soils, but our results of 0.3 – 0.9% mineralization d⁻¹ are well in line with the only previously published data, where 3% of the added chloroform was converted to CO₂ within 5 days in a sandy loam, corresponding to 0.6% mineralization d⁻¹ (Strand & Shippert 1986).

Soil characteristics

The various measurements of concentration, flux, formation and degradation of chloroform all pointed in the direction that the Hot Spots were the result of a higher gross formation of chloroform in the Hot Spot soil. Since the differences between Hot Spots and Low Spots could not be accounted for by visual observations in the field, measurements of various soil parameters were performed in order to narrow down the factors controlling the formation of Hot Spots. An overview

of these measurements is given in Table 4, together with a brief conclusion regarding the importance of each parameter.

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Table 4. Summary with full ranges of all parameters measured in order to gain insight into the factors controlling the highly elevated chloroform concentration in the Hot Spots. See the Materials and Methods section for further information on the various sample types. n is the total number of samples, in all cases distributed nearly or exactly identically between Hot Spots and Low Spots. The text should be consulted for further details leading to the brief conclusion concerning each parameter.

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Parameter	Location and sample type	n	Hot Spot	Low Spot	Conclusion
Identification of the CHCl ₃ Hot Spots	All sites: Soil air concentrations. NF and TH: Fluxes to the atmosphere and groundwater, formation and degradation of CHCl ₃ in soil in the laboratory.		High concentrations of CHCl ₃ in soil air and high fluxes to the atmosphere and groundwater. High formation but low degradation of CHCl ₃ in laboratory.	Much lower, but still increased conc. in soil air. Small positive fluxes to the atmosphere and groundwater. Low formation and degrad. of CHCl ₃ .	Higher gross and net formation of chloroform in Hot Spots, but also a small formation in the Low Spots.
O-horizon	TH: Thickness F+H-layer	32	3.5-20 cm	5-14 cm	No difference / significance
CO ₂	TH: Soil air	41	0.11-0.28 %	0.11-0.22 %	No difference / significance in NF. Slightly higher in TH Hot Spot?
	TH: Soil emission (August)	10	80-181 mg m ⁻² h ⁻¹	81-148 mg m ⁻² h ⁻¹	
	NF: Soil emission (June)	10	39-94 mg m ⁻² h ⁻¹	50-125 mg m ⁻² h ⁻¹	
	TH: Incubation of soil	8	231-373 mg m ⁻² h ⁻¹	210-333 mg m ⁻² h ⁻¹	
CH ₃ Cl	NF: Soil emission (June)	10	104-2400 ng m ⁻² h ⁻¹	580-4000 ng m ⁻² h ⁻¹	No difference / significance
pH	TH: pH _{H2O} in O-horizon	10	3.9-4.2	4.1-4.6	No difference / significance
	TH: pH _{H2O} in B-horizon	32	3.8-4.6	3.9-4.8	
	NF: pH _{H2O} in O-horizon	8	3.6-4.0	3.8-4.0	
Cl ⁻	TH: H ₂ O-extr. Cl ⁻ in B-hor.	32	3.9-9.0 mg kg ⁻¹ soil	2.1-10.9 mg kg ⁻¹ soil	No systematic variation / difference
	NF: Cl ⁻ in F-layer	6	50-217 mg kg ⁻¹ soil	242-285 mg kg ⁻¹ soil	
	NF: Cl ⁻ in H-layer	6	37-185 mg kg ⁻¹ soil	4-61 mg kg ⁻¹ soil	
Fe	TH: H ₂ O-extr. Fe in B-hor.	32	0.12-3.5 mg kg ⁻¹ soil	0.23-3.5 mg kg ⁻¹ soil	No difference / significance
Chlorination degree of SOM	NF: TOCl in F-layer	6	0.41-0.69 g kg ⁻¹	0.53-0.61 g kg ⁻¹	Slightly higher in Hot Spot H-horizon?
	NF: TOCl in H-layer	6	0.40-0.81 g kg ⁻¹	0.49-0.56 g kg ⁻¹	
Quality of SOM (chem. chlorination)	NF: CHCl ₃ formed, F-hor.	6	0.14-0.19 g kg ⁻¹	0.13-0.22 g kg ⁻¹	No difference between Hot Spots and Low Spots
	NF: CHCl ₃ formed H-hor.	6	0.31-0.36 g kg ⁻¹	0.33-0.36 g kg ⁻¹	

General microbial activity (CO₂)

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CO₂ was measured in the soil air samples as well as in the flux chambers as an estimate of the general microbiological activity in the soil. It was the only parameter that showed a statistically significant relationship with the chloroform concentration. This positive relationship, even though statistically significant, is pretty poor, though, (Figure 6) and furthermore the incubation study with soil from TH showed no differences in CO₂-production between Hot and Low Spot soil. In NF, no relationship between chloroform and CO₂ seemed to occur, at all. The general microbial activity is therefore not the single important parameter with regards to chloroform concentration in the soil.

General soil parameters

The thickness of the organic horizon was measured since this is where the main chlorinating activity including chloroform formation has been found to occur in forest soil (Asplund *et al.*, 1993; Hoekstra *et al.* 1998a; Haselmann *et al.* 2000; Albers *et al.* Submitted). The thickness of the organic layer was varying from 3.5-20 cm, which is a quite substantial variation, but no difference in thickness or type of organic horizon was found between the Hot Spots and the Low Spots.

pH was measured, since both haloperoxidase enzymes and the natural unspecific chlorination processes in soil have been shown to be pH-dependent (Asplund *et al.* 1993; Vollenbroek *et al.* 1995; Öberg *et al.* 1996; Sheng & Gold 1997) and the abiotic formation pathway seems to be very dependent on pH as well (Huber *et al.* 2009). The pH was acidic at all sites and at all sampling points, but again, no difference could be observed between Hot and Low Spots.

Chloride is a substrate in the natural chlorination reactions including the formation of chloroform and we therefore measured the chloride concentrations in both the organic and minerogenic horizons. Again, despite a large variation in the dataset, no systematic variation towards neither higher nor lower concentrations in the Hot Spots was found.

Water extractable iron was measured in the top soil, since an abiotic formation pathway of chloroform, which includes Fe(III) in the reaction, has recently been demonstrated in the laboratory (Huber *et al.* 2009). Other approaches for measuring iron (e.g. extraction with oxalate) could have been taken and the water-extractable iron is only a minor portion of the total iron. When the different soil samples are very similar regarding soil texture and pH as in our case, the approach gives an idea, though, whether or not iron could be an important parameter for the occurrence of chloroform Hot Spots and in this case it seemed not to be so. One possible hypothesis is however, that a sort of “background” abiotic formation of chloroform occurs in both Hot and Low Spots. This formation could then explain the concentration of ~10 times the atmospheric background in the Low Spot areas, while microorganisms are responsible of the differences between the Hot and the Low Spots. This hypothesis could be interesting to test in future studies.

Total organic halogens

If the current hypothesis is true, that the chloroform formation is a result of unspecific chlorination caused by chlorinating enzymes (see e.g. Hoekstra *et al.* (1998a)), one might expect that the chloroform Hot Spots were the results of a general increase in chlorinating activity and that this

increased activity was also reflected in a higher total organic chlorine concentration in the soil. Whereas for the F-horizons no difference was seen between Hot and Low Spot, this could actually seem to be the case for the H-horizon, where on average 2 times as much TOCl was present in the
595 Hot Spot soil compared to the Low Spot soil (Table 3). This difference is however diminished, when the TOCl-concentration is recalculated on a SOM-basis, meaning that the difference in the degree of chlorination of the organic matter is not very large between Hot and Low Spot. The large range of TOCl-values in especially the Hot Spot, makes it even harder to conclude that the SOM in the Hot Spot H-horizon is actually more chlorinated. It could be noted, however, that the sample
600 with the highest chloroform-concentration in the A_h-horizon (2.5 µg (kg soil)⁻¹) was the sample that showed the highest amount of TOCl in the H-horizon (812 µg (g SOM)⁻¹). To finally conclude on this, it would take a larger number of samples to be analyzed. It should be mentioned here also, that in addition to the unspecific halogenation mechanism, TOCl is also suggested to be formed by incorporation of demethylated fungal chlorinated anisyl metabolites (CAMs) into SOM (Öberg et al. 1997; Hjelm et al. 1999). Since CAMs are formed in a specific intracellular halogenation
605 reaction, this formation pathway for TOCl will not lead to the formation of chloroform and will therefore blur the relationship between TOCl and chloroform in soil.

Bromine and iodine were almost exclusively present as organic halogen and in all cases there was too little inorganic bromine and iodine present to be quantified. This is well in line with previously
610 published data on bromine and iodine speciation in soil (Maw & Kempton 1982) and probably reflects the preference of the halogenating enzymes for bromine and iodine, which both have a lower oxidation potential than chlorine (Sheng & Gold 1997). The relatively high TOCl/TICl ratios (in all cases >1) show that there is a substantial chlorination taking place in the soils. This seems however, to be the case in both Hot and Low Spot soil and more samples and preferentially a more
615 specific assay for determining unspecific chlorinating activity in the soils would be necessary for more insight into how chloroform is formed along with the macromolecular halogen, and whether general chlorinating activity can explain the differences between Hot and Low Spots. Comparing with the literature, our findings of ~600 µg TOCl (g SOM)⁻¹ and an additional ~100 µg TOBr+TOI (g SOM)⁻¹ falls within the values found for O-horizons of temperate coniferous forests. TOX values
620 of such soils have been reported to typically vary from 100-1000 µg TOCl (g SOM)⁻¹ (Asplund et al. 1989; Hjelm et al. 1995; Johansson et al. 2001). How much of the TOCl reported in these studies actually being Br and I is unknown, since the TOCl was estimated from the group parameter TOX.

Our results show that a significant pool of organic Br and I exists in the soil, and future studies should preferentially determine not just TOX, but rather the individual halogens, if possible.

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Chemical chlorination

It is well known from the literature on chlorination of drinking water that the amount of chloroform formed upon chlorination of organic matter varies not only with the quantity but also the quality of the organic matter (e.g. Reckhow et al. 1990). Since no difference in soil or vegetation was visible
630 between Hot and Low Spots, no major differences in chloroform precursors and hence chloroform formation potentials between Hot and Low Spot soil were expected. To exclude this possibility, Hot and Low Spot soil samples were chlorinated at a relatively low chlorine concentration. A difference between F- and H-horizon samples, but not between sites, was found, and hence it seems that the difference between Hot and Low Spot is not the quality of the organic matter from which the
635 chloroform is expected to be derived.

Conclusions

In conclusion, we have confirmed previous findings, that natural chloroform is formed in the soils of temperate coniferous forests and furthermore we have shown that the spatial variation in
640 chloroform concentration in the soils, which exceeds two orders of magnitude within single stands, is not randomly distributed in the forest floor, with points of high chloroform concentrations located close to each other in “Hot Spot” areas of varying sizes. Chloroform concentrations, emissions and formation rates are all highest in these Hot Spots, and the distributions of the Hot Spots were found to be unchanged within a two-year period. This is important to bear in mind, both when interpreting
645 the various published studies on natural chloroform in forests but also when designing future experiments. Furthermore our results show that one should be very careful to use small datasets for calculating natural chloroform emissions to the atmosphere on a global, regional or even local scale. Regarding the mechanism behind the formation of chloroform, we eliminated a number of causal relations between the chloroform Hot Spots and various parameters, potentially important for the
650 formation of these Hot Spots. The spatial pattern, in addition to the positive influence of both soil temperature and soil moisture, supports the hypothesis that fungi are likely to be involved in the formation. On the other hand more than one mode of formation might co-exist and one possibility is that abiotic formation could cause a background concentration and that this background concentration is what we see in the Low Spots. Fungal activity could then explain the highly
655 elevated concentrations in the Hot Spots. To fully conclude on this, further studies are needed. These should preferably manage to quantify chlorinating enzymes / chlorination potential of soils, more specifically than analyzing the TOCl contents of the soils or, if possible, to identify the responsible fungal species.

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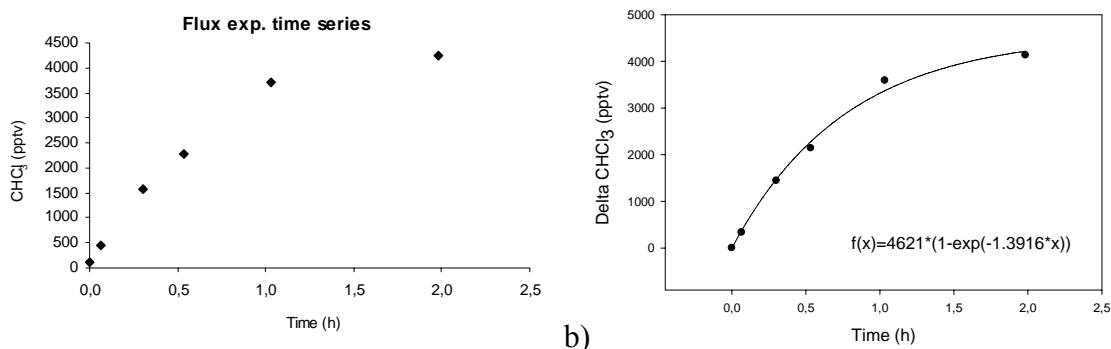
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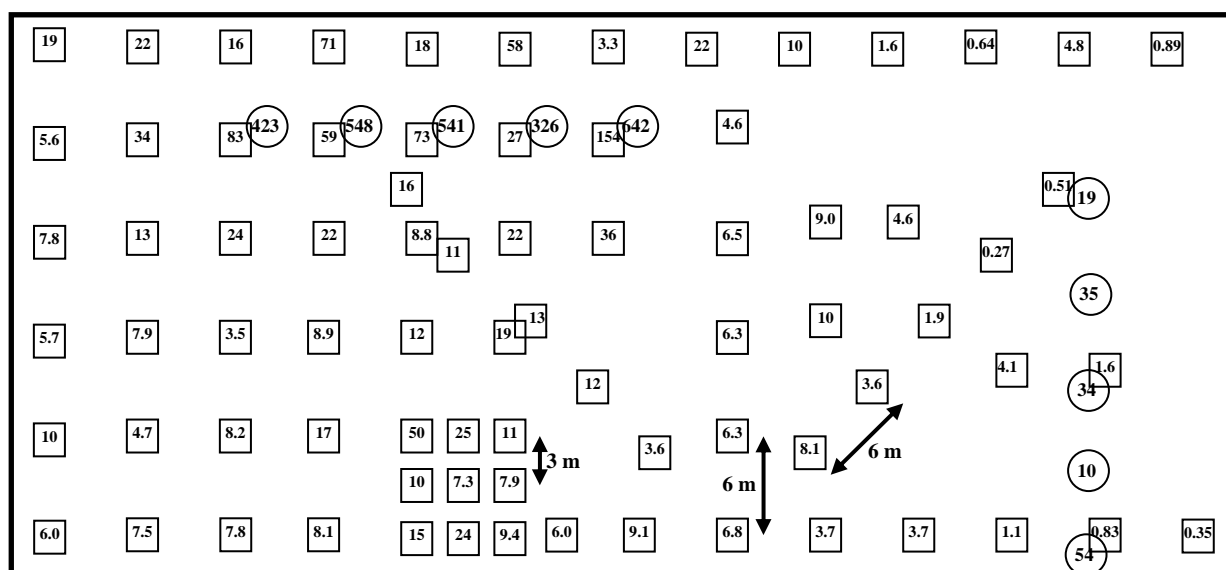
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Supplementary Material:

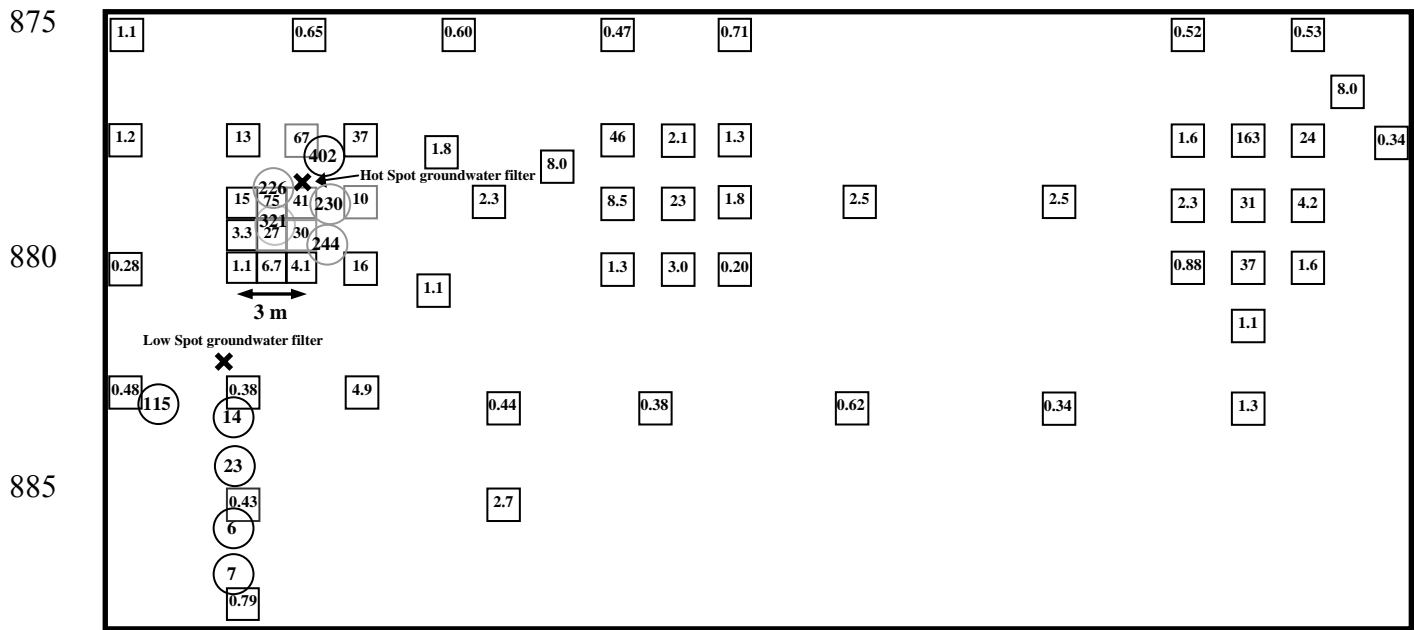


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Supplementary Figure S1. a) Time series for a static chamber flux-measurement in Tisvilde Hegn. The concentration in the steel canister was determined at the beginning of the experiment and then five times during the 2 hour incubation. b) Best fit of an exponential rise to maximum function for the changes compared to the initial atmospheric concentration. $Y = 4621 * (1 - e^{-1.3916X})$, where Y is the change in chloroform concentration and X is the incubation time in hours.



Supplementary Figure S2. Sample location and chloroform concentration in NF. Values in squares are the chloroform concentrations (ppbv) measured in soil air at 40 cm depth in autumn 2007 and spring 2008. Values in circles are the chloroform fluxes (ng m⁻² h⁻¹) measured in June 2009.



Supplementary Figure S3. Sample location and chloroform concentration in TH. Values in squares are the chloroform concentrations (ppbv) measured in soil air at 40 cm depth in spring 2007. Values in circles are the chloroform fluxes (ng m⁻² h⁻¹) measured in June 2009. The locations of the filters for abstraction of the upper groundwater below Hot and Low Spots are marked with crosses.

III**Formation, fate and leaching of natural chloroform in coniferous forest
soil**

Christian N. Albers, Troels Laier, Ole S. Jacobsen
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1 = Minor

2 = Substantially

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|---|---|
| a) Basic formulation of the project leading to the published work | 2 |
| b) Strategy for the project / choice and development of methods | 2 |
| c) Implementation of the project / empirical work | 2 |
| d) Analysis, interpretation and discussions | 2 |

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Formation, fate and leaching of natural chloroform in coniferous forest soil

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Abstract

Leaching of natural chloroform from forest soil to groundwater was followed by regular analysis of soil air and groundwater from multilevel wells at four different sites in Denmark for a period of up
10 to four years. Significant seasonal variation in chloroform was observed in soil air 0.5 m below surface ranging at one site from 120 ppb by volume in summer to 20 ppb during winter. With depth, the seasonal variation diminished gradually, ranging from 30 ppb in summer to 20 ppb during winter, near the groundwater table. Chloroform in the shallowest groundwater ranged from 0.5–1.5 $\mu\text{g L}^{-1}$ at one site to 2–5 $\mu\text{g L}^{-1}$ at another site showing no clear correlation with season. Comparing
15 changes in chloroform in soil air versus depth with on-site recorded meteorological data indicated that a clear relation appear between rain events and leaching of chloroform.

Chloroform in soil air co-varied with CO_2 given a delay of 3–4 weeks providing a strong evidence for its biological origin. The delay in chloroform change relative to CO_2 was observed both in nature and in laboratory incubation experiments. Sorption of chloroform to soils, examined using
20 $^{14}\text{C-CHCl}_3$ correlate with organic matter content, being high in the upper organic rich soils and low in the deeper more minerogenic soils. The marked decrease in chloroform in soil with depth may in part be due to microbial degradation which was shown to occur at all depths by laboratory tests using $^{14}\text{C-CHCl}_3$.

25 **Keywords:** Natural chloroform, seasonal variation, leaching, fate, sorption, forest

Introduction

In Denmark, a maximum of $1 \mu\text{g L}^{-1}$ of volatile halogenated hydrocarbons including chloroform is allowed for groundwater extracted for drinking water supply. Recently, however, Danish authorities agreed to raise the maximum allowed chloroform concentration to $10 \mu\text{g L}^{-1}$, provided reasonable
30 evidence concerning a natural source can be established. More knowledge on the production and subsequent fate of chloroform in soil is therefore of great interest.

Chloroform (CHCl_3) is a volatile organic pollutant originating from direct industrial production and as a by-product in various chlorination processes. Only 10–25% of the flux of $0.3\text{--}1.0 \text{ Tg yr}^{-1}$ to the atmosphere can be accounted for by human activity, and the remaining $\geq 75\%$ must come from
35 various natural sources (Laternus *et al.*, 2002; Cox *et al.*, 2003; McCulloch, 2003; Worton *et al.*, 2006). These natural sources have not been fully identified, though, and the modes of formation are still on a rather hypothetical level. One identified terrestrial source is the soils of temperate coniferous forests (e.g. Hoekstra *et al.*, 1998a; Laternus *et al.*, 2000; Haselmann *et al.*, 2000; Albers *et al.*, 2008; Albers *et al.*, submitted), and besides emission to the atmosphere (Hoekstra *et al.*,
40 2001; Dimmer *et al.*, 2001; Albers *et al.*, submitted), a leaching of chloroform to the groundwater seems to occur in temperate coniferous forests (Laternus *et al.*, 2000; Albers *et al.*, 2008). Laier *et al.* (2005) found that natural chloroform in groundwater beneath coniferous forests could be distinguished by its stable carbon isotopic ratio, $\delta^{13}\text{C}$: $-13 \text{--} -27\%$, from industrial chloroform having $\delta^{13}\text{C}$: $-46 \text{--} -63\%$. Albers *et al.* (2008) showed that the natural chloroform could escape
45 throughout the unsaturated zone and enter the upper groundwater in $\mu\text{g L}^{-1}$ -concentrations, which corresponded well with the measured soil air concentrations just above the groundwater table.

The previous studies (Hoekstra *et al.*, 1998a; Laternus *et al.*, 2000; Albers *et al.*, 2008) on natural chloroform in soil air and groundwater merely provided snapshots of chloroform distribution versus
50 depth, leaving the authors to speculate to what degree, and by what mechanism natural chloroform affects groundwater quality. Therefore, it was decided to study and map how chloroform is formed in forest soils in greater detail and how it leaches to groundwater. Having performed detailed mapping of the spatial variation of chloroform in soils (Albers *et al.*, submitted) we now focus on changes in vertical distribution of chloroform versus depth with time. For this purpose we regularly
55 sampled and analysed groundwater and soil air from multilevel wells located in four different temperate coniferous forests during periods of 2 to 4 years. Chloroform plus other chemical

constituents were determined in the soil air and groundwater. In addition, precipitation, soil temperature and groundwater levels were monitored on an hourly basis at the different locations in order to narrow down factors of importance for the production and leaching of chloroform.

60 Production, sorption and degradation of chloroform were also studied in the laboratory, in order to provide an understanding of the processes that may affect the leaching of chloroform to the groundwater.

Materials and Methods

65 *Study sites and installations*

The sites for studying vertical distribution of chloroform were selected among forest areas, where relatively high chloroform concentrations ($1\text{--}12\ \mu\text{g L}^{-1}$) were observed in shallow groundwater examined regularly by the national groundwater quality monitoring program. The four forests are all coniferous plantations named (abbreviations, GPS locations): Tisvilde Hegn (TH, $12^{\circ}03'40''$ E, $56^{\circ}02'22''$ N); Viborg Hedeplantage (VH, $9^{\circ}22'14''$ E, $56^{\circ}25'37''$ N); Liseborg Plantage (LP, $9^{\circ}21'33''$ E, $56^{\circ}25'42''$ N) and Nordre Feldborg (NF, $8^{\circ}57'01''$ E, $56^{\circ}22'51''$ N) (Fig. 1). Soil types and vegetation have been reported previously (Albers *et al.*, submitted). The soil at all study sites is sandy and the top soil is acidic and covered with an organic layer, ~ 10 cm thick. In TH, no moss or underwood is present due to dense plantation while at the other more open sites, the soil is partly
75 covered with mosses.

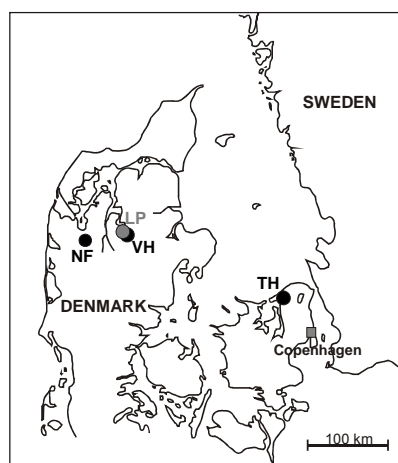


Fig. 1. Map showing the location of the four study sites.

At each site, a 4–6 m deep multilevel well was established during 2005–2007 using a 10 cm diameter hand auger. Six to eight 5 cm brass filters fitted with 6 mm PE tubing were then placed from the bottom and up to 0.3 m (TH + NF) or 0.5 m (VH + LP) below surface at specific intervals, with one to three filters being below the groundwater level. The sandy sediment from the drilling was back filled into the borehole around the filters, which were separated by a 10 cm bentonite plug (Albers *et al.*, 2008). An extra well for logging the groundwater level and for water sampling was established 2–5 meters from the multilevel well. Each well has a 1-meter screen below the apparent water level and the water level was monitored by a water level logger (HOBO U20-001-04, ONSET, MA, US). Atmospheric temperature and soil temperature (15 cm depth) were recorded by sensors installed near the multilevel wells. Precipitation was also recorded by a rain gauge (Pronamic, Silkeborg, DK) in a nearby open area at three of the sites (TH; VH; NF). Soil moisture at 15 and 50 cm depth next to the TH profile, was determined with a TDR-probe connected to a data logger (S-SMC-M005 and H21-002, Onset, MA, US).

95 **Field studies**

Soil samples collected during drilling of the multilevel well were used for chemical and textural analysis. pH was determined in a 2:1 water:soil slurry. Water content was determined by drying for 24 h at 105°C. The content of soil organic matter (SOM) was determined as loss on ignition (2 h, 550°C). Soil texture was determined by sieving with weighing of the fractions >2 mm (gravel), 0.6–2 mm (coarse sand), 0.2–0.6 mm (medium sand), 0.063–0.2 mm (fine sand) and <0.063 mm (silt and clay).

Sampling and analysis. The TH profile was monitored regularly (every 1–4 weeks) during two years. The VH and LP profiles were monitored regularly (every 3–6 weeks) during two years and then less regularly (every 4–16 weeks) during another two years. The NF profile was monitored regularly (every 3–6 weeks) during 8 months and then less regularly (every 4–16 weeks) during another two years. Due to problems with the upper filter (0.5 m depth) in LP, data from this filter could not be used and instead two additional filters (0.3 and 0.5 m depth) were installed after ~1½ year of monitoring.

Groundwater was sampled from the filters below the groundwater table using a peristaltic pump. A minimum of 1 L was pumped to waste before sampling in 125 mL screw bottles. The bottle placed

in a 1 L container was filled from the bottom allowing the steady flow of water to fill up the container before closing the bottle under water. The samples were taken to the laboratory and analyzed according to the procedure described by Busenberg and Plummer (1992). Field measurements of dissolved oxygen content and pH were performed along with groundwater
115 sampling.
Soil air was sampled from the filters in the unsaturated zone and from the atmosphere just above the forest floor in steel cylinders using a membrane pump (Albers *et al.*, submitted). Gas samples were analyzed for chloroform and CFCs and other C₁-organohalogens using the procedure described by Busenberg and Plummer (1992). The limit of quantification (LOQ) for chloroform in gas samples
120 was 10 pptv (10⁻¹² vol/vol) and for water samples 0.2 ng L⁻¹. For soil air samples, the total uncertainty of sampling and analysis is below 3% (Albers *et al.*, submitted). For water samples, the uncertainty of the analysis is slightly higher than for air samples.
Gas samples were also analyzed with respect to CO₂ and O₂ on a gas chromatograph equipped with a TCD detector (Albers *et al.*, submitted).

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Laboratory tests

In order to gain insight into the processes, which determines the vertical and seasonal patterns observed in the field, a few laboratory studies were conducted to estimate 1) Chloroform net production, 2) Chloroform sorption, and 3) Chloroform degradation.
130 **Net production of chloroform.** Soil in push-cores (H: 18 cm and Ø: 6 cm) were sampled in triplicates in NF, March 2007 and stored at 5°C for 14 days. The soil cores were then split into three visually distinguishable soil layers; the L-horizon (fresh litter, 0 to ~1 cm depth), the F-horizon (partly degraded litter, ~1 to ~7 cm) and the A_h-horizon (organic enriched minerogenic horizon, ~7 to 18 cm). Each layer was incubated at 15°C in a closed 1 L glass jar fitted with a valve for
135 sampling as described in Albers *et al.* (submitted). The air surrounding the incubated soils was analyzed for CHCl₃, CO₂ and O₂ every 7 days for the first 28 days and then every 10–14 days. To avoid anoxia, 25 mL pure O₂ were added to each jar after each sub-sampling of headspace gas. One of the L-horizons started to leak after two weeks of incubation and was therefore not included in any calculations of either CO₂- or chloroform-production.
140 **Sorption study.** 9 x 2–8 g of each soil (wet weight, depending on SOM-content) were transferred to a 15 mL glass vial with aluminium-lined lid and added 5 mL H₂O + 0.5 mL 1M NaN₃ (to avoid

microbial degradation of chloroform). After 24 hours of shaking, 50–5000 μL aqueous $^{14}\text{C-CHCl}_3$ (ARC Inc., St. Louis, MO, radiochemical purity >99%) solution was added and the vials were filled to the top with H_2O (2800–280000 DPM per glass corresponding to a final concentration of ~ 5 , ~ 50 and $\sim 500 \mu\text{g L}^{-1}$, in triplicates). A pilot study indicated that equilibrium between soil and water was almost achieved after 6 hours and that it was complete within the analytical error in less than 70 hours, which was then chosen for the duration of the following experiments. After these 70 hours of shaking, the samples were centrifuged (1000g, 15 min.) and the ^{14}C -activity in the water was determined by Liquid Scintillation Counting (LSC). 3 x 3 blanks with no soil added were treated similarly to account for any losses due to evaporation or sorption to the vials, but no significant losses were found. Sorption was described using Freundlich-isotherms:

$$C_s = K_F \times C_w^n,$$

where C_s is the concentration of chloroform in the soil in $\mu\text{g kg}^{-1}$, C_w is the concentration in the liquid in $\mu\text{g L}^{-1}$, K_F is the Freundlich coefficient in L kg^{-1} and n describes the non-linearity of the isotherm.

Mineralization study. Different soil fractions (10 g homogenized O-horizon and 15 g 2 mm-sieved minerogenic soil, respectively) were placed in 600 mL glass jars and 2 mL H_2O or 2 mL NaN_3 (10%, for semi-sterile controls) were added to the A- and B-horizons. All jars were equipped with a small glass container for CO_2 -absorbing liquid (3 ml 1M NaOH) and 1 mL $^{14}\text{C-CHCl}_3$ solution (75000 DPM mL^{-1} , corresponding to 100–150 $\mu\text{g kg}^{-1}$ soil wet weight) was then added to the soil in each jar. The lid was closed immediately after the addition of $^{14}\text{C-CHCl}_3$ and the mineralization of chloroform was then followed for 28 days, according to the method described in Albers *et al.* (submitted). In brief, the NaOH was changed every 4–10 days and the $^{14}\text{CO}_2$ produced by the mineralization of $^{14}\text{C-CHCl}_3$ was separated from dissolved $^{14}\text{C-CHCl}_3$ by precipitation with BaCl_2 (Albers *et al.*, submitted). The mineralization study was performed at 10°C , and with total soil moisture of 80% in the organic soil and 11–39% in the minerogenic soils.

Results and Discussion

Field data

170 *Site descriptions*

Texture, soil organic matter (SOM) content and pH determined on soil samples, collected during multilevel drillings, is shown in Fig. 2 and Appendix Table A1. The pH varies from 4 to 5 except at TH, where pH increases gradually with depth from around 4 to almost 8, most likely due to increasing carbonate content in the soil at TH, especially below 4 m depth. The lithology of all well profiles shows mostly medium to fine sand, with higher silt/clay content in the upper one meter, particularly for the NF profile. In LP, the sand seems to be even coarser than at the other three profiles.

The oxygen content in soil air remains high (>19%) throughout the unsaturated zone at all four locations. Furthermore, dissolved oxygen is close to saturation ($\sim 10 \text{ mg L}^{-1}$) in the shallow groundwater.

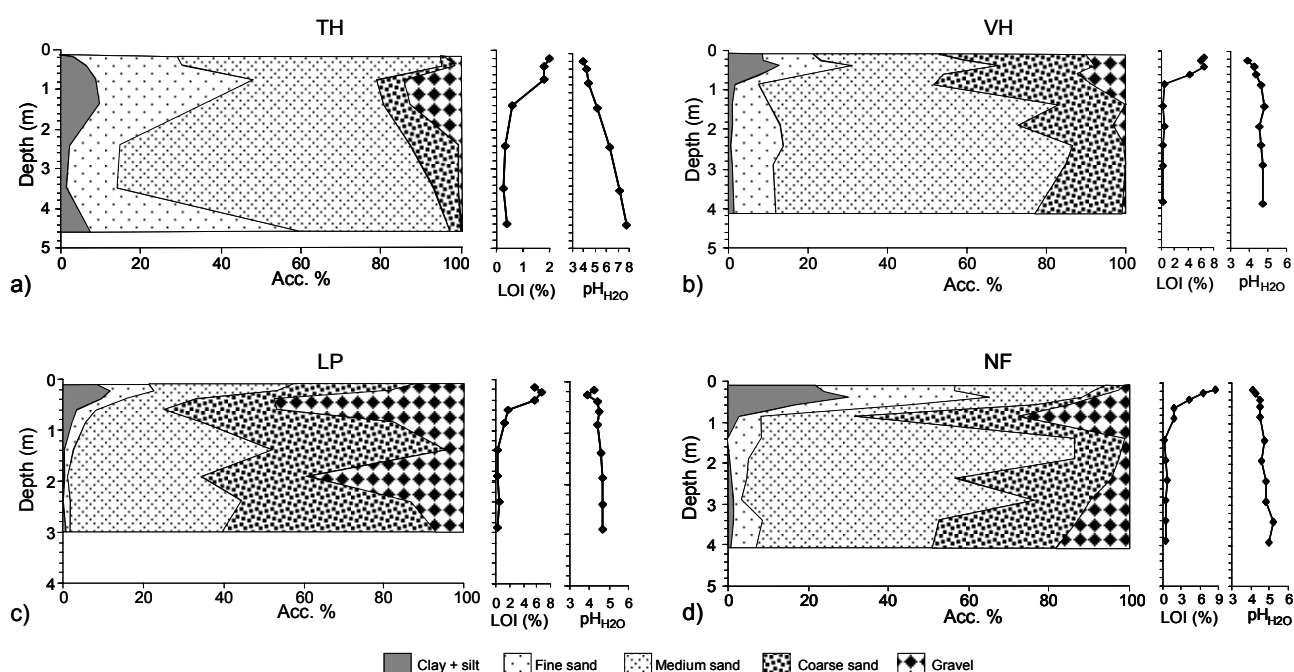


Fig. 2. Texture and other basic data for the minerogenic horizons of the four profiles. The texture is shown as the accumulated percent of each size fraction, starting with the finest material (Clay + silt). Note that the scale on the second axis is different for the LP profile. The exact sampling depths and all data values are gathered in Appendix Table A1.

Vertical and seasonal variations

Great variations in chloroform concentrations can be observed between sites, depths and seasons (Figs. 3–5). We will start by describing the variations observed between the four sites. Despite the apparent similarities of the four sites regarding vegetation and soil types, the vertical and temporal distributions of chloroform are very different. During summertime, chloroform concentrations are

much higher in TH and LP than in the other two profiles, but also the shapes of the vertical concentration curves are different between the profiles (Fig. 3). During summer, there is an increase in chloroform during the first ½–1 m and then a more or less steady decrease down the profile in TH and LP. In VH, where the highest filter was placed at 0.5 m depth and any increase from 0.3 to 0.5 m therefore could not be observed, a similar decrease is seen, although the starting concentration was somewhat lower. In NF, the concentration in the top soil is much lower than in the other profiles, and here the chloroform concentration increases slightly down the profile during both summer and winter.

Three studies on the vertical distribution of natural chloroform in forest soil have previously been reported, and they all showed a somewhat similar trend with maximum concentrations at ~1 m depth and then a decrease further down the profile (Laternus *et al.*, 2000; Hoekstra *et al.*, 2001; Albers *et al.*, 2008). This is very similar to what we observe for the TH, LP and possibly VH profiles. Albers *et al.* (2008) included two neighbouring profiles, one of which was located in a small (~30 m²) area with high chloroform production and one in an area with low production. The profile from the low production area showed a somewhat similar trend during summer to what is seen for the NF profile, with a slight increase down the profile, possibly caused by diffusion of chloroform from neighbouring areas with higher concentrations. The presence of areas with both high and low concentrations of chloroform was recently reported for all four study sites (Albers *et al.*, submitted) and while the TH, VH and LP profiles are all located in areas with high production rates, the NF profile is located right on the border between an area with high and low chloroform concentrations in the soil. This possibly explains the very different shape of the chloroform concentration profile in NF, which may be significantly influenced by the neighbouring area with high chloroform concentrations, especially in deeper parts of the profile.

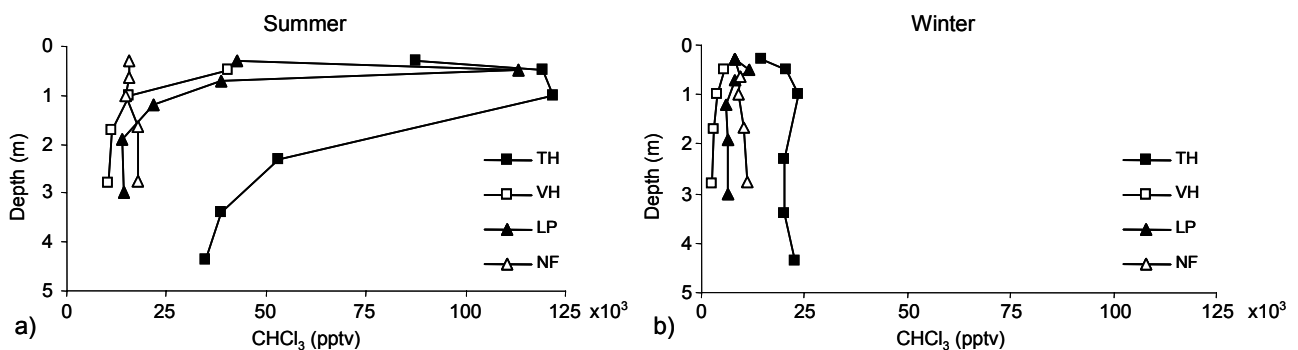


Fig. 3. Snapshots of chloroform concentrations throughout the unsaturated zones. a) August 2007, b) February 2008.

220 The concentrations of chloroform in the four profiles were monitored to varying degrees and during different time lengths as described in the Materials and Methods section. This procedure made it possible to observe both short term changes in concentration caused e.g. by sudden changes in temperature and soil moisture, and the long term variation due to seasonal changes and year to year variations.

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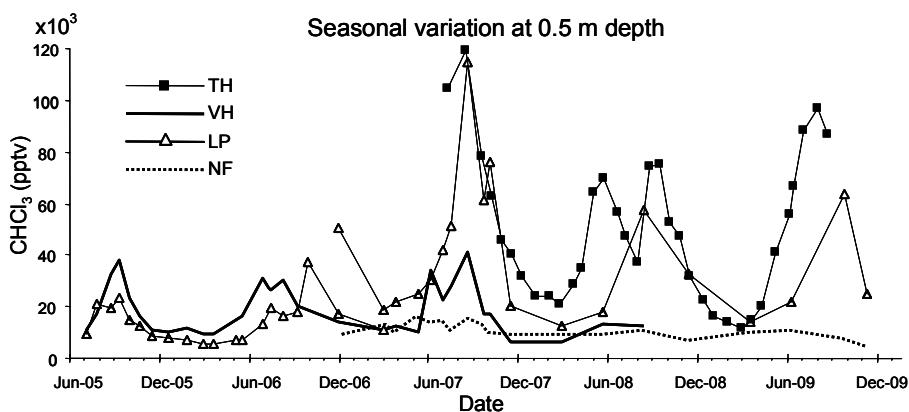
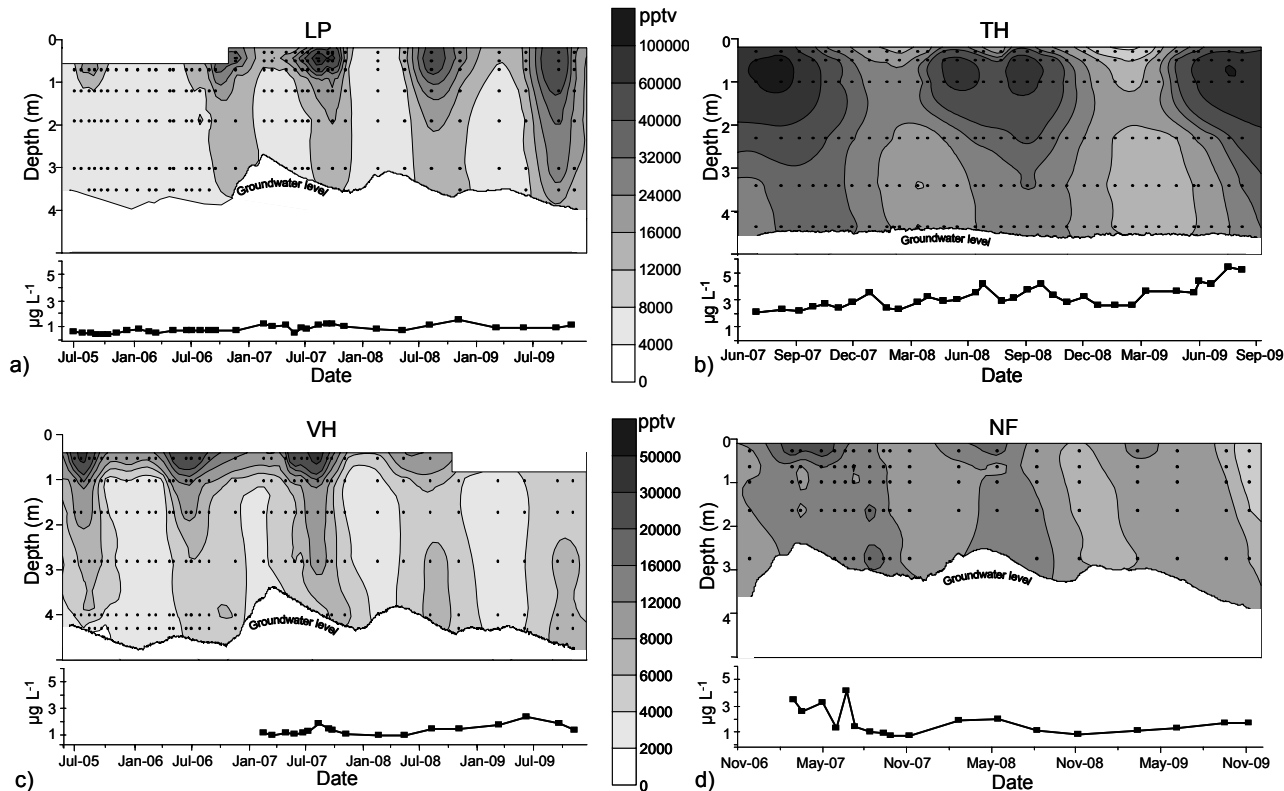


Fig. 4. Seasonal variation in chloroform concentration at 0.5 m depth in the four profiles. For clarity, the data marks are left out for the VH and NF filters, since they were sampled at the same dates as the LP filter, although the 0.5 m filter in NF was installed at a later time and in VH, the upper filter was blocked during the last ~1 year of sampling. In LP, the 0.5 m filter was not installed until autumn 2006, and data from the filter at 0.7 m depth is therefore shown for the first ~1½ year period, with a short overlap in time with the 0.5 m filter.

All four profiles show clear seasonal variation, with highest chloroform concentrations during the summer period (Figs. 4 and 5). The increase in concentration during spring and summer is substantial in the upper layers (Figs. 4 and 5), but less so at greater depths (Fig. 5). Furthermore, there appear to be a slight delay down the profile (Fig. 5), which probably relates to transport of chloroform from a shallow source.

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240 **Fig. 5.** Chloroform throughout the unsaturated zone of the four profiles. Linear kriging was used to estimate values between sampling dates and depths, represented by dots. In LP, the two upper filters were not installed until autumn 2006 and in VH, the upper filter was blocked during the last ~1 year of sampling (blank areas). Note that the chloroform scale is different in TH and LP, compared to VH and NF. The groundwater level and the chloroform concentration in the shallowest groundwater are shown below.

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The clear seasonal variations observed just above the groundwater table in the TH, VH and LP profiles are not reflected in the shallowest groundwater (Fig. 5). Elevated chloroform concentrations do seem to occur after summer 2007 (VH and NF) and summer 2008 (LP), but no clear seasonal variation exists. In TH, an increase from ~2 to ~5 $\mu\text{g L}^{-1}$ was noted during the 2 year period of observation (Fig. 5). Apparently, the chloroform containing water is being mixed, as it reaches the groundwater, to a degree, which almost completely masks any seasonal trends. Based on calculations using Henry's Law, near equilibrium does exist for chloroform in soil air and groundwater, though (Table 1). In three of the profiles, the concentration in the groundwater is slightly higher than expected from the analysis of the soil air from above the groundwater table.

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255 This would further imply that either the equilibrium between soil water and soil air is not fully established or it is shifted towards higher concentration in soil water as compared to pure water, due to e.g. the presence of dissolved organic matter. Laboratory experiments would be needed in order

to determine the exact equilibrium between soil air and soil water. An alternative explanation could be the influence of laterally flowing groundwater. Only in LP, the upper filter was usually right
 260 below the groundwater table. In the other profiles, a few decimetres often separated the groundwater table and the filter used to abstract the shallow groundwater. The chloroform concentration could then be influenced by unknown chloroform concentrations upstream, which may be very different due to the huge spatial variation in chloroform production (Albers *et al.*, 2008; Albers *et al.*, submitted).

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Table 1. Comparison of measured chloroform concentrations ($\mu\text{g L}^{-1}$) in the shallow groundwater with calculated soil water concentrations in the deepest part of the unsaturated zone. Using the dimensionless Henry's Law constant for chloroform (Görgényi *et al.*, 2002), one can calculate the chloroform concentration in the soil water from the concentration in the soil air, taking the soil temperature into account (Albers *et al.*, 2008). Averages for the whole
 270 monitoring periods are given.

Site	TH	VH	LP	NF
CHCl_3 soil water (calc.)	2.0	0.39	0.79	1.0
CHCl_3 groundwater	3.2	0.97	0.83	1.6

Based on calculated net-infiltration to the groundwater it is possible to estimate the net-flux of chloroform to the groundwater on each of the four localities: In the area where the TH profile is located, the net-infiltration has been calculated to $\sim 250 \text{ mm year}^{-1}$ and around the other three sites
 275 to $\sim 500 \text{ mm year}^{-1}$ (Højberg *et al.*, 2006). As the average chloroform concentration in the uppermost groundwater in TH is $\sim 3 \mu\text{g L}^{-1}$ and $\sim 1 \mu\text{g L}^{-1}$ at the other stations, this implies a total input of $\sim 0.75 \text{ mg m}^{-2} \text{ year}^{-1}$ around the TH profile and $\sim 0.50 \text{ mg m}^{-2} \text{ year}^{-1}$ around the other profiles. This can be compared to the flux to the atmosphere, recently measured summer and winter in areas of high and low chloroform production around the TH and NF profiles (Albers *et al.*,
 280 submitted). The calculations are complicated due to the fact that the TH profile is located in a high production area and the NF profile is on the border between areas of high and low productivity. However, using an average between emissions from high and low production areas in NF, a yearly emission is estimated to be ~ 10 and $\sim 4 \text{ mg m}^{-2}$ around the TH and NF profiles, respectively. This indicates that the amount of chloroform leaching to the groundwater is a minor but not insignificant
 285 part of the total chloroform formed in the top soil (roughly around 10% at both sites).

Prior to our study, variation in natural chloroform in forest soil (10 cm below surface) recorded by monthly sampling during one year also indicated a seasonal trend, with highest concentrations

during spring and early summer (Haselmann *et al.*, 2002). Their maximum concentration found was
290 ~100 pptv in April, which is 2–3 orders of magnitude lower than maximum concentrations found by
us. Lower concentrations should be expected according to the present findings (Fig. 3) considering
their shallower sampling depth. However, the very low concentrations of Haselmann *et al.* (2002)
suggest that they have sampled an area with very low chloroform production. The patchy
distribution of chloroform production described by Albers *et al.* (submitted) emphasise, that great
295 caution should be taken comparing published data from seemingly identical environments.

In TH, the chloroform concentration was also determined in the air just above the forest floor (20
cm height) at each sampling date (Fig. 6). This concentration would be expected to depend partly on
the concentration gradient from soil to air, but also parameters like temperature and soil moisture
300 would be important for the emission from the soil. Finally the wind will be important for how fast
the chloroform is diluted with air from above the canopy. Apparently, there is a somewhat similar
seasonal variation above and below the forest floor. There also seem to be relatively greater
differences between summer and winter above than below, as expected if higher temperature and
lower soil moisture should lead to relatively higher emissions. There are, however, also data points,
305 for which the two curves do not co-vary. One reason for this is probably the wind conditions, which
can vary greatly on a daily basis and at the same time will have a major influence on the chloroform
concentration in the atmosphere, but not in the soil air at 30 cm depth. On one occasion in late May
2008, the chloroform concentration was particularly high at 20 cm height (423 pptv, Fig. 6). The
local weather that day was sunny, relatively warm (21°C) and with almost no wind (average speed
310 <2 m s⁻¹). At the same time, the soil was rather dry (~5.5% water vol/vol at 15 cm depth), all in all
conditions promoting a high chloroform concentration below the tree canopy.

Although not as sophisticated as the regular measuring of chloroform and other volatile
organohalogens performed in e.g. the Advanced Global Atmospheric Gases Experiment (AGAGE,
2010), the data presented here, illustrate that significant temporal variations in atmospheric
315 chloroform may exist on a local scale, and that a number of parameters, which influence both the
emissions from soil as well as the later mixing in the air, are important. The variations in
atmospheric chloroform are greatest from April to November, while during winter time, the
concentration in the air above the forest floor was typically ~20 pptv, slightly above the
atmospheric average of 10–15 pptv for the Northern Hemisphere (Worton *et al.*, 2006; AGAGE,
320 2010).

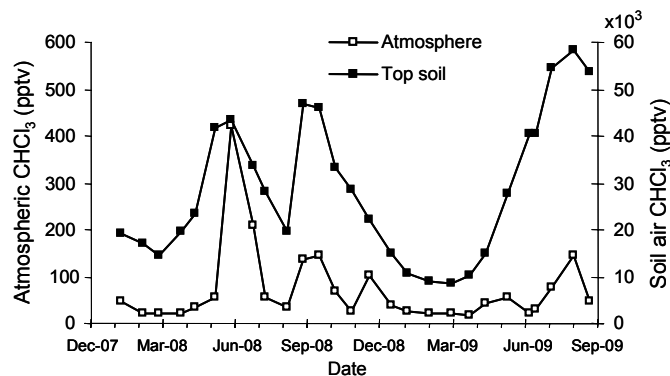


Fig. 6. Seasonal variation in chloroform above (20 cm height) and below (30 cm depth) the forest floor in TH.

325 Besides chloroform, the CO₂ concentration, considered to be a good indicator of microbial activity, was determined in the air samples. CO₂-release from microbial activity can be lowered by both decreasing temperatures and low soil moisture content, but on the other hand responds quickly to both increases in temperature and rewetting of soil (Borken *et al.*, 2003). As expected for two microbially derived products, CO₂ and chloroform co-vary at shallow depth, where the highest

330 microbial activity is expected to occur, in the TH profile, (Fig. 7a). The drought in the early summer 2008 (sunny, warm and only 38 mm rain in 66 days), e.g., resulted in a decrease in both CO₂ and chloroform concentrations. This change was possibly due to a decrease in microbial activity in combination with re-equilibration and evaporation. After rewetting of the soil, the concentrations of both CO₂ and chloroform started to increase again but while the CO₂ is responding within less than

335 a week to the rain, it takes more than three weeks before the chloroform concentration starts to rise. A similar pattern was observed after a dry period the following early summer of 2009 (Fig. 7b). The delay of chloroform relative to CO₂ was also observed as a response to the warming of the soil during early spring of both 2008 and 2009. This apparent general slower response to increased temperature and soil moisture for chloroform relative to general microbial activity was not so

340 obvious for the other three profiles due to less frequent sampling. A delay was clearly observed in the laboratory, though, using soils from both NF and TH as discussed later.

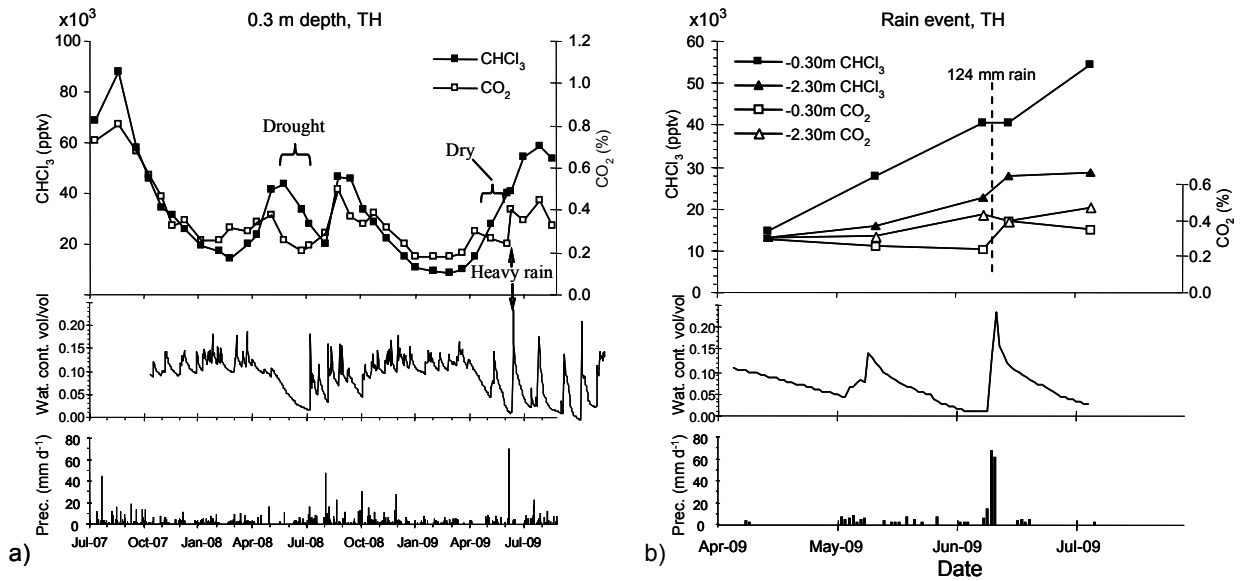


Fig. 7. a) Chloroform and CO₂ concentrations at 30 cm depth in TH. The soil moisture (TDR) at 15 cm depth next to the profile and the daily precipitation in the area is also shown. CO₂ appears to respond quicker than chloroform to both increase and decrease in soil moisture and temperature. b) Chloroform and CO₂ concentrations at 30 and 230 cm depth around a heavy rain event (124 mm rain in 30 hours) in June 2009, ending a rather dry period with only 32 mm rain in several small events during a 32 days period. CO₂ production is responding immediately to the rain. Chloroform appears to have leached to greater depths during the rain event.

A relatively dry period in TH in early summer 2009, resulting in decreasing CO₂ concentrations despite a rise in soil temperature, was ended by a heavy rain event with 124 mm rain in 30 hours. Air samples throughout the profile were taken two days before and five days after the beginning of the rain event, showing some noticeable features (Fig. 7b). In the top soil (30 cm depth) the CO₂ concentration responded quickly as expected, rising by 67% after the rewetting of the soil, while the chloroform concentration was at first unchanged. Deeper down the profile, the CO₂ concentration decreased slightly, while the chloroform concentration increased as an immediate response to the rain event. At 2.3 m depth, the increase in chloroform was 23%. Since the total uncertainty of sampling and analysis is very low (< 3%) (Albers *et al.*, submitted), the observed changes cannot be caused by errors during sampling or analysis.

The slight decrease in CO₂ concentration was possibly due to the infiltration of water from higher soil horizons containing less CO₂, while the rise in chloroform concentration most likely originates from the higher concentrations of chloroform in the top 1 meter. This indicates that infiltrating water plays an important role in the leaching of chloroform, as also discussed below.

365

Laboratory studies

Incubation of forest soil

The results of incubation of three different sections of 18 cm soil columns, based on texture, show significant differences in chloroform production between different layers (Fig. 8). Furthermore, there is an apparent lag time, before significant net production of chloroform takes place (Fig. 8a). As mentioned above, there appears to be a general delay in chloroform production relative to the CO₂ for a period of 3–4 weeks before responding to more favourable conditions concerning microbial activity. The laboratory incubation study showed the exact same trend; the soil, that was sampled in the late winter time and furthermore kept three weeks at 5°C before the incubation at 15°C, showed only a small net production of chloroform for the first ~4 weeks followed by an up to 20 fold increase in the net production rate during the following two weeks and then a similar or slightly decreasing production during the following 3½ weeks. This pattern was most pronounced for the F-horizons, but was also visible in all the A_h-horizons and in one of the L-horizons. In another incubation study with soil from TH, we observed a similar lag time, with a slightly shorter duration of ~3 weeks (data not shown). Such long lag time is possibly the result of a rather slow growing biomass, which has to increase before it produces the enzymes responsible for the production of chloroform. It would be too speculative to say more about the nature of this biomass, but it does suggest that the recently proposed abiotic pathway for chloroform production (Huber *et al.*, 2009) is not responsible for the major part of the chloroform production in forest soil, since a lag time would not be expected for this reaction pathway.

One implication of the discovery of this lag time is that when making soil incubations for studying production of natural chloroform, the soil should either not be kept too cold before incubation, or incubation should be carried out for several weeks time. Haselmann *et al.* (2000), e.g., incubated forest soil for just one week after having stored it at -18°C and this procedure could possibly have led to an underestimation of chloroform production in the soil.

We calculated the net production from the release of chloroform to the head space after the 4-week equilibration period, and found that, on a m²-basis, ~68% of the net production came from the F-horizons, while ~30% was released from the A_h-horizon and only 1–2% came from the L-horizon (Fig. 8c). When calculated on a soil weight basis, the F-horizon showed by far the highest chloroform production (Fig. 8d). This distribution between horizons was consistent between soil cores, while there were major differences in the total release from each soil core. CO₂-release,

which may be viewed as a general indicator of microbial activity in the soil, showed much less pronounced differences between both soil cores and between horizons (Fig. 8b).

400 The total chloroform release of $30\text{--}40 \mu\text{g m}^{-2} \text{d}^{-1}$ must be viewed as a minimum value, due to potential sorption to soil of part of the chloroform. Test studies (data not shown) showed that with the setup used in this experiment, apparent equilibration between soil and air in the glass jars was achieved relatively fast (~ 2 days) and that $\sim 50\%$ of the chloroform would be dissolved in soil water or sorbed to soil for the F- and A_h -horizons, whereas much less is anticipated to be present in the

405 soil in the incubated L-horizon due to its low mass, relative to the other soil horizons. Since only the F- and A_h -horizons seem to contribute significantly to the total production, the actual average net production may be around twice the average release to the air of $30\text{--}40 \mu\text{g m}^{-2} \text{d}^{-1}$. Fluxes of chloroform to the atmosphere, measured in June 2009 using a flux chamber next to the sample locations of the incubated soils, showed an average flux of $\sim 48 \mu\text{g m}^{-2} \text{d}^{-1}$ (Albers *et al.*, submitted).

410 The soil emission of chloroform measured in the field and the production measured in the laboratory then seem to be in the same order of magnitude, although the net production apparently is slightly higher. Apart from general uncertainties in comparing laboratory and field measurements, this could either be caused by the high spatial variation of natural chloroform production in the area and/or because in nature not all the formed chloroform is released by direct emissions from soil to

415 air. Part of the chloroform will leach towards the groundwater (Fig. 5) and part if of it is also likely to be taken up by roots and subsequently be emitted to the atmosphere from the vegetation.

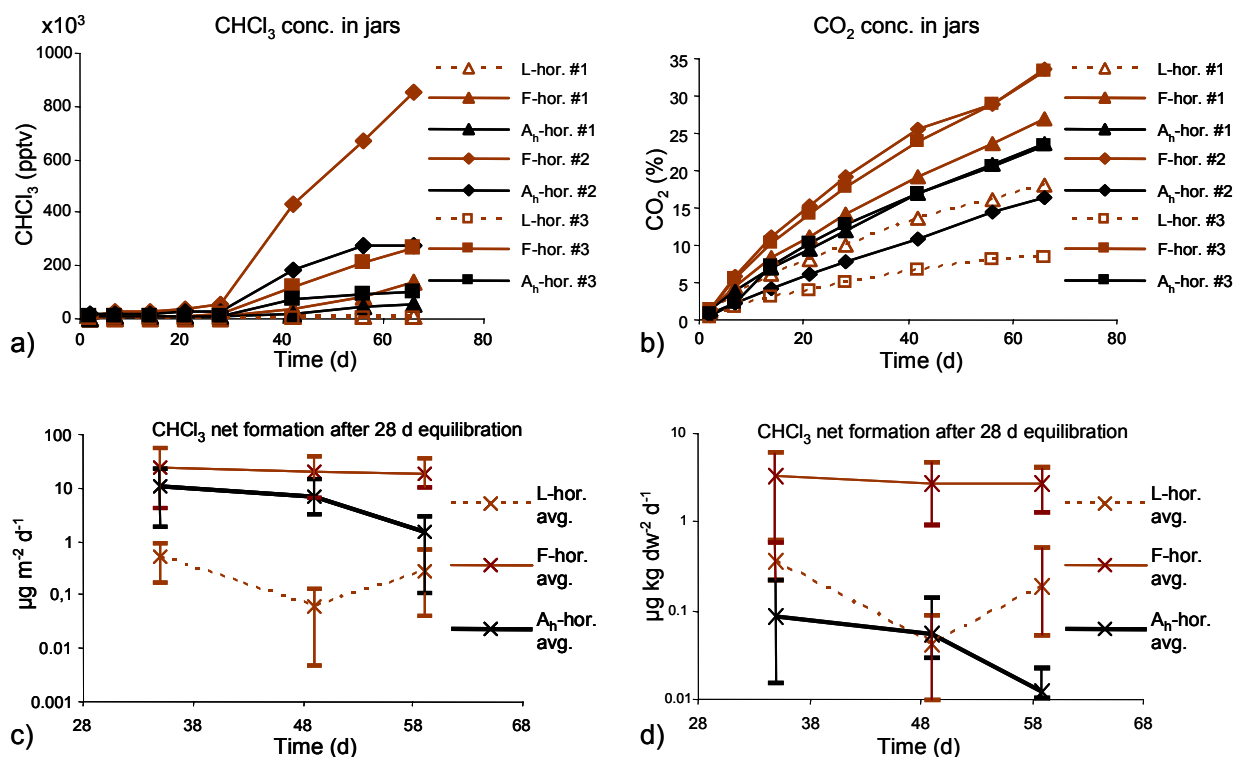


Fig. 8. a) Chloroform concentration in the head space of the incubation jars with soils sampled in NF. All replicates are shown. b) Head space CO₂-concentration. All replicates are shown. c) Chloroform net production on m² basis, after the lag time of four weeks at 15°C, based on chloroform liberated after day 28 from the soil to the air of the incubation jars in a). Error bars are the range for the three replicate soil samples. Note the semi-logarithmic scale. d) Same as c), but on a soil dry weight basis.

425 Sorption of chloroform to forest soil

Sorption to soil can be of major importance for the fate of organic compounds in soil. Previous studies have shown that organic matter is important for the sorption of chloroform (Wilson *et al.*, 1981; Grathwohl, 1990), but these studies were conducted with soils not directly comparable with our forest soil (Wilson *et al.*, 1981) or as a vapour-phase sorption experiment (Grathwohl, 1990), difficult to use in the prediction of a soil/water-partitioning coefficient. We therefore conducted a simple batch-equilibrium sorption experiment applying ¹⁴C-chloroform on four soils representing the major classes of soil in the study sites; O-horizon (present and similar in all four study sites), A-horizon (present in NF, VH and LP), B-horizon (present in all four study sites) and C-horizon (present and similar in all four study sites) (Appendix Table A1). These experiments showed that organic matter content appear to control the partitioning between soil and water (Fig. 9b), although inorganic constituents could have an influence in the C-horizon, as has also been observed previously for soils low in organic matter (Farell & Reinhard, 1994; Riley *et al.*, 2005). One

implication of this is that in the O-horizon, sorption will have a major influence on the fate of chloroform, possibly delaying both evaporation and leaching of the compound. In the A-horizon there will also be a small retardation of leaching processes, but in the B-horizon, and especially in the C-horizon, sorption is of minor influence, and most of the chloroform will be dissolved in water or present in the gas phase, and easily leach or diffuse towards the groundwater. Since the partitioning of chloroform between water and air is 10–15 fold in favour of the dissolved state at normal temperate soil temperature (Görgényi *et al.*, 2002), the major mass of chloroform in sub-horizons will be present as a water solute, except under very dry conditions. This is obviously important, when trying to understand and predict the migration of chloroform into groundwater. The most obvious hypothesis would then be that this movement will mainly occur with the chloroform dissolved in water. This is supported by the field measurements of chloroform before and after a heavy rain event, where the massive transport of water towards deeper horizons e.g. led to a 23% increase in chloroform concentration within a few days at 2.3 m depth (Fig. 7b).

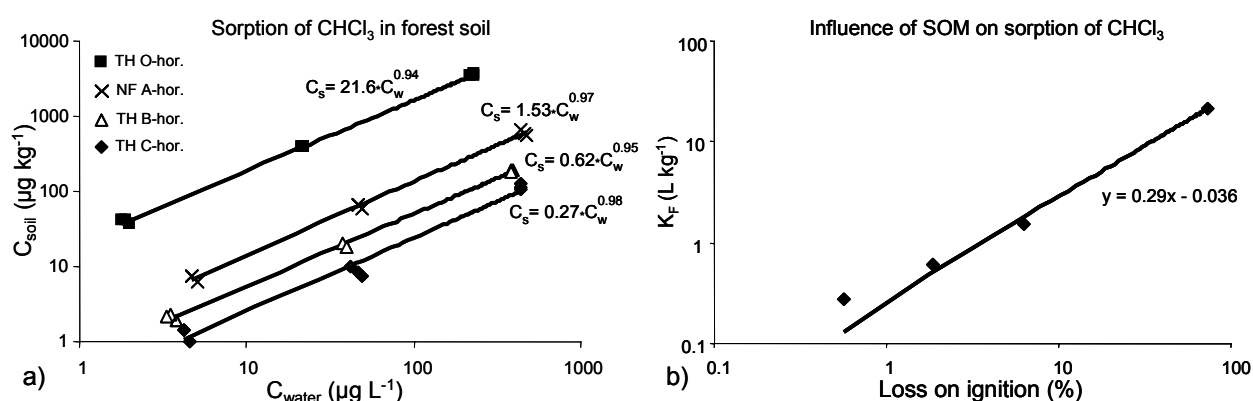


Fig. 9. a) Sorption study with ¹⁴C-CHCl₃ and four soils from NF and TH. All replicates are shown and used to calculate the best fit to the Freundlich equation (also shown). b) Relationship (linear correlation) between SOM-content and the sorption coefficient, K_F. For both figures, note the double logarithmic scale.

Mineralization study

Transformation, abiotic and/or biotic, is another potentially important process for the fate of the chloroform formed in the soil. While chloroform is known to be degraded by OH⁻-radicals in the atmosphere, giving an average atmospheric half-life of ~6 months (Laternus *et al.*, 2002), the only reported abiotic degradation mechanism for chloroform in soil is reductive dehalogenation (Pecher *et al.*, 2002; Kenneke & Weber, 2003). Since oxic conditions, in combination with relatively coarse soil material leaving room for diffusion (Fig. 2), prevail throughout the unsaturated zone and even

in the shallow groundwater in all four profiles, abiotic degradation of chloroform is not expected to
465 occur in the investigated system. The same can be said for reductive dehalogenation
(halorespiration), which is a well known biotic degradation pathway for several halogenated
compounds, including chloroform (Gupta *et al.*, 1996a & b). Except perhaps for anoxic microniches
in the organic horizon that might occur at wet conditions (Van der Lee *et al.*, 1999), aerobic
conditions can be expected to always exist in the four profiles and only aerobic degradation
470 pathways will be relevant. The only reported aerobic transformation mechanism for chloroform is
co-metabolism by different oxygenase-expressing bacteria. Strand & Shippert (1986) found that
~3% of the added chloroform was oxidized to carbon dioxide within five days in an Indianola sandy
loam soil and Albers *et al.* (submitted) found similar mineralization rates in forest top soils. No
other studies on aerobic degradation in natural soils exist, but several isolated oxygenase-expressing
475 bacteria or mixed cultures are known to co-metabolize chloroform (e.g. Bartnicki & Castro, 1994;
Alvarez-Cohen *et al.*, 1992; Chang & Alvarez-Cohen, 1996). The degradation pathway seems to be
 $\text{CHCl}_3 \rightarrow \text{HOCCl}_3 \rightarrow \text{O=CCl}_2 \rightarrow \text{HCO}_3^-$, with the chlorine released as HCl (Bartnicki & Castro,
1994), or in other words complete mineralization of the chloroform molecule. To see if any
mineralization of chloroform took place in our soil, we performed a mineralization study that
480 revealed initial rates of 0.14–1.0% mineralization of the added $^{14}\text{C-CHCl}_3$ per day (Fig. 10).
Mineralization rates were highest in the organic horizon, lower in the A- and B-horizons and lowest
in the C-horizon. The addition of azide (a microbial inhibitor) resulted in an almost complete lack
of mineralization, indicating strongly that the mineralization is biotic. On the basis of this
experiment it can be concluded that aerobic degradation of chloroform exists in the forest soil, and
485 it is possible that the decrease in concentration down the profiles in TH, VH and LP (Fig. 3a) is a
result of biotic degradation, rather than sorption or diffusion processes. Biotic degradation would
then be a third sink of the chloroform formed in forest soil, besides emissions to the atmosphere and
leaching to the groundwater. The significance of this third sink would depend on the residence time
for chloroform in each of the soil horizons, since different mineralization rates exist (Fig. 10b).

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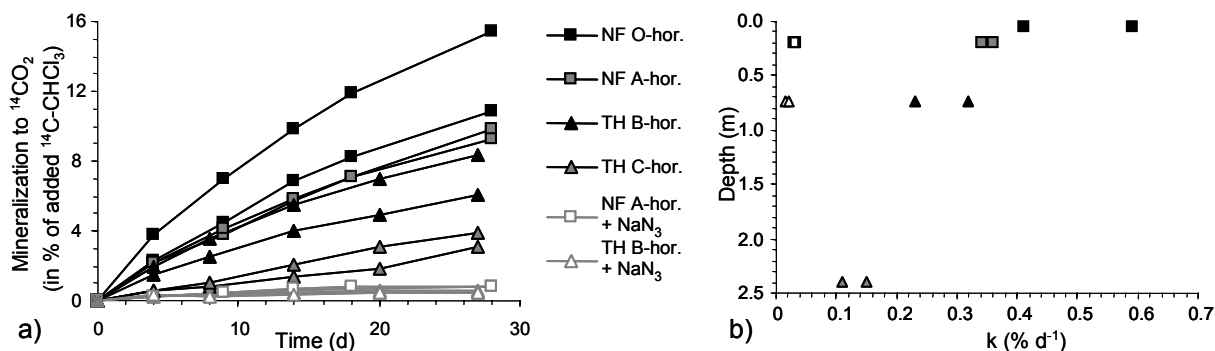


Fig. 10. Mineralization of $^{14}\text{C-CHCl}_3$ in different soil horizons sampled in NF or TH. Sterilized (NaN_3) soil was used only for the A- and B-horizons. a) Accumulated release of $^{14}\text{CO}_2$ from mineralization of $^{14}\text{C-CHCl}_3$. The duplicate soil samples are shown. b) Mineralization rate according to the depth of which the soil was sampled. First order degradation kinetics was assumed. Squares are soil from NF, triangles soil from TH. Open symbols are NaN_3 -sterilized soil samples.

Conclusions

Solid evidence for the occurrence of natural chloroform in groundwater at the $\mu\text{g L}^{-1}$ level has been established through regular analysis of soil air and groundwater from multilevel wells for periods of up to four years. The formation of natural chloroform is related to biological processes in the organic rich top soils, particularly the F-horizon, showing a pronounced seasonal variation. Sorption, particularly to organic rich soils, and microbial degradation at all levels in the unsaturated zone preclude a simple description of the leaching of chloroform, though approximately ten percent of the chloroform produced appear to end up in the groundwater.

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Appendix A

510 Table A1

Texture and other basic data for the minerogenic horizons of the four profiles. ? = not determined.

Site	Sample depth (m)	Horizon	Clay + silt (%)	Fine sand (%)	Medium sand (%)	Coarse sand (%)	Gravel (%)	Loss on ignition	pH _{H2O}
TH	0.1–0.3	E	2.9	26	66	1.4	3.6	2.0	3.9
TH	0.3–0.5	B	6.5	24	65	3.4	1.4	1.8	4.2
TH	0.5–1.0	B	8.9	39	31	7.0	14	1.8	4.4
TH	1.0–1.8	C	9.6	26	45	6.8	13	0.57	5.2
TH	1.8–3.0	C	2.2	13	72	12	0.55	0.32	6.3
TH	3.0–4.0	C	1.3	13	79	6.0	0.37	0.24	7.1
TH	4.0–4.8	C	7.7	52	38	2.5	0.40	0.38	7.8
VH	0.1–0.2	A _h	8.5	13	31	22	25	6.6	?
VH	0.2–0.3	A _h	9.0	14	36	31	10	6.0	3.9
VH	0.3–0.5	A _h	13	18	37	24	7.8	6.6	4.3
VH	0.5–0.7	B _h	8.0	12	34	34	12	4.4	4.4
VH	0.7–1.0	B	1.6	6.1	44	40	8.2	0.57	4.6
VH	1.3–1.5	C	0.70	9.6	73	17	0.18	0.23	4.8
VH	1.8–2.0	C	1.0	12	60	24	3.4	0.46	4.5
VH	2.3–2.5	C	0.65	13	73	13	0.58	0.27	4.6
VH	2.8–3.0	C	1.0	10	74	15	0.25	0.21	4.7
VH	3.7–4.0	C	1.2	11	65	22	1.3	0.24	4.7
LP	0.1–0.2	A _h /E	8.53	13	36	31	11	5.8	4.2
LP	0.2–0.3	A _h	11.7	11	31	28	18	6.6	3.9
LP	0.3–0.5	A _h	9.41	6.6	17	20	47	5.6	4.4
LP	0.5–0.7	B	3.40	4.7	17	28	46	1.9	4.5
LP	0.7–1.0	B	2.11	3.4	29	48	18	1.2	4.4
LP	1.3–1.5	C	0.33	2.2	50	43	4.4	0.34	4.6
LP	1.8–2.0	C	0.27	1.2	33	26	40	0.37	4.7
LP	2.3–2.5	C	0.18	1.5	43	42	13	0.40	4.7
LP	2.8–3.0	C	0.73	0.77	38	54	6	0.25	4.7
NF	0.1–0.2	A _h	21.9	35	37	4.8	1.9	8.6	4.1
NF	0.2–0.3	A _h	23.6	33	35	6.2	2.0	6.6	4.3
NF	0.3–0.5	A	30.4	35	23	3.8	7.8	4.3	4.5
NF	0.5–0.7	B	16.7	29	31	8.4	15	1.9	4.5
NF	0.7–1.0	B	3.22	5.3	23	41	28	1.8	4.5
NF	1.3–1.5	C	0.21	8.3	78	13	0.70	0.32	4.7
NF	1.8–2.0	C	0.56	4.9	81	11	1.7	0.35	4.6
NF	2.3–2.5	C	0.69	4.2	52	39	4.3	0.71	4.8
NF	2.8–3.0	C	1.36	2.0	73	14	9.5	0.46	4.8
NF	3.3–3.5	C	1.40	7.2	44	35	13	0.39	5.2
NF	3.8–4.0	C	0.95	5.9	44	31	18	0.43	5.0

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IV

Methodological problems in determining TCAA in soil – the discovery of novel natural trichloroacetyl containing compounds and their interference with a common method for determining TCAA in soil and vegetation

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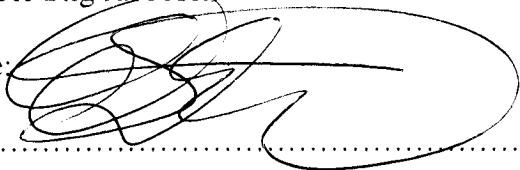
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| a) Basic formulation of the project leading to the published work | 2 |
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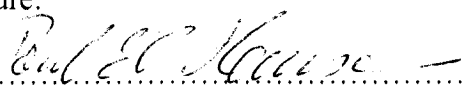
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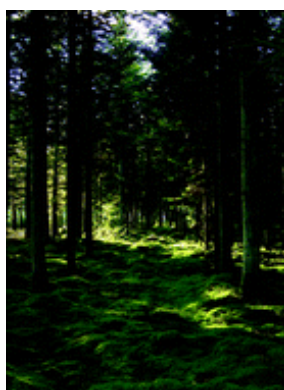
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Trichloroacetic acid (TCAA) is a pollutant with several sources and is also formed naturally in soil. We show that almost all investigated environmental compartments (soil, soil water, groundwater, spruce needles and throughfall, but not rain) contain compounds, which make false positives in the thermal decarboxylation method often used for determination of TCAA in environmental samples. The compounds are dominating quantitatively over TCAA in soil, soil water and groundwater, while TCAA is dominating in needle and throughfall samples. The compounds behave differently from TCAA with regard to the velocity and the pH-dependence of the chloroform release. We did not manage to reveal the whole chemical structure of the compounds, but a trichloroacetyl group seems to be the only plausible structure giving rise to CHCl_3 both upon heating and under alkaline conditions. Besides the trichloroacetyl group, the compounds did in general contain a carboxylic acid group, although in needle and throughfall samples, trichloroacetyl compounds with a neutral charge at pH 7.5 co-existed with the carboxylic acids. Trichloroacetyl groups in humic substances and possibly other macromolecular structures contribute to the major portion of the total trichloroacetyl- CHCl_3 in topsoil, but smaller molecules with less UV-VIS absorption seem to constitute the major part of trichloroacetyl- CHCl_3 in soil water and groundwater. The trichloroacetyl containing compounds are most likely naturally occurring compounds formed in the natural chlorination processes in soil, but additional studies are needed to substantiate this hypothesis.



Environmental impact

The widely occurring pollutant trichloroacetic acid (TCAA), a previous herbicide and a present secondary atmospheric air pollutant, as well as a product of natural chlorination processes in soil, was investigated in various compartments of a temperate coniferous forest environment. We found that a popular analytical method, which determines TCAA indirectly, co-determines a class of not previously described possibly natural compounds, containing a trichloroacetyl group. This paper is therefore both a presentation of these compounds as widespread in nature as well as a guidance of which method to be used for environmental studies on TCAA. Furthermore, a small guidance to where care should be taken, when considering previously published environmental concentrations of TCAA, is given.

35 **Introduction**

Trichloroacetic acid (TCAA) is a widespread secondary atmospheric air pollutant^{1,2} formed in the atmospheric degradation of 1,1,1-trichloroethane and tetrachloroethene.^{3,4} TCAA has, however, also been found in old preindustrial glacier ice^{5,6} and furthermore, TCAA has been shown to be formed naturally in forest soils.⁷ Several authors have reported the presence of TCAA in a wide range of
40 soils in concentrations, which cannot be explained by the atmospheric deposition alone and recently, very high concentrations, up to ~2 mg/kg dry weight (dw), were reported for a Scottish forest soil.⁸ Since TCAA is known to sorb very little to the soil,⁹ easily leach through different soil types¹⁰ and furthermore is quickly mineralized by microorganisms in forest soil, with a half life as low as 24 h reported,^{11,12} such high TCAA concentrations could possibly indicate a large formation
45 potential in the soil,

Since agreement on the degree and significance of natural formation of TCAA in the terrestrial environment has not yet been achieved, we wanted to investigate the presence and behaviour of TCAA in a coniferous forest environment. Basically two main methods for the analysis of low concentrations of TCAA in environmental samples exist. The traditional method is based on
50 esterification and subsequent analysis on either GC/MS or GC/ECD.^{1,13,14} This method is substance specific but includes a time consuming multistep procedure. During the last 20 years, a less time consuming method has become popular, in which TCAA is decarboxylated upon heating and the formed CHCl_3 subsequently is analyzed on a gas chromatograph coupled to e.g. a mass spectrometer (MS) or for low detection limits to an electron capture detector (ECD).^{13,15,16} To
55 investigate the presence and formation of TCAA in the forest environment, we decided to use thermal decarboxylation followed by GC/ECD-analysis of the chloroform formed in the decarboxylation process. The yield of chloroform upon heating has been shown by several authors to be close to 100%, when TCAA is heated for 90 minutes at 90°C or more at a pH between 3 and 8.^{12,15,17,18} In the present paper, however, we present the presence of a group of presumably natural
60 compounds, interfering with this thermal decarboxylation method in various types of environmental samples.

Experimental

65 Environmental samples

Soil, soil water, groundwater, needle and precipitation samples were collected in a mixed coniferous forest near Tisvilde (12°03'40" E, 56°02'22" N) in Eastern Denmark in the years 2007-2009. In addition, groundwater was sampled from a spruce forest in Feldborg (8°57'01" E, 56°22'51" N) and from a mixed coniferous forest near Viborg (9°21'33" E, 56°25'42" N), both located in Western
70 Denmark, in 2009. All sites are coniferous forests with sparse vegetation below the trees and the soils are all sandy with an organic horizon 5-25 cm thick on top of the sandy soil. Soil was sampled from the top soil with steel cylinders (H: 30 cm and Ø 6 cm) and either extracted with water within 24 h or stored frozen until analysis. The soil was homogenized either by sieving (minerogenic soil) or in a coffee mill (organic horizon) before extraction with water. In the field,
75 soil water was abstracted from the soil with 4 suction cells (Prenart Teflon-quartz, Prenart, DK) placed at 0.5-3.4 m depth. The soil water was collected in evacuated (300-400 hPa, semi continuously suction) glass bottles connected to the cells with polyethylene tubing. Groundwater was sampled through 5 cm brass filters placed in the upper groundwater as described earlier.¹⁹ Needles were taken from spruce trees (*Picea abies*). Twigs were cut off and the two-year-old needle
80 class was chosen for further analysis. After removal from the twigs, the needles were crushed to fine powder under liquid nitrogen in an agate mortar. Precipitation was sampled both from openings in the forest (rain) and below the vegetation (throughfall). A stainless steel funnel was placed at 1.5 m height and connected through polyethylene tubing to a glass bottle placed in the dark below the soil surface.

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Chemical analyses

TCAA, trichloroacetaldehyde (chloral), 1,1,1-trichloroacetone, 3-hydroxy-4,4,4-trichlorobutyric acid and cis-3,4,4,4-tetrachlorocrotonic acid standards were all of analytical quality and purchased from Sigma-Aldrich (Steinheim, Germany). [2-¹⁴C]-TCAA was purchased from Moravek
90 Biochemicals (Brea, California), with a radiochemical purity of ≥99.5%. All water used was MiliQ-water that had been heated to 100°C and afterwards purged with N₂ to remove all CHCl₃, TCAA and other compounds liberating CHCl₃ upon heating.

TCAA was extracted from soil and needles by shaking 5 gram soil/needle samples with 50 ml water in glass bottles overnight. The slurries were then centrifuged (3000 g, 10 min.) and the supernatant

95 analyzed similarly to soil water, groundwater and precipitation samples: 2 x 12.5 ml sample were transferred to 13 ml glass vials equipped with PTFE-lined lids, preliminary found not to adsorb chloroform or TCAA. The content of one of the vials was then diluted to 125 ml and analyzed for chloroform as it was. The other vial was heated to 100°C for 90 min. to convert TCAA to CHCl₃. After heating, the vial was shaken for at least 20 h at 10°C to ascertain that all formed CHCl₃ had
100 partitioned into the aqueous phase. Preliminary experiments with CHCl₃ showed that a negligible amount of CHCl₃ (<2%) was lost by heating and subsequently re-equilibrating the vial at 10°C. For the analysis of base-hydrolyzable CCl₃-compounds, 12.5 ml sample was transferred to a third vial and added 200 µl 6M NaOH and shaken (2h, 10°C) to convert trichloroacetyl containing compounds to CHCl₃. The heated vial and the vial added NaOH were then diluted to 125 ml and
105 analyzed for CHCl₃. TCAA and trichloroacetyl containing compounds were quantified from the difference between CHCl₃ in the treated and the non-treated vial.

CHCl₃ was analyzed quantitatively by purge & trap GC/ECD as previously described,¹⁹ with limits of quantification (LOQ) of ~0.0005 µg/l. The LOQ for TCAA varied with the background CHCl₃ concentration. Groundwater and soil water samples were purged with N₂ (1h, 20°C) to remove most
110 of the background CHCl₃ and the LOQ for CHCl₃ from either TCAA or trichloroacetyl containing compounds was in general ~0.01 µg/l in groundwater, soil water and precipitation samples and ~0.1 µg/kg wet weight (ww) for soil and needle samples.

As a surrogate for the relative concentration of dissolved humic substances in groundwater samples, soil water samples and soil extracts, the UV-VIS absorption of various solutions was determined on
115 a Shimadzu UV-1800 (Shimadzu Corp., Kyoto, JP) after appropriate dilution with MilliQ-H₂O. Solid phase extraction (SPE) of TCAA and trichloroacetyl containing compounds was done using commercial Oasis[®] MAX SPE columns (Waters Corp., Milford, MA). After pre-equilibration with 1 ml MeOH, 1 ml H₂O and 1 ml 0.5M PO₄-buffer (pH 7.5), the sample was slowly loaded to the column (1-2 ml min⁻¹). When the loading was completed, 1 ml 0.5M PO₄-buffer (pH 7.5) was
120 slowly eluted through the column to ensure the attachment of all carboxylic acids to the anion exchanger. The column was then eluted very slowly with 1 ml MeOH to elute neutral compounds and then with 1 ml MeOH/H₂O/HNO₃ (80/17/3%, pH 0.6) to elute carboxylic acids. pH 0.6 should be low enough to elute most organic acids, and even worked for TCAA, which is a rather strong acid (pK_A = 0.7).

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Results and discussion

Preliminary tests

At environmental pH, TCAA is deprotonated and the sorption of deprotonated TCAA to soil has been shown to be low.⁹ Batch equilibrium studies with [2-¹⁴C]-TCAA confirmed that partitioning of TCAA into soil or needle matrices seemed not to be of major concern (K_d from 0.01 in C-horizon to 1.0 in the organic top horizon). With the applied water:soil and water:needle ratios of 10:1, at least 90% and in most cases close to 100% of the TCAA present in the sample would be expected to be present in the aqueous phase, and consequently the extraction of TCAA from the various environmental matrices with water seemed applicable. In the literature, examples of the influence of environmental matrices on the recovery of TCAA in the thermal decarboxylation procedure exist,^{15,16} but as also pointed out by these authors, this is most likely due mainly to partitioning of the formed CHCl_3 into soil, needles or other matrices. To avoid partitioning of the formed CHCl_3 into soil or needles we decided to extract TCAA from the environmental matrices before performing the thermal decarboxylation, and from spiked samples, this revealed recoveries of 95-101% with standard deviations of only a few percent at environmentally relevant pH-values, Fig. 1a. At alkaline pH, TCAA is not decarboxylated upon heating, and this stabilization at low proton concentration has an influence on the standard thermal decarboxylation procedure down to a pH of ~ 8 .¹² We could confirm this stabilization, as no CHCl_3 was released upon heating at alkaline pH, neither in pure water nor in soil extracts (Fig. 1a).

Preliminary investigations of soil extracts, needle extracts and groundwater showed the presence of CHCl_3 released upon heating in all samples investigated. Since the decarboxylation procedure is an indirect analysis of TCAA, we were worried that other compounds could interfere. A group of compounds, which could give false positives in the thermal decarboxylation procedure, is trichloroacetyl compounds, which contain a CCl_3 -group next to a carbonyl group. Such compounds, with chloral and 1,1,1-trichloroacetone as well known examples, are also thermolabile and release CHCl_3 upon heating.²⁰ We performed a test with chloral and 1,1,1-trichloroacetone using the same procedure as for TCAA and found that at pH=7.5, CHCl_3 -yield was almost 100% (Fig. 1a). To ascertain that it indeed was TCAA we measured, when heating various environmental water extracts and groundwater samples, we needed a test where only compounds containing the CCl_3 -C=O structural element and not TCAA would release CHCl_3 . As known from the haloform reaction, CHCl_3 is released from CCl_3 -groups sitting next to carbonyl groups when the pH is

alkaline,²¹ while TCAA is stable at high pH (Fig. 1a). A quick test with NaOH added to solutions of either TCAA or chloral to give a pH ~12.5, confirmed that chloroform was released quantitatively from chloral and 1,1,1-trichloroacetone, while TCAA released no chloroform at alkaline pH (Fig. 1b). There was no difference between solutions made in pure water or in soil extracts, so water soluble soil constituents did not influence CHCl₃-release from chloral or the lack of CHCl₃-release from TCAA.

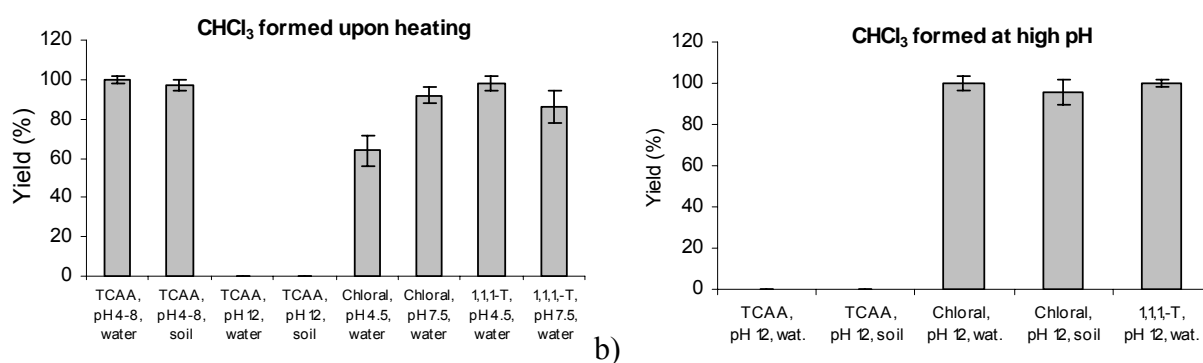


Fig. 1 (a) CHCl₃ yield in the standard decarboxylation procedure (100°C, 90 min.) at different pH values. TCAA was dissolved in water and soil extracts at different pH values in the interval 4-8 and showed no effect of pH within this interval but at alkaline pH no CHCl₃ was formed from TCAA. Trichloroacetyl containing compounds, exemplified by chloral and 1,1,1-trichloroacetone (1,1,1-T), were heated at pH 4.5 and 7.5. (b) CHCl₃ released after 2 hours at 10°C at alkaline pH, as a method to distinguish between TCAA and compounds containing a CCl₃-group next to a carbonyl-group, exemplified by chloral and 1,1,1-trichloroacetone (1,1,1-T). TCAA and chloral were dissolved in both water (“wat.”) and soil extracts (“soil”). Error bars are in all cases standard deviations of three replicates, except for TCAA at pH 4-8 (water and soil extract) where 6 samples with varying pH were analyzed.

Detection of trichloroacetyl containing compounds in environmental samples

NaOH was added to various environmental samples, which liberated CHCl₃ upon heating (standard TCAA decarboxylation procedure), to give a pH of ~12 and left at 10°C for 2 hours. This treatment would, as seen in Fig. 1b, not result in release of CHCl₃ from TCAA, only from base-hydrolyzable compounds like chloral and other compounds containing a CCl₃-group next to a carbonyl group. Such compounds with unknown structure will from now on be termed trichloroacetyl-CHCl₃. In Table 1, the results from the analyses of various environmental samples are presented. Each sample was analyzed for background CHCl₃ (no treatment), CHCl₃ formed upon heating (100°C, 90 min., from now on termed heat-CHCl₃) and CHCl₃ formed under alkaline conditions (pH adjusted to 12 with NaOH, 10°C, 120 min. (trichloroacetyl-CHCl₃)).

Table 1 Analysis of trichloroacetyl-CHCl₃ (CHCl₃ released after 2 h at pH12 and 10°C subtracted a background concentration of chloroform) and heat-CHCl₃ (standard TCAA-procedure, 100°C, 90 min. at the noted pH, subtracted a background concentration of chloroform) in various environmental aqueous samples and water extracts. The concentration range in the investigated samples/extracts is presented and furthermore the molar ratio between CHCl₃ released at alkaline pH and the CHCl₃ released upon heating is shown. A ratio of 1 or higher indicates that all CHCl₃ released upon heating could come from trichloroacetyl-CHCl₃. Soil and groundwater samples were heated at their natural pH, while precipitation and needle extract samples were heated at pH 7.5, adjusted with a phosphate-buffer.

Sample type	No. of independent samples	Trichloroacetyl-CHCl ₃ (µg/l)	Heating pH	Heat-CHCl ₃ (µg/l)	Ratio (Trichloroacetyl-CHCl ₃ / Heat-CHCl ₃)	Interpretation
TCAA commercial standard, 1 µg/l	-	<0.01	5	1	0	TCAA ^a
Chloral commercial standard, 1 µg/l	-	1	7	1	1.15	CCl ₃ -C=O ^c
1,1,1-T commercial standard, 1 µg/l	-	1	7	1	1.11	CCl ₃ -C=O ^c
Soil water from suction cells	4	0.3-9	7-8	0.4-8	0.88-1.38	CCl ₃ -C=O ^c
Soil extracts	6	0.7-7	3.7-4.3	0.3-3	2.13-3.50	CCl ₃ -C=O ^c
Unpolluted forest groundwater	9	0.1-1.0	4.5-5.1	0.1-0.7	1.03-3.94	CCl ₃ -C=O ^c
TCAA-polluted groundwater, 0.7 µg/l	1	<0.01	7	1	0	TCAA ^a
Rainwater, TCAA ~0.1 µg/l	3	<0.01	7.5	0.1-0.2	0	TCAA ^a
Throughfall from coniferous forest	3	0.02-0.5	7.5	0.2-4	0.03-0.23	Mix ^b
Needle extracts	4	0.1-1.3	7.5	2-10	0.05-0.20	Mix ^b

^aTCAA only. ^b Mixture of TCAA and trichloroacetyl compounds. ^cProbably trichloroacetyl compounds only

As shown in Table 1, minimally the same amount of CHCl₃ was formed when soil and groundwater samples were added NaOH as when they were heated at their natural pH. Heating at pH 12 released no more CHCl₃ than just keeping the sample at pH12 and 10°C for two hours. Furthermore, heating at neutral pH (standard TCAA procedure) followed by addition of NaOH (trichloroacetyl-CHCl₃ procedure) resulted in exactly the same CHCl₃-release as when just adding NaOH, indicating strongly that all the CHCl₃ released upon heating of the soil and groundwater samples was derived from unknown trichloroacetyl containing compounds and that no or very little TCAA co-existed with these. Both throughfall and needle samples contained mainly heat-hydrolyzable TCAA, with trichloroacetyl containing compounds constituting less than 25% of the total CHCl₃ released upon heating. The remaining more than 75% CHCl₃ released upon heating most likely originates from TCAA, since we are aware of no other compounds that release CHCl₃ only upon heating and not by making the pH highly alkaline. The presence of TCAA in rain and throughfall samples was confirmed by GC/MS analysis after methylation with acidic methanol (results not shown).

For both soil and groundwater samples, we observed that the CHCl₃ released upon heating was pH-dependent, with almost the same amount released when heating at a pH-value between 6 and 7.5 as when adding NaOH, while less CHCl₃ was released when heating at acidic pH. A test with one soil extract adjusted to different pH-values in the interval 1.3-7.5 and heated to 100°C for 90 min. showed a strong pH-dependence and also within the natural variation in pH between soils, this trend

could be seen (Fig. 2a). The pH effect for the groundwater samples at their natural pH was less pronounced, but decreasing the pH to 1.3, clearly diminished the amount of CHCl_3 released upon heating. The effect of changing pH was also seen for chloral (Fig. 1a and ¹⁸), whereas 1,1,1-trichloroacetone was completely converted at both pH 4.5 and 7.5 (Fig. 1a). Since the conversion of TCAA upon heating (100°C, 90 min.) is complete within the pH-interval 1.3-7.5 (Fig. 2a and ¹²), this strongly indicates that the CHCl_3 formed by heating groundwater and soil-derived samples originates from the trichloroacetyl compound(s) and that these, like chloral and 1,1,1-trichloroacetone, are both base- and heat-hydrolyzable, although only partly hydrolyzed upon heating at acidic pH, with only ~10% released as CHCl_3 at pH = 1.3.

We also investigated the influence of the time of heating for both a natural sample and for the TCAA standard, Fig. 2b. It is clear that the unidentified compound(s) in the groundwater sample is converted much faster to CHCl_3 than TCAA. On the other hand only ~90% of the total base-hydrolyzable CCl_3 could be liberated as CHCl_3 upon heating at pH 7.5 and this seemed to be a repeatable phenomenon also found in soil extracts. This is a first indication that the environmental samples contain not only a single trichloroacetyl compound, but rather a sum of compounds all containing a base-hydrolyzable CCl_3 -group but with different chemical properties.

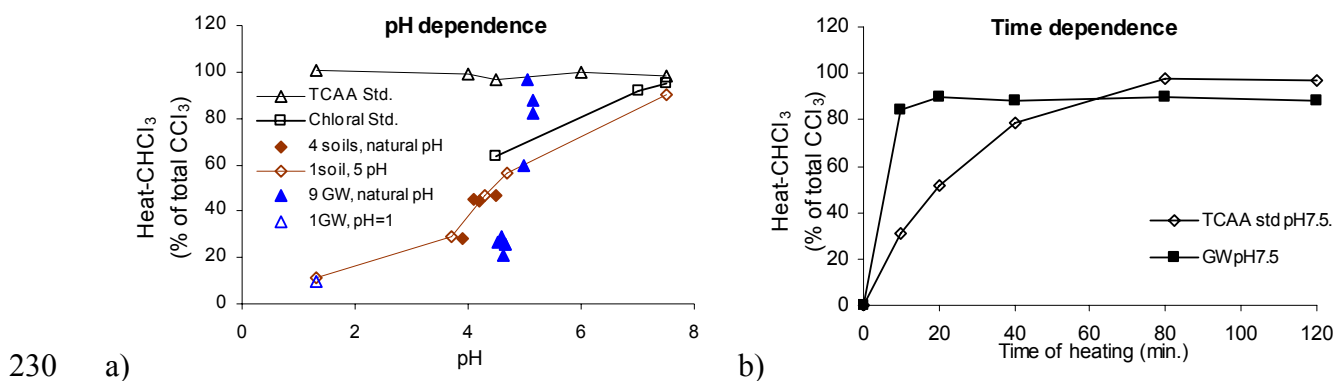
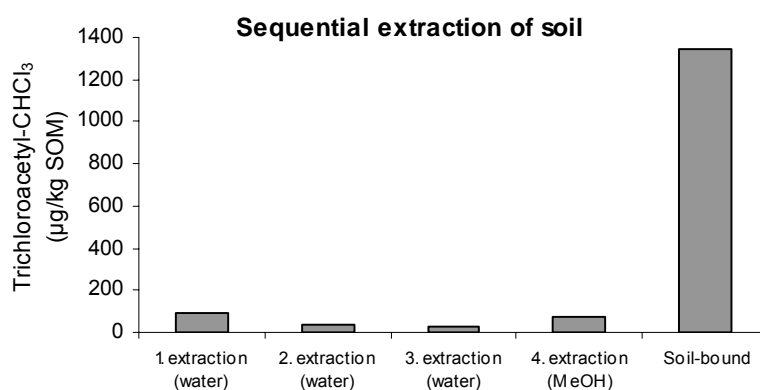


Fig. 2 (a) Influence of pH on the release of CHCl_3 from TCAA, chloral and unknown CCl_3 -compounds in environmental samples upon heating (100°C, 90 min.). pH was adjusted with either PO_4 -buffers (pH 3.7-7.5) or with concentrated HNO_3 (pH1). “Soil” means aqueous extracts of forest soil. “GW” is forest groundwater samples from Feldborg and Viborg. For the environmental samples, total CCl_3 refers to the CHCl_3 formed under alkaline conditions. The strong dependence on pH shows that CHCl_3 formed upon heating of the soil and groundwater samples was not derived from TCAA. (b) Time-dependence of the conversion of TCAA and unknown CCl_3 -compounds in groundwater to CHCl_3 upon heating at pH 7.5. “GW” is a forest groundwater sample from Viborg.

Since adjusting pH to 12 seemed to better quantify the trichloroacetyl-CHCl₃ than heating, and
240 since furthermore, the alkali method was more convenient and was not influenced by TCAA, if
present, we chose this for further investigations of trichloroacetyl containing compounds in
environmental samples. To optimize the method, a time test was performed with a soil extract
containing ~1 µg/l trichloroacetyl-CHCl₃. The test revealed that 60% of the maximum CHCl₃-
release was obtained after 15 minutes and full hydrolysis was obtained within ~1 hour with no
245 additional release of CHCl₃ in the following 6 hours. A reaction time of 2 hours was chosen for the
following samples.

Trichloroacetyl-CHCl₃ in SOM

The preceding findings of trichloroacetyl-CHCl₃ were all performed with aqueous samples or
250 aqueous extracts, indicating that the yet unidentified compound(s) was water soluble. A simple test
was set up to see whether all trichloroacetyl-CHCl₃ in soil is water extractable; organic top soil was
sequentially extracted, three times with water and then once with MeOH. Each extract was analyzed
for trichloroacetyl-CHCl₃. After the MeOH-extraction, the soil was washed with water and 0.1M
NaOH was added to the soil, to test if any non-extractable trichloroacetyl-CHCl₃ was left in the soil.
255 A typical example of such sequential extraction is shown in Fig. 3. In that example, only ~6% of the
total trichloroacetyl-CHCl₃ was released from the soil in the first extraction, ~2% in each of the
following two water-extractions and then ~4% in the MeOH extraction. More than 85% of the total
trichloroacetyl containing compound(s) was still present in the soil after the sequential extraction,
indicating the presence of a relatively large pool of trichloroacetyl-CHCl₃ in the soil organic matter
260 (SOM) not extractable with water or MeOH.



265 **Fig. 3** Trichloroacetyl-CHCl₃ in sequential extracts of soil from the organic H-horizon in Feldborg. Solvent was either pure water or 80% MeOH. Soil bound is the trichloroacetyl-CHCl₃ liberated from the soil when 0.1M NaOH is added after the sequential extraction.

The soil-bound trichloroacetyl-CHCl₃ is likely to be CCl₃-C=O structural elements in
 270 macromolecular parts of the soil organic matter since it is not extractable with either water or MeOH. One likely candidate for such macromolecular molecules is humic substances (HS), which consist mainly of polycarboxylic humic and fulvic acids.^{22,23} These acids can be extracted with 0.1M NaOH and SOM-content of the H-horizon was found to decrease with 29% after extraction with 0.1M NaOH, so, as in most soils, humic and fulvic acids were important though not single
 275 constituents of the SOM. Since humic and fulvic acids are slightly extractable with water, and are present in both soil water and groundwater, the trichloroacetyl-CHCl₃ we had found in the different environmental compartments could in principle be structural elements in humic and fulvic acids. Aromatic structures are ubiquitous structural elements in humic and fulvic acids from soil^{22,23} and a simple approach to compare the content of these acids in different samples is to measure the UV-
 280 absorption at 254 nm (A_{254}) and the specific colour at 465 nm (A_{465}), although some variation exists between humic substances (HS) from different sources.²² The ratio between trichloroacetyl-CHCl₃ concentration and A_{254} and A_{465} in various samples and extracts is presented in Table 2. Assuming that all CHCl₃ liberated, when the soil residue is treated with 0.1M NaOH, originates from humic and fulvic acids, would give maximum ratios of ~0.2 and 1.5 for trichloroacetyl-CHCl₃/ A_{254} and trichloroacetyl-CHCl₃/ A_{465} , respectively. This is close to the ratio in the second and third water
 285 extract in the sequential extraction, where the continued extraction of trichloroacetyl-CHCl₃ might then be due to extraction of trichloroacetyl-CHCl₃ in humic structures. In the first water extract, in the MeOH extract and especially in all investigated suction cell and groundwater samples, the

290 higher ratios indicate that trichloroacetyl-CHCl₃ in humic structures do not constitute the main fraction of the total trichloroacetyl-CHCl₃.

295 **Table 2** Ratio between trichloroacetyl-CHCl₃ concentration and absorption at 254 nm or 465 nm, as a substitute for content of dissolved humic substances (HS). A₂₅₄ is mainly due to aromatic residues. A₄₆₅ is mainly due to coloured moieties of HS. A₄₆₅ was below detection limit in some of the groundwater samples from Viborg and Feldborg, denoted with a ?.

Sample type	Ratio (µg/l / A ₂₅₄)	Avg. for sample type	Ratio (µg/l / A ₄₆₅)	Avg. for sample type	Mainly HS-CCl ₃ ?
Whole soil washed with water	0.17-0.18	~0.17	1.2-1.5	~1.3	Yes (assumed)
Whole soil washed with water and MeOH	0.15		1.0		
1. water extract of soil	0.54-0.88	~0.72	4.9-9.9	~7.8	Probably not
2. + 3. water extract of soil	0.23-0.28	~0.26	1.8-2.0	~1.9	Probably
MeOH extract of soil	0.55	0.55	8.0	8.0	Probably not
Soil water from suction cells	2-11	~5	68-495	~182	Probably not
Groundwater (Viborg), pH 4.6-4.7	7.5-17	~17	388-?	>500	Probably not
Groundwater (Feldborg), pH 4.9-5.2	15-29		?-?		
Groundwater (Tisvilde), pH 6.1-8.7	1.4-6.3	~3	19-113	~46	Probably not

Behaviour of trichloroacetyl containing compounds in solid phase extraction

300 To investigate whether the trichloroacetyl-CHCl₃ in environmental samples consisted of neutral compound(s) like chloral and trichloroacetone, we performed solid phase extraction on pre-fabricated columns (Waters Oasis[®] MAX), which consist of a hydrophobic polymer with a quaternary amine anionic exchanger incorporated. Such a column will capture both non-ionic compounds and carboxylic acids, and when the column is afterwards flushed with a pH 7.5 buffer, organic compounds that carry no charge at that pH will be fixed on the hydrophobic resin, while organic compounds with a carboxylic acid group will attach to the anion exchanger. The non-ionic compounds can then be eluted with pure MeOH, and afterwards the carboxylic acids can be eluted with 80% methanol, acidified to a pH below the pK_a of the acid. In Table 3, the results from solid phase extraction on various chemical standards and environmental samples containing trichloroacetyl-CHCl₃ are presented.

310 **Table 3** Recovery of standards and trichloroacetyl-CHCl₃ in environmental samples in the fraction with either non-ionic
 compounds or carboxylic acids obtained from solid phase extraction. Trichloroacetyl-CHCl₃ was analyzed in samples
 before the preconcentration and in each of the two eluates after the preconcentration. Throughfall and needle extracts
 contained TCAA apart from the trichloroacetyl containing compound(s). The recovery of TCAA in these samples was
 determined by also heating the acidic eluates after adjustment of pH to 7.5 and found to be only slightly lower than the
 315 recovery of the TCAA standard (results not shown).

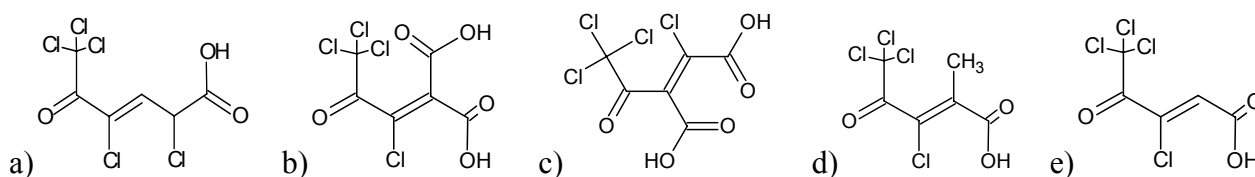
Sample	No. of samples	Non-carboxylic acid (% recovery in MeOH eluate at pH 7.6)	Carboxylic acid (% recovery in MeOH/H ₂ O eluate at pH 0.6)	Total recovery (%)
TCAA std.	1	3	95	98
Chloral std.	1	86	2	88
1,1,1-trichloroacetone std.	1	95	0	95
Chloroform std.	1	43	3	46
Soil water from suction cell	4	0-4	88-98	89-99
Soil extract	2	0	43-50	43-50
Forest groundwater	3	1-4	61-74	63-78
Throughfall from conif. forest	1	17	40	57
Spruce needle extract	3	10-26	23-34	44-55

TCAA eluates as expected for a carboxylic acid and chloral, 1,1,1-trichloroacetone and CHCl₃
 eluates as expected for non-ionic compounds. The low yield of CHCl₃ is most likely at least partly
 due to evaporation during the solid phase extraction, since it is not designed for volatile compounds.
 320 The trichloroacetyl-CHCl₃ in the soil water from the suction cells, soil extracts and groundwater did
 not elute with MeOH at neutral pH, while most (suction cell and groundwater) or half (soil extracts)
 of the trichloroacetyl-CHCl₃ eluted with acidified MeOH/H₂O as expected for carboxylic acids. No
 additional trichloroacetyl-CHCl₃ could be eluted when more than one ml eluent was applied, and
 the missing trichloroacetyl-CHCl₃ seemed to be lost during loading of the soil extract. The low
 325 yield when soil extracts were preconcentrated was a repeatable problem, which must be due either
 to an unknown matrix effect or to trichloroacetyl-CHCl₃ located in and therefore lost with high
 molecular weight compounds (>6-8 kDa), that, according to the supplier of the columns, cannot be
 trapped. Laboratory experiments with soil extracts and solutions of purified humic acid (HA) from
 the forest soil, showed that more than 75% of the HA was not recovered on the solid phase
 330 extraction columns and that the suction cells did decrease the HA-content as much as 50% – most
 likely because of filtration, since according to the supplier of the suction cells, the cells do not
 adsorb dissolved organic matter. This could then also explain the better recovery of trichloroacetyl
 containing compound(s) from the soil water sampled with suction cells, since it had already been
 exposed to a size-exclusion. Furthermore, this also indicates that the trichloroacetyl-CHCl₃
 335 concentration in the forest soil pore water could be higher than what was estimated using the
 suction cells for sampling, since some of the macromolecular/colloidal trichloroacetyl-CHCl₃ is

probably lost. Also when spruce/pine throughfall and spruce needle extracts were preconcentrated, the yields of trichloroacetyl-CHCl₃ were low (50-57%), while the recovery of TCAA present in these samples was only slightly lower than the almost full recovery of TCAA in the standard solution. Interestingly, 23-47% of the recovered trichloroacetyl-CHCl₃ in throughfall and needle extracts was eluted with the pure MeOH, indicating the presence of non-ionic (at pH 7.5) trichloroacetyl containing compound(s) as well as trichloroacetyl containing compound(s) containing a carboxylic acid group in these samples.

345 **Conclusions on the chemical structure of trichloroacetyl containing compounds**

The major part of trichloroacetyl-CHCl₃ in top soil seems to be present in non extractable compounds or in compounds only extractable with NaOH, possibly humic substances. Although the comparison between trichloroacetyl-CHCl₃-concentration and A₂₅₄ only gives a rough estimate, it seems likely that most of the trichloroacetyl-CHCl₃ in soil pore water and in groundwater consists of easily leachable substances with a CCl₃-C=O group and a carboxylic acid group. Such compounds have not previously been published as naturally occurring, but in two cases, compounds with both these structural elements have been identified as products from the chlorination of HS or polyphenolic model compounds of HS in the laboratory, when the concentration of chlorinating agent was low and the pH was below neutral,^{24,25} Fig. 4. It is possible that the natural unspecific chlorination processes, previously shown to take place in forest soil²⁶⁻²⁹ can result in the same or similar acids, as was also previously hypothesized.²⁸



360 **Fig. 4** The six previously published structures fulfilling the structural elements of trichloroacetyl containing compounds that we have identified in environmental samples. (a) from ²⁴, (b)-(e) from ²⁵. The compounds were formed during artificial chlorination with low chlorine dose and only at pH-values below neutral.

It has been suggested, that compounds containing the structural elements CCl₃-C-OH and CCl₃-C-Cl would also decompose and release CHCl₃ at high pH.^{25,30} This hypothesis was tested with two commercial standards containing such structural elements (3-hydroxy-4,4,4,-trichlorobutyric acid and cis-3,4,4,4-tetrachlorocrotonic acid). Neither of these compounds produced detectable amounts

of CHCl_3 when heated to 100°C or when dissolved in 0.1M NaOH , so the only plausible structural element to give CHCl_3 at alkaline pH remains to be $\text{CCl}_3\text{-C=O}$. It should be mentioned, though, that trichloroimines ($\text{CCl}_3\text{-C=N}$) also release CHCl_3 in alkaline solution,²¹ but that this rare structural element should be present in the various environmental samples seems highly unlikely.

Relevance of trichloroacetyl- CHCl_3 to the interpretation of previously published studies

The discovery of trichloroacetyl containing compounds as widespread in the forest environment will have some implications for the interpretation of previously published studies, in which TCAA was analyzed by the thermal decarboxylation method. Analysis of TCAA in environmental samples by decarboxylation was first used by Frank *et al.* (1990) who reported the analysis of 25 needle samples using both the standard derivatization method and what should subsequently be known as the standard thermal decarboxylation method¹³. The two methods showed a high degree of positive relationship although decarboxylation on average, but not always, led to determination of slightly higher TCAA concentrations in the needles. Even though decarboxylation in one occasion led to 70% higher TCAA-concentration, the concluding remarks on the use of the thermal decarboxylation method for needle samples as being “sufficient for routine determination”¹³ seems justified. As we will now show, this fairly soft statement of Frank *et al.*, that the timesaving decarboxylation method can be used for routine analyses of needle samples, led to a series of research articles where different authors used this method for samples from various environmental compartments (Table 4). This was done without comparing to a substance specific method and except for Reeves *et al.* (2000) and Plümacher & Renner (1993), also without referring to the original method development by Frank *et al.*, blurring the fact that the method was a “routine method” for TCAA in needles. In Table 4, a complete list of the papers published in international journals where the thermal decarboxylation method was used for environmental samples without comparison with the more substance specific derivatisation method, is shown.

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Table 4 Articles in international journals using the thermal decarboxylation method for TCAA in environmental samples without comparing with the more substance specific derivatization method. The type of sample that was analyzed with the decarboxylation method is also shown.

Publication	Sample type
Dickey <i>et al.</i> (2005) ¹⁶	Soil
Stidson <i>et al.</i> (2004) ⁸	Soil, rain, needles, streamwater
Stidson <i>et al.</i> (2004) ³¹	Needles
Heal <i>et al.</i> (2003) ³²	Rain & cloud water
Reeves <i>et al.</i> (2000) ³³	Needles
Weissflog <i>et al.</i> (2004) ³⁴	Freshwater
Weissflog <i>et al.</i> (2003) ³⁵	Needles
Weissflog <i>et al.</i> (2001) ³⁶	Needles
Weissflog <i>et al.</i> (1999) ³⁷	Needles and soil
Plümacher & Schröder (1994) ³⁸	Needles
Plümacher & Renner (1993) ¹⁵	Needles, rain and throughfall

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Based on the results published by Frank *et al.* (1990) in combination with our results in this paper, it seems as though using the thermal decarboxylation method for needle and rain water samples will only result in minor (up to ~25%) overestimations of TCAA concentration (Table 1). A recently published statement like “It is unlikely that chloroform precursors (except TCA) are present in conifer needles, so the decarboxylation method might be the method of choice for analysis of vegetation”³⁹ should, with the present knowledge, be considered critically, however. Using the method for throughfall samples might lead to similar overestimation, while there is a risk that using thermal decarboxylation for soil samples might determine trichloroacetyl containing compounds, only. The fact that some interfering moiety could exist in soil has previously been discussed,^{8,16} but the fact that also aqueous extracts of soil can contain mainly non-TCAA-derived CHCl₃ liberated upon heating, is a new and important perspective.

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Apart from Frank *et al.* (1990), two additional papers have compared the thermal decarboxylation method with the derivatization method. Matucha *et al.* (2006) compared the two methods using three needle samples and one laboratory soil sample that had all been previously spiked with TCAA and found good agreement between the two methods.¹² Unfortunately the authors did not analyze any environmental samples, and hence this study is not very informative on the possible use of the decarboxylation method for such samples. Fillibeck *et al.* (1995) compared the two principal methods for TCAA analysis for several rain and throughfall samples.⁴⁰ The ratio between TCAA determined from derivatization and thermal decarboxylation ranged from ~0.14 to ~8. This very large range unfortunately makes it impossible to interpret if any trichloroacetyl-CHCl₃ existed in e.g. some of the throughfall samples, but in most of the precipitation samples, trichloroacetyl

420 containing compounds appear not to have been major contributors to the total CHCl_3 liberated, similarly to what we found for precipitation samples (Table 1).

425 Dickey *et al.* (2005) compared "TCAA"-concentrations in natural coniferous forest soil samples determined by heating of either whole soil samples or of soil extracts, to see if the standard procedure of extracting the TCAA with water underestimates the whole soil TCAA concentration because of sorption or other binding of the TCAA to the soil. The authors show that less than 20% of the CHCl_3 released when heating the whole soil samples is released when heating the soil extracts. The authors suggested that the underestimation by extracting with water was due to binding of TCAA to ion exchange sites in the soil and hence low extraction efficiency. In light of our results a much simpler explanation might exist, namely that Dickey *et al.* (2005) have partly or 430 fully been measuring trichloroacetyl- CHCl_3 when heating the forest soil as well as the aqueous soil extracts. Their low yields of "TCAA" in the soil extract fits very fine to the results in Fig. 3, with only a small part of the trichloroacetyl- CHCl_3 extracted with water, the rest being structural elements of humic substances or other organic soil constituents. Dickey *et al.* (2005) did actually test if a commercial humic acid released CHCl_3 upon heating and found no such release. Humic 435 acids are however extracted from soil or lignite with a high pH solution (most often 0.1M NaOH) and any trichloroacetyl- CHCl_3 originally present in the humic acid would therefore have been lost during the extraction and purification. All in all it seems very likely that Dickey *et al.* (2005) did not measure TCAA, but rather trichloroacetyl containing compounds, in the coniferous forest samples, which could also explain the relatively high "TCAA"-concentrations they found in otherwise 440 unpolluted samples. Such artefact in the TCAA-determination is probably also the reason of the very high "TCAA"-concentrations (up to ~ 2 mg/kg dw) in soil determined by the same research group using the whole soil method of Dickey *et al.*⁸ The risk that interfering moieties could exist in the soil was actually discussed by the authors⁸, but unfortunately these reservations have not been mentioned when the "TCAA"-concentrations were later cited.^{16,41}

445 In contrast to the thermal decarboxylation procedure for TCAA in soil, analysis of soil TCAA using derivatization instead of thermal decarboxylation will most likely lead to reliable results. Hoekstra *et al.* (1999) analyzed TCAA in two samples of temperate coniferous forest soil using the esterification procedure and found concentrations from 0.2 – 0.3 $\mu\text{g}/\text{kg dw}$.¹⁴ Peters (2003) similarly 450 used esterification and found TCAA concentrations in soils of temperate forests from <0.05 – 1.9

455 $\mu\text{g}/\text{kg}$ dw, except for one site that contained up to $12 \mu\text{g}/\text{kg}$ ⁴² and finally Scott *et al.* (2005) measured TCAA concentrations from $<0.8 - 4 \mu\text{g}/\text{kg}$ and in one case $10 \mu\text{g}/\text{kg}$ in temperate soils from Canada and England using esterification.² In addition, Frank (1988) observed concentrations of TCAA in forest soil of $20 - 380 \mu\text{g}/\text{kg}$, but did not state the analytical method used.⁴³ In a personal communication, the author states that these measurements were conducted with esterification of the TCAA and that the TCAA found in the soil most likely derived from atmospheric deposition and run-off from leaf-surfaces into the soil.⁴⁴

460 From this it seems clear that TCAA does occasionally exist in small concentrations in unpolluted soil. Similarly, it is of course possible, that a small amount of TCAA could have been present in some of the soil samples we analyzed, but with the presence of water extractable trichloroacetyl- CHCl_3 in concentrations of $10 - 70 \mu\text{g}/\text{kg}$ dw, values of less than 10 – 20% of this would have been impossible to determine as a difference between CHCl_3 released upon adding NaOH and upon heating at pH 7.5. This approach was possible for the needle and throughfall samples that contained much more TCAA than trichloroacetyl- CHCl_3 , but not for soil samples. We can therefore not rule out that small amounts of TCAA could be present in the forest soil, but when using the thermal decarboxylation method on this sample type, most if not all of the liberated CHCl_3 comes from trichloroacetyl containing compounds.

Conclusions

470 We have shown that trichloroacetyl compounds are widespread in the forest environment. This is important to consider, before one uses the thermal decarboxylation method to determine TCAA in environmental samples and is also important to keep in mind when interpreting previous published studies, where that analytical method has been used. If the decarboxylation method is to be used for the determination of TCAA, this is best done at low pH, as the interference from trichloroacetyl compounds is minimized. Determination of trichloroacetyl compounds by conversion to CHCl_3 can be done accurately at pH = 12.

480 The origin of the trichloroacetyl compounds is probably natural and the formation could be linked to the natural formation of CHCl_3 and TCAA, known to take place in forest soil. Future studies are needed to support this hypothesis and it would also be interesting to see in a future study, if the compounds are present outside the forest environment. Furthermore, a full structural identification and isolation of the easily leachable non-humic trichloroacetyl containing compounds, would be necessary, to determine if the compounds have (eco)toxicological relevance.

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Occurrence and fate of naturally formed trichloromethyl compounds in coniferous forests

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Christian Nyrop Albers has contributed to the project as described below:

0 = No contribution

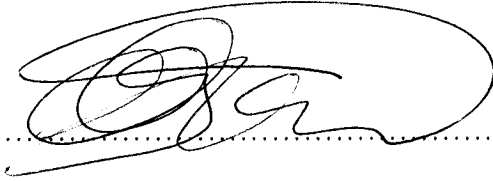
1 = Minor

2 = Substantially

- | | |
|---|---|
| a) Basic formulation of the project leading to the published work | 2 |
| b) Strategy for the project / choice and development of methods | 2 |
| c) Implementation of the project / empirical work | 2 |
| d) Analysis, interpretation and discussions | 2 |


Date: *23 02 2010*

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Occurrence and fate of naturally formed trichloromethyl compounds in coniferous forests

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10 Abstract

Pollution with organochlorines has received major attention due to various environmental effects, but it is now increasingly recognized, that they also take part in biogeochemical cycles and that natural background concentrations exist for several chlorinated compounds. We here report the natural occurrence and cycling of organic compounds with a trichloromethyl moiety in common.

15 The study areas are temperate coniferous forests. Trichloromethyl compounds can be found in all compartments of the forests (groundwater, soil, vegetation, throughfall), but not all compounds in all compartments. The atmospheric input of trichloromethyl compounds is found to be minor, with significant contributions for trichloroacetic acid (TCAA), only.

20 In top soil, where the formation of the compounds is expected to occur, there is a clear positive relationship between chloroform and trichloroacetyl containing compounds. Other positive relations occur, which in combination with chlorination experiments performed in the laboratory, point to the fact that all the trichloromethyl compounds may be formed concurrently in the soil, and their subsequent fates then differ due to different physical, chemical and biological properties. TCAA cannot be detected in soil and groundwater, but sorption and mineralization experiments performed
25 in the laboratory in combination with analyses of vegetation, show that TCAA is probably formed in the top soil and then either taken up by the vegetation or mineralized fast in the soil. Based on this and previous studies, a conceptual model for the natural cycling of trichloromethyl compounds in forests is proposed.

30 *Keywords:* Natural organochlorine, chloroform, trichloroacetic acid, forest, soil, groundwater

1. Introduction

During the last twenty years, the general perception that the occurrence of chlorinated organic compounds in nature is mainly the result of human activity has been challenged. Natural organic compounds containing especially chlorine but also other halogens have been found in various ecosystems (for most recent reviews see Öberg, 2003; Gribble, 2003; Laternus et al., 2005; Clarke et al., 2009). A portion of these studies have been carried out in temperate coniferous forests, where the majority of soil chlorine is organically bound (Öberg, 2003; Albers et al., Submitted a) and where especially chlorine in macromolecular structures, but also smaller identifiable compounds, have been found in the soil. Chloroform (CHCl_3), one of the most common groundwater pollutants in the Western World (e.g. Carter et al., 2008), is one compound, whose natural formation has received attention in a number of studies (e.g. Hoekstra et al., 1998; Laternus et al., 2000; Hoekstra et al., 2001; Dimmer et al. 2001; Svensson et al., 2007; Albers et al., 2008a). The formation of CHCl_3 in the soil seems to be associated with microbial activity (Haselmann et al., 2000; Albers et al., Submitted a, b). Furthermore, it has been made probable that an abiotic formation of CHCl_3 may exist in soil (Huber et al., 2009). The current hypothesis on the biotic formation of CHCl_3 involves the excretion of fungal chloroperoxidases, which have been detected in forest soil (Asplund et al., 1993), and the subsequent formation of HOCl or other oxidized chlorine species (Hoekstra et al., 1998). The oxidized chlorine will react unspecifically with soil organic matter (SOM), probably in a somewhat similar way, as has been shown to occur during chlorination of drinking water and paper pulp (Rook, 1977; Kringstad and Lindström, 1984; Deborde and von Gunten, 2008). Various organic structures can function as precursors for the formation of CHCl_3 . By fractionation of dissolved organic matter, humic substances (HS) in general and humic acids (HAs) in particular have been shown to form high amounts of CHCl_3 during chlorination (Singer, 1999; Reckhow et al., 2008). HAs are not present in very high concentrations in natural waters, but in soil, they typically constitute the major part of HS, which again constitute 30-75% of SOM (Stevenson, 1994; Swift, 1996). Plenty of suitable organic matter must therefore be expected to be present for the formation of CHCl_3 in forest soil. From literature on chlorination of drinking water, it is well known, that besides CHCl_3 , TCAA is formed as the major disinfection by-product (e.g. De Leer et al., 1985; Reckhow et al., 1990; Reckhow et al., 2008). TCAA was once a widely used herbicide and its toxicity is of concern especially in the terrestrial environment, where predicted no effect concentrations as low as $2.4 \mu\text{g}/\text{kg dw}$ have been calculated for coniferous trees (Ahlers et al., 2003) and risk quotients above one can be calculated for a number of locations (Lewis et al., 2004).

TCAA, formed from various chlorinated air pollutants in needles of coniferous tree species, has through biomonitoring studies been connected to observed phytotoxic effects (e.g. Frank, 1991; Weissflog et al., 2001). It is therefore important to determine background concentrations of TCAA, if these are formed along with CHCl_3 in the soil. Some studies have indeed suggested the natural formation of TCAA in forest soil (Hoekstra et al., 1999; Fahimi et al., 2003; Matucha et al., 2007), and the presence of presumably natural TCAA in forest soil has been shown (Hoekstra et al., 1999; Stidson et al., 2004). Beside CHCl_3 and TCAA, a third kind of compound containing a trichloromethyl group is known from the chemical chlorination of HS, namely compounds containing a trichloroacetyl ($\text{CCl}_3\text{-C=O}$) group (Figure 1) (Boyce and Hornig, 1983; De Leer et al., 1985). Recently this structural element was found in significant concentrations in various forest compartments (Albers et al., 2010). In the same paper it was made probable, that on several occasions, trichloroacetyl containing compounds had by mistake been interpreted as being TCAA due to the application of an analytical method not specifically detecting TCAA but rather the CHCl_3 formed upon heating.

In conclusion it seems very likely that an unspecific chlorination occurs in forest soil, and that this chlorination among other things leads to the formation of CHCl_3 . Trichloroacetyl containing compounds are also likely to be formed in forest soil, but the quantities and processes concerning this formation are largely unknown. TCAA would be expected to be formed along with CHCl_3 and trichloroacetyl containing compounds, but currently there is reasonable doubt concerning the origin and quantity of TCAA in forest soil.

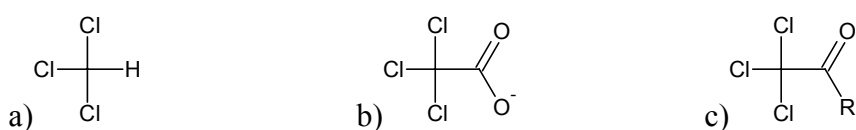


Figure 1. The trichloromethyl compounds of interest in this study. a) Chloroform (CHCl_3). b) Trichloroacetic acid (TCAA). c) The trichloroacetyl-group of trichloroacetyl containing compounds. This group liberates CHCl_3 at alkaline pH, and in cases where this CHCl_3 is quantified, the term “trichloroacetyl- CHCl_3 ”, which really means “ CHCl_3 originating from trichloroacetyl containing compounds”, will be used. R may be either an H-atom (= chloral) or a C-atom as part of an unknown larger molecular structure.

The purpose of our study was to investigate occurrence, fate and possible relationships between the different trichloromethyl compounds, previously proposed to be formed in forest soil. In particular we wanted to look more into the formation and fate of trichloroacetyl containing compounds, whose occurrence in forest soil was recently reported and which are likely to play an important role in the

95 formation of CHCl_3 . We included several environmental compartments in our study (precipitation,
vegetation, soil from several depths and groundwater) to get a better picture of the cycling and fate
of the compounds in the forest ecosystem. Finally we conducted some laboratory experiments to
look more into the factors and processes influencing the formation and fate of trichloromethyl
compounds in general, and TCAA in particular.

100

2. Materials and Methods

2.1. Study sites

Four forests were chosen as study sites because they were previously found to contain significant
amounts of natural CHCl_3 and trichloroacetyl containing compounds (Albers et al. 2010; Albers et
105 al., Submitted a). All four forests are coniferous plantations on sandy soils, located in a maritime
temperate climate in Denmark, and at all four locations, the sandy soil is covered with an organic
layer (O-horizon), 5–25 cm thick. Names of study sites (abbreviation; GPS-coordinates): Tisvilde
Hegn (TH; 12°04' E, 56°02' N), Viborg Hedeplantage (VH; 9°22' E, 56°26' N), Nordre Feldborg
(NF; 8°57' E, 56°23' N), and Liseborg Plantage (LP; 9°22' E, 56°26' N). All locations have been
110 thoroughly described previously (Albers et al., 2010; Albers et al. Submitted a, b). Soil was
sampled from additionally two locations, both situated in the Jura Mountains in Switzerland. The
forest site near Les Bayards (6°32' E, 46°54' N) is a mixed stand of Beech (*Fagus sylvatica*) and
Norway Spruce (*Picea abies*), and so is the forest near Chaumont (6°59' E, 47°02' N). At both
locations the small organic layer of 0–10 cm thickness is located on top of a thin clayey layer mixed
115 with stones from the underlying limestone sedimentary rock.

2.2. Environmental samples

Soil was sampled from the top soil with steel cylinders (H: 30 cm and Ø: 6 cm), divided into visible
horizons and either extracted within 24 hours or stored frozen until analysis. The soil was
120 homogenized either by sieving (minerogenic soil) or in a coffee mill (organic horizons) before
extraction with water (2:1 water:soil slurry, 24 hours shaking at 10°C in the dark followed by
centrifugation). On one occasion, soil was also sampled to a depth of 4.8 m in TH and 2.8 m in VH
(see Albers et al. (Submitted b) for procedure and basic soil parameters).

125 In the field, soil water was abstracted from soil in TH with suction cells (Prenart Teflon-quartz,
Prenart, DK) placed at 0.5–4.3 m depth and collected in evacuated (300–400 hPa, semi
continuously suction) glass bottles connected to the cells with polyethylene tubing. Groundwater
was sampled through 5 cm brass filters placed in the upper groundwater as described earlier (Albers
et al., 2008a; Albers et al., Submitted b).

130 Needles were taken from spruce trees (*Picea abies*). Twigs were cut off and the two-year-old needle
class was chosen for further analysis. After removal from the twigs, the needles were crushed to
fine powder under liquid nitrogen in an agate mortar and 5 g needles were extracted with 50 mL
water for 5 hours. Spruce xylem was taken from the outer 5 cm of the stem at 1.4 m height using an
increment borer (Haglöf Sweden, Långsele, Sweden). Xylem could not be crushed under liquid
135 nitrogen and was therefore divided into fine particles in a coffee mill and then extracted in the same
way as the needles. Precipitation was sampled both from clearings in the forest (rain) and below the
vegetation (throughfall). Stainless steel funnels were placed at 1.5 m height and connected through
polyethylene tubing to glass bottles placed in the dark below the soil surface.

2.3. Chemical analyses

140 Commercial TCAA was of analytical quality and purchased from Sigma-Aldrich (Steinheim,
Germany). [2-¹⁴C]-TCAA was purchased from Moravек Biochemicals (Brea, California), with a
radiochemical purity of ≥99.5%. All water used was Milli-Q water that had been heated to 100°C
and afterwards purged with N₂ to remove all CHCl₃, TCAA and other compounds liberating CHCl₃
upon heating.

145 Trichloroacetyl-CHCl₃ and TCAA was analyzed as described previously (Albers et al., 2010), with
TCAA determined from the difference between CHCl₃ released upon heating to 100°C for 2 hours
at pH 7.5 (TCAA + trichloroacetyl-CHCl₃) and CHCl₃ released without heating at alkaline pH
(trichloroacetyl-CHCl₃ only). Furthermore TCAA was determined in selected samples in a
compound specific commercial analysis performed by Eurofins Denmark. Briefly, the method used
150 by Eurofins was pre-concentration of TCAA and internal standard on OASIS solid phase extraction,
elution with MeOH and methylation of TCAA in MeOH/BF₃ at 70°C. Methylated TCAA was then
extracted into hexane and analyzed by GC/MS. The limit of quantification (LOQ) was given as 0.01
µg/L.

CHCl₃ in aqueous samples was analyzed by purge & trap as described previously (Albers et al.,
155 2008a; Albers et al., Submitted a).

Soil pH was measured in a 2:1 water:soil slurry. pH and oxygen in groundwater samples were
analyzed in a flow-through cell in the field.

Total dissolved organic carbon (DOC) in precipitation and soil water samples was determined on an
Elementar liquiTOC (Hanau, Germany).

160

2.4. Laboratory experiments

Sorption studies: 8 x 2–9 g of each soil (depending on SOM-content) were transferred to a 15 mL
glass vial (with teflon-liner in the lid) and 5 mL H₂O + 0.5 mL 1M NaN₃ (to avoid microbial
degradation of TCAA) was added. After 24 hours of shaking, TCAA and [2-¹⁴C]-TCAA solution
165 was added and the vials were filled to the top with H₂O. Duplicate concentrations of 1, 10, 100 and
1000 µg/L total TCAA was then achieved, with ¹⁴C-activity ranging from 840–8400 DPM/mL.
After another 70 hours of shaking, the samples were centrifuged (1000g, 15 min.) and the ¹⁴C-
activity in the water was determined by Liquid Scintillation Counting (LSC). For each TCAA-
concentration, two blanks with no soil added were treated similarly to account for any losses
170 through e.g. sorption to the vial. Sorption was described using the Freundlich-isotherms:

$$C_s = K_F \times C_w^n$$

C_s is the concentration of TCAA in soil or HS in µg/kg, C_w is the concentration in the liquid in
µg/L, K_F is the Freundlich coefficient in L/kg and n describes the non-linearity of the isotherm.

175 *Mineralization studies:* Triplicate soil cores (H: 30 cm, Ø: 6 cm) were sampled in NH and TH, and
divided into visible soil horizons (top soil study) or from 0–4.8 m in TH (depth study).

Mineralization of [2-¹⁴C]-TCAA in the soils was determined by measuring release of ¹⁴CO₂ with
0.5M NaOH as a CO₂-trap. 5 g homogenized O-horizon or 10 g 2mm sieved mineral soil was
weighed into 200 mL glass jars and equilibrated for three days at 10°C. One or two mL [2-¹⁴C]-
180 TCAA solution (25000 DPM/mL) was then added to the soil resulting in a TCAA concentration of
~7 µg/kg soil. After addition of the TCAA-solution, the soils contained 82–90% moisture (O-
horizons) or 20–39% moisture (mineral soils) almost reaching water holding capacity for most of
the soils. Each microcosm was equipped with a small glass vial containing 2 mL 0.5M NaOH to
trap CO₂. The NaOH-solution was renewed every 6, 24, 48, 72 and 168 h (top soil experiment) or
185 every 5, 24, 48, 96, 168 and 264 h (depth experiment) and the ¹⁴C-activity in the NaOH-solution

was determined by LSC. All mineralization experiments were done in triplicates at 10°C in the dark. For three subsamples, half of the two mL TCAA-solution was replaced with one mL 1M NaN₃ (microbial inhibitor) to test if the mineralization was caused by microorganisms.

190 *Chlorination studies:* To check that the soil in the forests could work as a precursor for formation of both CHCl₃, TCAA and trichloroacetyl containing compounds, and to see any differences between soil layers and sites, three subhorizons of the O-horizon in NF were chlorinated together with one mixed O-horizon from TH and two humic acid (HA) fractions extracted from F- and H-horizons from TH. In NF, soil was sampled in five replicates from the top soil with steel cylinders (H: 15 cm and Ø: 6 cm) and the soil was divided into dead needles (litter- (L-) horizon), partly degraded plant material (fermentation- (F-) horizon) and dead plant material degraded to beyond recognition (humification- (H-) horizon). Larger roots and particles were removed, the samples were freeze-dried and then finally grinded to fine powder in a coffee mill followed by further grinding in an agate mortar. The L-horizon samples were pooled before chlorination while the other 10 NF-
195 samples were chlorinated individually. The TH samples (soil and HA) were sampled without replicates. The HA-fractions were extracted with 0.1M NaOH under an N₂-atmosphere and purified according to the IHSS standard procedure (Albers et al., 2008b; IHSS, 2009), with the exception that HNO₃ and KNO₃ were used instead of HCl and KCl.

The organic matter (SOM) content of the soil and HA samples was determined as loss on ignition
205 and soil or HA corresponding to 15.5 mg SOM was transferred to a 16 mL glass vial with a PTFE liner in the lid. Fourteen mL H₂O, 1.35 mL 1M PO₄-buffer (pH 4.0) and 155 µL 50mM NaOCl were added, resulting in 1 g/L SOM in slurry and [OCl⁻] = 0.5 mM. The vials were sealed and incubated for 24 hours at 10°C before the reaction was quenched with 100 µL 1M Na₂S₂O₃. 3 x 100 µL were taken for the analysis of CHCl₃, TCAA and trichloroacetyl-CHCl₃, respectively. Two vials
210 with no soil added were included as controls and treated similarly to the sample vials. Small amounts of CHCl₃ were found in these control vials and this CHCl₃ was subtracted from the CHCl₃ found in the chlorinated soil samples. No TCAA or trichloroacetyl-CHCl₃ was detected in the controls.

215 **2.5. Statistical software**

Simple regressions on TCAA concentration in throughfall samples (dependent variable) and trichloroacetyl-CHCl₃ and TCAA concentrations in throughfall and rain samples (independent variables) were performed using KyPlot Version 2.0 (KyensLab Inc.). Since data did not show a normal distribution, the non-parametric Spearman's Rank Correlation Coefficient was used to
220 determine if correlations were significant. A similar analysis was carried out for soil bound trichloroacetyl-CHCl₃ (dependent variable) and total soil organic chlorine (independent variable).

3. Results and Discussion

3.1. Reliability of the applied analytical methods

225 Because of the recently published uncertainties in determining TCAA in soil and other environmental compartments by thermal decarboxylation and subsequent analysis of the formed CHCl₃ (Albers et al., 2010), we will spend a moment discussing the accuracy when using exactly that method in this study. Trichloroacetyl containing compounds will also form CHCl₃ upon heating, and when the heating is performed at neutral pH, almost quantitatively (Albers et al.,
230 2010). Each time a sample was heated for TCAA analysis, a similar sample was therefore treated with alkali, to determine trichloroacetyl-CHCl₃, without determining TCAA, as suggested previously (Albers et al., 2010). To test if this was indeed a reliable way to determine TCAA, we had a number of different samples analyzed in a commercial laboratory, where a compound specific method for the determination of TCAA was used (Table 1). The same samples were analyzed using
235 thermal decarboxylation in our laboratory, with the subtraction of both background CHCl₃ and trichloroacetyl-CHCl₃. The agreement between this analysis and the commercial analysis was in most cases good, but in a few cases, the thermal decarboxylation method gave slightly higher concentrations, even when corrected for trichloroacetyl-CHCl₃. The reason for this discrepancy is not obvious, since the most likely uncertainty in the thermal decarboxylation method is incomplete
240 conversion of trichloroacetyl containing compounds upon heating, which would rather lead to an underestimation of TCAA. Bearing these uncertainties in mind, the TCAA concentrations in precipitation and vegetation presented throughout the paper should be trustworthy, though.

Due to the high trichloroacetyl-CHCl₃ concentration in soil, and hence high limit of quantification (LOQ) for TCAA in this sample type, the decarboxylation method cannot be used to determine low concentrations of TCAA in soil (Albers et al., 2010). An interesting outcome of the commercial analyses is therefore the verification that the forest soil samples do not contain significant amounts of TCAA (Table 1).

Table 1. Comparison of the analytical method used for the determination of TCAA throughout this paper with substance-specific GC/MS analysis by a certified commercial laboratory. In the GC/ECD method, TCAA was quantified from the CHCl₃ formed upon heating at pH 7.5, subtracted a background concentration of trichloroacetyl-CHCl₃ (shown in the right column). The LOQ of the GC/ECD-method therefore varied with this background-CHCl₃. The LOQ of the GC/MS method was 0.01 µg/L and the limit therefore varied for solid samples depending on the ratio between sample and water used to extract TCAA from that sample. See section 2.3 for more details on the two analytical methods.

Sample type	No. indep. samples	TCAA (µg L ⁻¹ or µg (kg fw) ⁻¹)	TCAA (µg L ⁻¹ or µg (kg fw) ⁻¹)	Trichloroacetyl-CHCl ₃ (µg L ⁻¹ or µg (kg fw) ⁻¹)
		Direct on GC/MS	Indirect on GC/ECD	
Soil	2	<0.1	<3	12–14
Groundwater	2	<0.01	<0.1	0.35–0.99
Rain	1	0.05	0.09	<0.01
Spruce throughfall	3	0.1–4.0	0.2–4.1	0.04–1.2
Spruce needles	1	46	50	6.9
Spruce xylem	1	<2	1.6	0.89

The determination of trichloroacetyl containing compounds by treatment with alkali, and subsequent analysis of the CHCl₃ formed, has been shown to be quantitative and with no interference from other compounds like e.g. TCAA (Albers et al., 2010). The background concentration of CHCl₃ in the sample will, however, influence the detection limit since the larger the background, the higher the uncertainty in the difference between the two measurements. Samples with high CHCl₃ concentrations relative to trichloroacetyl-CHCl₃ were therefore purged with N₂, ensuring the LOQ to always be <0.1 µg/L and typically <0.01 µg/L in liquid samples.

3.2. Trichloromethyl compounds in aqueous compartments

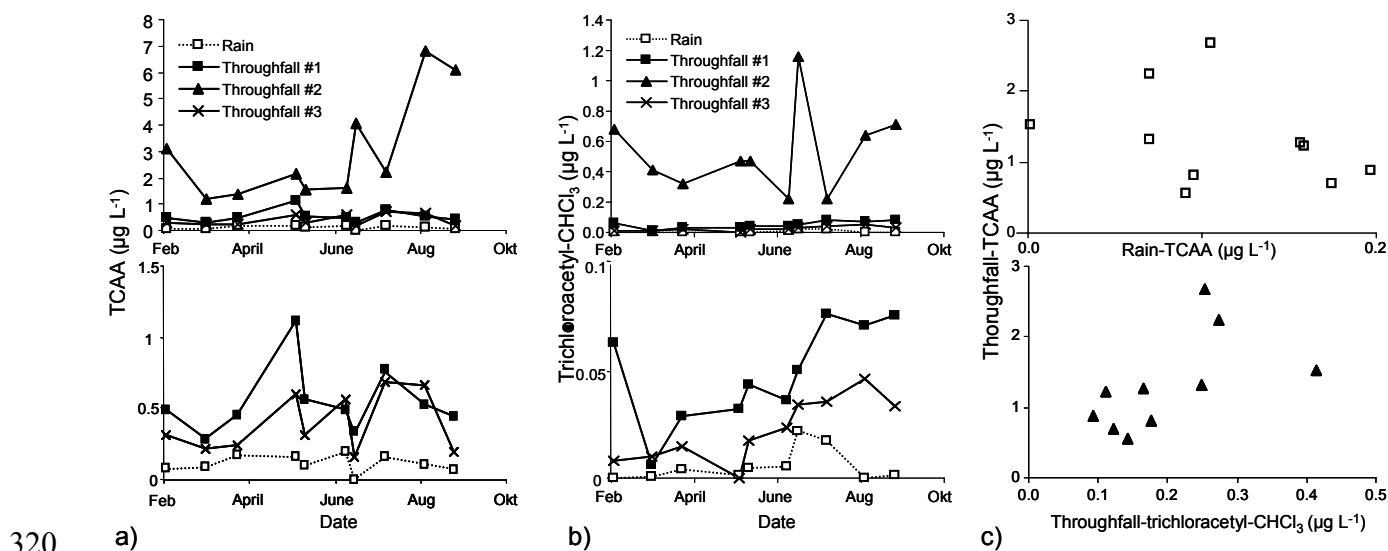
Precipitation samples were analyzed regularly for TCAA and trichloroacetyl-CHCl₃ during 7 months. While significant spatial and temporal variation was found for both compounds in the throughfall samples, no spatial and only minor temporal variation was found for the rain samples (Figure 1a & b). The average TCAA concentration in rain during the period was 0.11 µg/L and in

throughfall samples 1.3 $\mu\text{g/L}$, with a range of 0.39–3.0 $\mu\text{g/L}$ for the averages among the three replicate throughfall samplers. In rain, trichloroacetyl- CHCl_3 could normally not be detected and was in all cases $<0.02 \mu\text{g/L}$. In the throughfall samples, the average trichloroacetyl- CHCl_3 concentration was 0.20 $\mu\text{g/L}$, with a range of 0.025–0.53 $\mu\text{g/L}$ for the averages among the three replicates. The same throughfall sampler, which showed high TCAA concentrations (up to 6.8 $\mu\text{g/L}$ with an average of 3.0 $\mu\text{g/L}$), was also by far the sampler, showing the highest trichloroacetyl- CHCl_3 concentrations (up to 1.2 $\mu\text{g/L}$ with an average of 0.53 $\mu\text{g/L}$). Taking the average concentrations of TCAA and trichloroacetyl- CHCl_3 in throughfall at all sampling dates, there is, however, only a weak (although statistically significant) positive relationship between the two compounds (Figure 1c, closed triangles). Whether TCAA and trichloroacetyl containing compounds in throughfall have a common origin, therefore remains somewhat unclear. The formation in soil and subsequent translocation to and excretion from needles is a likely pathway for both compounds, though.

Increased concentrations of TCAA in throughfall have previously been reported (Reimann et al., 1996). Both dry and wet deposition of TCAA on the needles (Stidson et al., 2004) could cause this increased concentration and, since the needle cuticle seems not to be a barrier for TCAA (Cape et al., 2003), most likely also TCAA leached from the interior of the needles. No temporal relationship was found between TCAA in rain and TCAA in throughfall (Figure 1c, open squares). Since dry deposition of TCAA is very small ($\sim 1\%$) compared to wet deposition (Stidson et al., 2004), this suggests that only a minor portion of the TCAA in throughfall derives directly from the atmosphere, supporting leaching of TCAA from the interior of the needles. Conversion of volatile organochlorines like trichloroethene (TCE) and tetrachloroethene (PCE) to TCAA may occur inside both leaves of deciduous trees and conifer needles (Newman et al., 1997; Weissflog et al., 2007; Strycharz and Newman, 2009). All trees in the area are most likely exposed to similar concentrations of these substances, since no local pollution with these exist, but the huge variation between throughfall samplers points to a heterogeneous source of at least a part of the TCAA in throughfall. This is further substantiated by the different quantities of throughfall entering the three replicate throughfall samplers: The total precipitation in the area in the monitoring period was 421 mm, as determined in a rain gauge in one of the clearings. Of these, ~ 240 mm entered throughfall sampler #2 and #3 and ~ 150 mm entered throughfall sampler #1. This means that similar precipitation amounts entered the two samplers showing highest and lowest TCAA and trichloroacetyl- CHCl_3 concentrations, and that no relationship existed between possible pre-

concentration of wet and dry deposition of TCAA and concentrations of these compounds in throughfall. Furthermore, TCAA and trichloroacetyl-CHCl₃ concentrations were not higher in
 305 periods with little precipitation, where higher pre-concentration of any dry deposition would be expected.

The exact speciation of trichloroacetyl containing compounds in throughfall is unknown. They were previously shown to be a mixture of compounds with and without a carboxylic acid group, although
 310 compounds containing an acid group were dominating (Albers et al., 2010). The only plausible trichloroacetyl containing compound, which could be derived from the atmosphere is chloral, an intermediate in the atmospheric degradation of 1,1,1-trichloroethane and biotic degradation of trichloroethene. Chloral contains no carboxylic acid group and could constitute only a small part of the trichloroacetyl containing compounds in throughfall. The most likely origin of the majority of
 315 trichloroacetyl-CHCl₃ in throughfall seems not to be atmospheric then, but rather trichloroacetyl containing compounds taken up through the transpiration system and subsequently excreted from the needles during rain events. The occurrence of trichloroacetyl containing compounds in soil is discussed in another section of the paper.



320 Figure 2. Concentration of a) TCAA and b) Trichloroacetyl-CHCl₃ in rain and in pine/spruce throughfall in TH (February to August 2009). Only the average of the three replicates is shown for the rain samples, since no significant variation was found between replicates. Throughfall #2 is left out below, to better distinguish between the other three time series. c) An apparent weak positive relationship ($R^2 = 0.32$, $p=0.03$) between TCAA and trichloroacetyl-CHCl₃ in
 325 throughfall (closed triangles) exists, while there is no relationship between TCAA in throughfall and TCAA in rain (open squares). Each data point represents the average of all samples from one date.

Trichloroacetyl-CHCl₃ was determined in soil water in TH, using two different approaches; suction cells and extraction of soil with water (Figure 3a). The two methods are showing a similar vertical trend, but extraction of soil with water leads to ~4 times higher estimates of trichloroacetyl-CHCl₃ in soil water. Neither of the two methods is necessarily providing the correct concentration. Using suction cells, the concentration may be underestimated, since, if present in macromolecular, supramolecular or colloidal structures, the suction cup may filter off some of the trichloroacetyl-CHCl₃ (Albers et al., 2010). Extraction with water may on the other hand lead to an overestimation, if sorption processes are important for these compounds. In the TH profile, no matter what the exact concentration in the soil water is, the concentration of dissolved/aqueous trichloroacetyl-CHCl₃ seems to be highest at ~0.5 m depth and then to decrease down the unsaturated zone (Figure 3a). In the groundwater, it is hardly detectable, which is probably due to the increasing pH. Trichloroacetyl containing compounds are rather stable at acidic pH, while they are hydrolyzed at alkaline pH (e.g. Fuson and Bull, 1934), and a pH above 7, which is found below ~4 m depth, will therefore lead to a relatively fast hydrolysis of the compounds. Since trichloroacetyl containing compounds in soil and groundwater seem to be hydrolyzed at somewhat similar rates as chloral (Albers et al., 2010), the half-lives previously found for this compound (~14 days at pH 7.7 and ~3 days at pH 8.8, at 21°C (Koudjonou et al., 2008)) might give an idea of the half-life of trichloroacetyl containing compounds in deeper parts of the TH profile, although the temperature is somewhat lower there (8-10°C). The hydrolysis of trichloroacetyl containing compounds results in the liberation of CHCl₃, but while the CHCl₃ concentration does peak at a slightly lower depth (~1 m), also the CHCl₃ concentration is decreasing down the profile (Figure 3a). As previously discussed (Albers et al., Submitted b), this decrease might be caused by microbial mineralization and other processes occurring in the soil, and is therefore not necessarily in disagreement with the hypothesis that parts of the trichloroacetyl containing compounds are converted to CHCl₃ during leaching. In the VH site, where the pH is low (4–5) throughout the unsaturated zone, a decrease in trichloroacetyl-CHCl₃ concentration is also observable down the profile, but here, a significant amount reaches the groundwater, resulting in a concentration of ~2 µg/L (Figure 3b).

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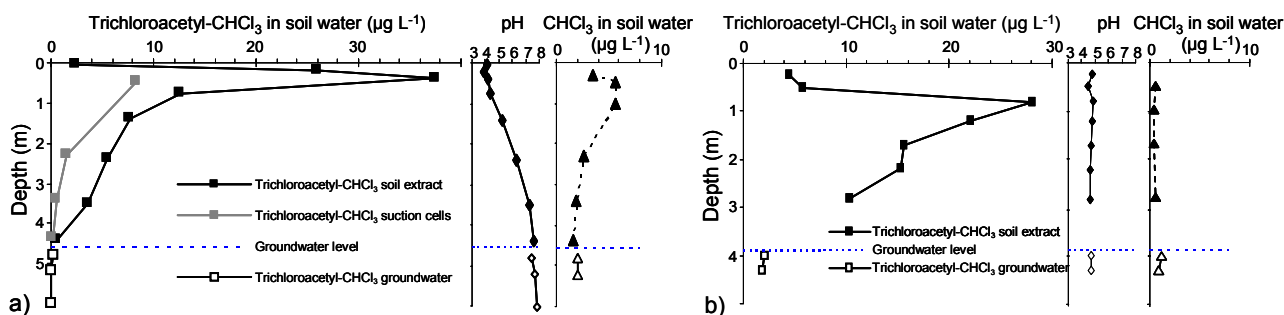
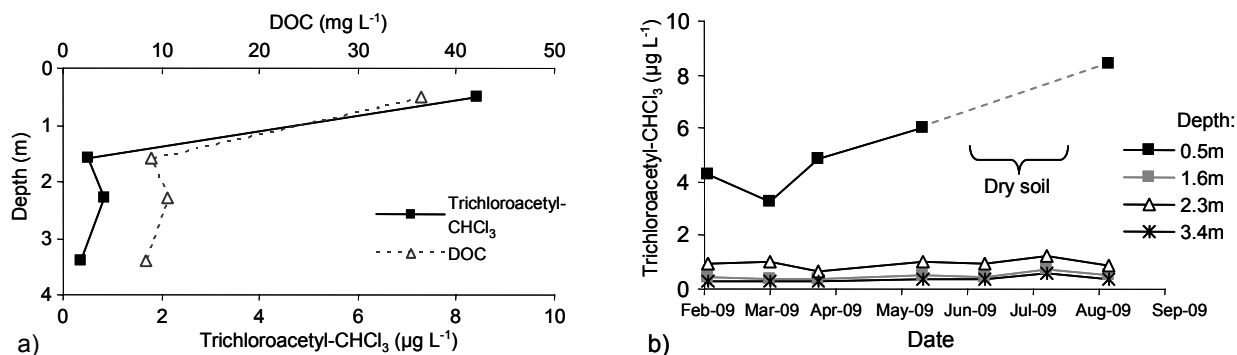


Figure 3. a) Trichloroacetyl-CHCl₃ in soil water in a soil profile in July in TH. Trichloroacetyl-CHCl₃ in the unsaturated zone was analyzed either directly in soil water (abstracted with suction cells) or calculated from aqueous soil extracts, assuming all water extractable trichloroacetyl containing compounds were present in the soil water (black line).
 360 Trichloroacetyl-CHCl₃ in groundwater was measured directly in the groundwater samples. The pH in soil and groundwater is also shown and furthermore the CHCl₃ concentrations for the same profile and sampling date, reported by (Albers et al., 2008a). CHCl₃ in soil water was calculated from measurements of CHCl₃ in soil air (unsaturated zone) or analyzed directly (groundwater samples). b) Trichloroacetyl-CHCl₃ in soil water in a similar soil profile in November in VH. Suction cells were not installed in the VH site. CHCl₃ in soil water was calculated from soil air samples from the same date (Albers et al., 2008a).
 365

The concentration of trichloroacetyl-CHCl₃ in soil water abstracted with suction cells was monitored during a 7-month period in TH in 2009 (Figure 4b). A rather dry period in June and July, led to the lack of samples from 0.5 m depth in that period, but apart from this, some seasonal trend
 370 seemed to occur at this depth, with increasing concentration from ~3.3 μg/L in March to 8.4 μg/L in August. This is similar to increases in CHCl₃ concentration observed from winter to summer in similar systems (Albers et al., Submitted b). The CHCl₃ concentration seems to vary according to microbial activity, which again varies with temperature and soil moisture. For CHCl₃ there is a smaller but still significant seasonal variation in deeper parts of the soil profiles (Albers et al.,
 375 Submitted b), but this variation is not observed for trichloroacetyl containing compounds (Figure 4b).

As discussed above and in (Albers et al., 2010), abstraction of soil water with the applied suction cells may underestimate soil water trichloroacetyl-CHCl₃, since, according to the supplier of the cells, compounds with a molecular size >6-8 kDa are lost by filtration. This might e.g. lead to the
 380 loss of as much as 50% of DOC in soil extracts and solutions of purified humic acids (Albers et al., 2010). Since a significant part of DOC in soil extracts is macromolecular structures like e.g. humic substances, and since some of the trichloroacetyl-CHCl₃ may be part of such structures, the concentrations in Figure 4b should be considered minimum values. Although the ratio of DOC:trichloroacetyl-CHCl₃ is not a constant in the soil water abstracted with suction cells, some

385 relationship could seem to exist between these two parameters (Figure 4a), further indicating that at least some of the trichloroacetyl containing compounds are not small single compounds, but rather part of larger organic structures.



390 Figure 4. a) Typical concentration profile of trichloroacetyl-CHCl₃ in the suction cells in TH. The DOC-content is also shown. b) Time series of trichloroacetyl-CHCl₃ in soil water from February to August, 2009. Soil water was sampled in four depths with the suction cells. At two occasions in June and July, no sample was present at 0.5 m depth, probably due to low soil moisture.

395 As shown (Figure 3), trichloroacetyl containing compounds may reach the groundwater, especially if the soil and groundwater is acidic (Figure 3b). At three occasions, 9 abstraction filters, containing acidic groundwater at the VH, LP and NF sites, were then analyzed for CHCl₃ and trichloroacetyl-CHCl₃ (Figure 5). CHCl₃ was present in all filters, in concentrations from 0.2–6.6 μg/L.

400 Trichloroacetyl-CHCl₃ was also present in all filters but at lower concentrations (0.05–1.1 μg/L). In most of the filters, there was a small tendency towards increasing concentrations from March to September, while no such tendency was observed for CHCl₃. Furthermore, no relationship between CHCl₃ and trichloroacetyl-CHCl₃ concentration seems to exist. This lack of relationship is not necessarily implying that there is no relationship between formations of the two compounds in the top soil, though, since the possible hydrolysis of trichloroacetyl containing compounds and
 405 subsequent release of CHCl₃ does not necessarily happen to similar degrees at similar depths at different sites.

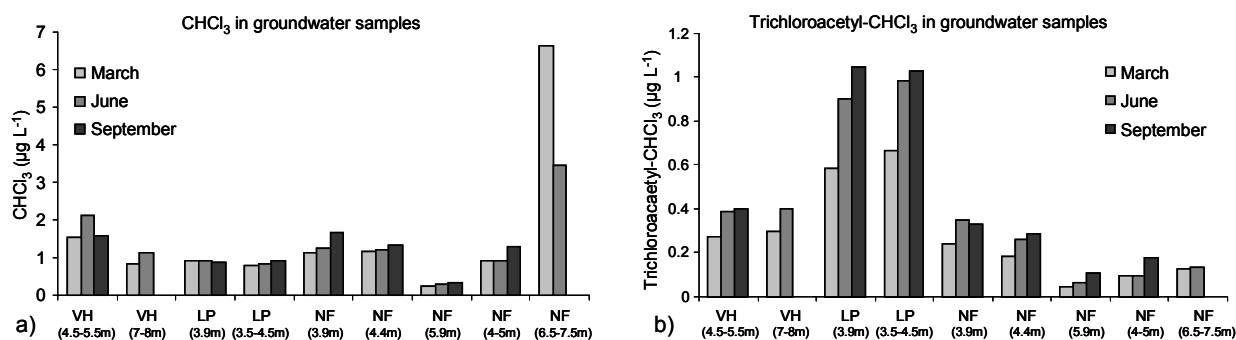


Figure 5. a) CHCl₃ and b) trichloroacetyl-CHCl₃ in groundwater below three CHCl₃-producing forests in Jylland, DK.

410 The limit of quantification for CHCl₃ was 0.0001 µg/L and for trichloroacetyl-CHCl₃ ~0.01 µg/L. The nine wells are all characterized by a rather low pH of 4.5–5.2, where trichloroacetyl containing compounds are expected to be more stable than at higher pH, as found in the groundwater in TH. At the last sampling date, no sample was taken from VH (7–8m) and NF (6.5–7.5m).

415 3.3. Trichloroacetyl containing compounds and CHCl₃ in soil

3.3.1. Water extractable

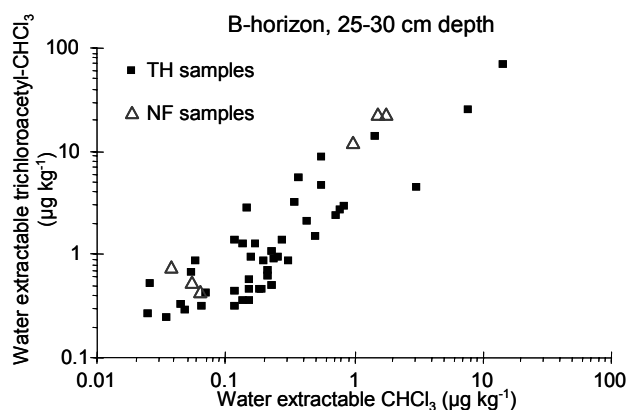
While no relationship exists between CHCl₃ and trichloroacetyl-CHCl₃ in groundwater (Figure 5), there is a clear positive relationship in samples taken at 30 cm depth (Figure 6). For the 44 TH samples, the best linear correlation ($y = 4.2x + 0.26$) had an R^2 of 0.94 (correlation not shown).

420 Despite a low number of samples from NF, the relationship seems to exist also for this site. On average, the trichloroacetyl-CHCl₃ concentration was ~6 times higher than the CHCl₃ concentration, but with a wide range of 1.4 to 20 times. The span in concentrations is much larger, though, ranging for CHCl₃ from 0.025–15 µg/kg and for trichloroacetyl-CHCl₃ from 0.24–68 µg/kg. All TH-samples were taken within a 20 x 70 m area, so this indicates a very large spatial

425 variation in the concentrations of both compounds. A similar large spatial variation was recently reported for CHCl₃ in soil air in the same area, where three CHCl₃ “Hot Spots” with sizes of 25–50 m² were found (Albers et al., Submitted a). The soil samples containing the highest concentrations of CHCl₃ and trichloroacetyl-CHCl₃ in Figure 6, were indeed sampled within these small areas of very high soil air CHCl₃ concentrations. This all in all points to a similar origin of trichloroacetyl

430 containing compounds and CHCl₃ in the top soil. They may either be formed in the same reactions, or trichloroacetyl containing compounds may be formed at first, and CHCl₃ is then liberated afterwards by hydrolysis of the trichloroacetyl groups. Both pathways are plausible for the unspecific chlorination of organic matter, since during chemical chlorination, trichloroacetyl containing compounds have been shown to be intermediates in the formation of CHCl₃, and

435 depending on conditions (pH and HOCl-concentration), some of the trichloroacetyl containing compounds may be stable and hence exist alongside with the CHCl_3 (Boyce and Hornig, 1983; De Leer et al., 1985).



440 Figure 6. Relationship between water extractable trichloroacetyl- CHCl_3 and CHCl_3 in soil from 25–30 cm depth in TH (44 samples) and NF (6 samples). Note the double logarithmic scale.

Both trichloroacetyl containing compounds and CHCl_3 in the soil were extracted with water. This solvent is not typically used for relatively hydrophobic compounds like CHCl_3 , since these tend to
445 sorb to the soil in aqueous slurries. For the soils in question, the partitioning coefficient between soil and water is ~ 0.6 for the TH samples and ~ 1.5 for the NF samples (Table 2). This means that the CHCl_3 concentrations in Figure 6 should be $\sim 30\%$ higher in the TH samples and $\sim 75\%$ higher in the NF samples, assuming equilibrium is established. The CHCl_3 concentrations in soil, reported here, should therefore be regarded as minimum values. For the trichloroacetyl containing
450 compounds, the partitioning coefficient between soil and water is unknown, but it is expected to be smaller than for CHCl_3 since they largely consist of carboxylic acids (Albers et al., 2010).

3.3.2. Soil bound trichloroacetyl- CHCl_3

More than 85% of the trichloroacetyl- CHCl_3 in an organic top soil sample was recently reported to
455 be unextractable with water and methanol and presumably be part of macromolecular/solid structures in the soil, e.g. humic acids (Albers et al., 2010). We determined the concentration of water extractable trichloroacetyl- CHCl_3 as well as trichloroacetyl- CHCl_3 bound to soil in a total of 42 samples from organic horizons of two Danish and two Swiss locations (Figure 7a). In general, there is a positive relationship between the two fractions of trichloroacetyl- CHCl_3 , at all locations,
460 but the fraction of total trichloroacetyl- CHCl_3 being extractable with water varies from $\sim 3\%$ in soil

from TH to ~20% in soil from the Jura Mountains. Some of the NF samples were divided into L-, F- and H- subhorizons (see section 2.4 for definitions). The six samples from the L-horizon (dead needles) were pooled before analysis, so the single L-horizon data point represents an average of all six samples, which were divided into horizons. The L-horizon is somewhat lower in especially soil-bound trichloroacetyl-CHCl₃ compared to the F- and H-horizons, while no significant difference can be seen between these two horizons.

Despite the variation between samples, it seems likely that the two fractions of the total trichloroacetyl-CHCl₃ are formed in the same chlorination process and therefore also both are related to the formation of CHCl₃ in soil, cf. the discussion in the previous section. Furthermore, the total concentration of trichloroacetyl-CHCl₃ seems to be up to 100 times the CHCl₃ concentration in the top soil, all of which can potentially turn into CHCl₃ with time. The rate of hydrolysis of these compounds under acidic conditions remains open for future studies, however.

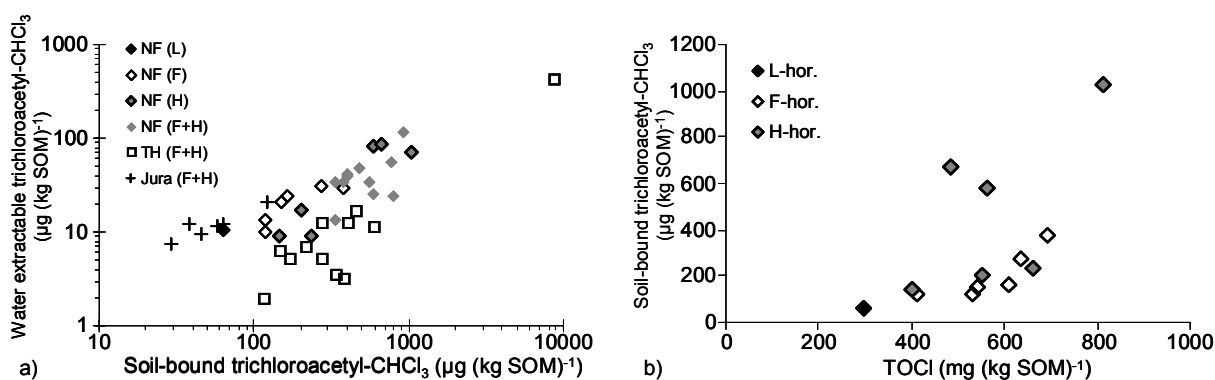


Figure 7. a) Relationship between water extractable and soil bound trichloroacetyl-CHCl₃ in two Danish (NF and TH) and two Swiss (both Jura) coniferous forests. The data are normalized to SOM-content. Note the double logarithmic scale b) Soil bound trichloroacetyl-CHCl₃ and total organic chlorine in 13 organic top soil samples from NF. TOCl data from Albers et al. (Submitted a). The positive relationship among all samples was statistically significant ($p = 0.01$ for Spearman's Rank Correlation), though a linear regression gives an R^2 of only 0.36.

The thirteen subhorizons of six NF soil cores, were also analyzed for total organic chlorine (TOCl), as reported elsewhere (Albers et al., Submitted a). The L-horizon certainly seems low in both soil-bound trichloroacetyl-CHCl₃ and TOCl, and in general there does seem to be a weak positive relationship between the two parameters. The low concentration in the L-horizon fits well with the hypothesis that both trichloromethyl compounds and TOCl are formed during unspecific chlorination. This chlorination is expected to occur to the highest degree during the degradation of

organic matter in the F-horizon, where also the highest CHCl_3 formation rates have been reported (Albers et al., Submitted b).

490 Soil bound trichloroacetyl- CHCl_3 seems to constitute only ~1% of TOCl, and is therefore not a main structural element of TOCl, whose speciation apart for a few percent in a characterized aromatic Cl-fraction (Dahlman et al., 1993; Hjelm and Asplund et al., 1995; Flodin et al., 1997), is largely unknown. Nevertheless, the $\text{CCl}_3\text{-C=O}$ group is interesting, since it, to our knowledge, is the first aliphatic structural element containing chlorine, which has been identified in the
495 macromolecular/solid fraction of SOM.

3.4. Chlorination of soil

To test the possibility for the various trichloromethyl compounds to be formed in an unspecific chlorination reaction between SOM and HOCl, which in soil may be formed from e.g.
500 chloroperoxidase enzymes, various organic soil horizons and SOM-fractions were chlorinated at field-like conditions (Figure 8). CHCl_3 , trichloroacetyl containing compounds and TCAA were all formed in all soils and SOM-fractions, and no major differences were found as a function of type of SOM added to the reaction. The H-horizon seemed to form more of all the trichloromethyl compounds than the L- and F-horizons, and the same was seen for the humic acid (HA) extracted
505 from the H-horizon as compared to the HA purified from the F-horizon. HA was found to be a slightly better precursor for the formation of all the trichloromethyl compounds than the whole soil, but since the purified HA fraction in different horizons of the soil in TH was found to constitute between 5–27% of SOM, precursors for all the compounds must exist in other SOM-fractions as well.

510 The relatively lower yield of CHCl_3 in the F-horizon might partly be due to a lower content of HA. Only ~5% of SOM could be extracted as HA from this horizon, while in the H-horizon 16% of SOM was recovered as HA. Furthermore HA from the H-horizon seems to be of better quality, with regards to its potential to form CHCl_3 . CHN-analysis and solid state ^{13}C -NMR spectroscopy did not reveal major differences between HA extracted from the F- and H-horizons, although the HA from
515 the H-horizon was slightly less aliphatic and contained more carboxylic acid and especially carbonyl structures (Supplementary Figure S1 and Table S1). The structural elements responsible for the differences in trichloromethyl forming potential therefore remain unknown, although one

could speculate that part of the carbonyl-groups in the H-horizon HA are methyl-ketones, which are efficient CHCl_3 -precursors through the haloform reaction (Fuson and Bull, 1934) or β -dicarbonyl groups, which are also very efficient CHCl_3 -precursors (Dickenson et al., 2008).

In all the chlorination reactions, trichloroacetyl containing compounds were dominating, but all three groups of trichloromethyl compounds were formed in concentrations of a similar order of magnitude. The concentration of chlorinating reagent (HOCl) relative to organic matter, although much lower than what is usually used in studies on the chlorination of drinking water (e.g. Singer, 1999; Reckhow et al., 2008), was most likely much higher in this experiment than what can be expected in the environment, where exo-enzymes can be expected to continuously produce a very small amount of chlorinating agent. The ratio between the various compounds are therefore likely to be different in natural soils, since e.g. the ratio of CHCl_3 to trichloroacetyl- CHCl_3 is increasing with increasing HOCl concentration (De Leer et al., 1985). The pH value is also very important for the relative distribution of trichloromethyl compounds (Boyce and Hornig, 1983), but the pH-value of 4, used for the chemical chlorination of soil and HA, is very close to what is found in the organic horizon at all four study sites.

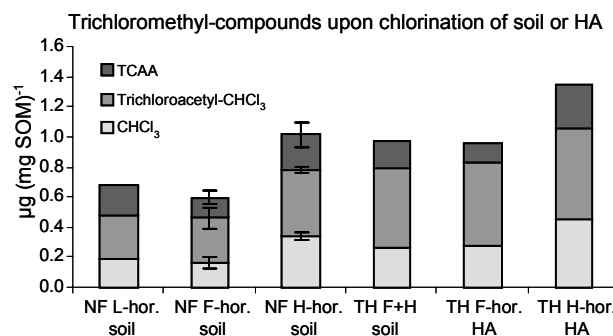


Figure 8. Chlorination of slurries of freeze-dried and powdered soil or HA. Chlorination conditions: $\text{SOM} = 1 \text{ g/L}$, $[\text{NaOCl}] = 0.5 \text{ mM}$, $T = 10^\circ\text{C}$. Reaction time = 24 h. Error bars for NF F- and H-horizons are std. deviation on 5 replicate soil samples. The L-horizon replicates from NF were pooled before chlorination and therefore contain no true replicates and no error bar is indicated. The samples from TH were all single samples. TCAA was determined as the difference between CHCl_3 liberated upon heating at pH 7.5 (TCAA + trichloroacetyl- CHCl_3) and CHCl_3 liberated upon addition of NaOH to pH 12 (trichloroacetyl- CHCl_3). It must therefore be viewed as minimum values, since the concentration is underestimated if not all trichloroacetyl containing compounds are converted to CHCl_3 during the heating procedure (*cf.* discussion in section 3.1).

The full molecular structure of the trichloroacetyl containing compounds formed during the chlorination was not investigated, but using solid phase extraction as previously described (Albers

et al., 2010), it was found that 28% of the trichloroacetyl containing compounds formed upon chlorination of H-horizon HA also contained a carboxylic acid group, while the remaining 72% did not. This is a relative low carboxylic acid fraction compared to soil and groundwater samples, where almost all water extractable trichloroacetyl containing compounds also contain a carboxylic acid group, and also lower than in throughfall and spruce needle extracts, where at least 50% of the trichloroacetyl containing compounds contain such acid group (Albers et al., 2010). The reason for this discrepancy is not obvious but would be interesting to investigate in future studies.

3.5. TCAA in spruce needles

As mentioned previously, TCAA was present in rather high concentrations in throughfall, and it was also found to be detectable in spruce needles in concentrations of 5–67 $\mu\text{g}/\text{kg}$ fw (Figure 9, second axis). At the same dates where needles were collected, we also collected two soil samples from ~15 cm depth at the base of the tree trunk. TCAA in forest soil and in groundwater was always below our analytical level of detection, but the soil water content of CHCl_3 and trichloroacetyl- CHCl_3 was estimated by aqueous extraction as described in a previous section. There seemed to be a positive relationship between both trichloroacetyl- CHCl_3 and CHCl_3 in the soil and TCAA in the needles (Figure 9). This may be an indication that, although TCAA was not detectable in the soil, it might still be formed there, and then quickly afterwards be either taken up by the vegetation or removed by sorption or degradation processes.

A few analyses of CHCl_3 , TCAA and trichloroacetyl- CHCl_3 were done on spruce xylem, revealing the presence of all compounds, but in rather low concentrations (CHCl_3 : 0.1–2 $\mu\text{g}/\text{kg}$ fw, TCAA: ~2 $\mu\text{g}/\text{kg}$ fw and trichloroacetyl- CHCl_3 : ~1 $\mu\text{g}/\text{kg}$ fw). Although more analyses and experiments are needed to finally conclude on this, the presence of all the trichloromethyl compounds in the xylem is a further indication that vegetation is a possible route of disappearance from the soil and that TCAA in needles may be an indicator of TCAA formation in the soil.

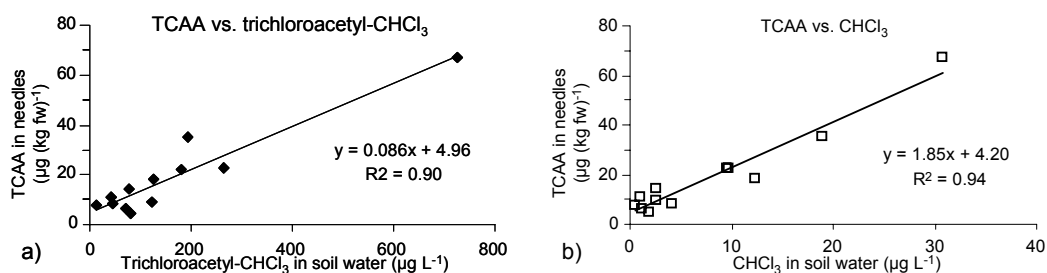


Figure 9. a) TCAA in spruce needles vs. water-extractable trichloroacetyl- CHCl_3 in soil (~15 cm depth) at the base of the trunk of the tree from which the needles were sampled. b) TCAA in spruce needles vs. water-extractable CHCl_3 in soil (~15 cm depth) at the base of the tree trunk.

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The point where the regression crosses the second axis in Figure 9 may be viewed as the TCAA concentration, which is not influenced by the presence of CHCl_3 or trichloroacetyl containing compounds in soil or in other words, the concentration of TCAA not influenced by the unspecific chlorination processes occurring in the soil. This “background” concentration could then be the concentration, which is caused by atmospheric deposition of TCAA and uptake and subsequent transformation of certain volatile organochlorines as discussed in a previous paragraph. This input of TCAA to the needles would not be expected to cause a huge difference between individual trees since the spatial variation in the input from the atmosphere is small compared to the large spatial variation in the formation and concentration of trichloromethyl compounds in soil, which we have shown in this and previous papers. Based on the two regressions in Figure 9, this “background” concentration seems, although somewhat uncertain due to the variation among samples, to be in the area of 4–5 $\mu\text{g}/\text{kg}$. In this case the “background” concentration of the compound of interest (TCAA) is then the anthropogenic input to the system, while the observed variation is caused by natural processes.

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3.6. Fate of TCAA in forest soil

3.6.1. Sorption

The expected formation of TCAA and the apparent uptake from soil by the vegetation combined with the fact that none or very little TCAA is present in the forest soil, suggests that TCAA is disappearing from the soil very quickly after its formation. The two most likely routes of disappearance, besides the uptake by roots, are through sorption or degradation processes. Sorption processes were assessed using a batch equilibrium setup. The sorption of TCAA shows a clear decrease with soil depth and decreasing SOM-content, but only a minor sorption was found for all

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600 tested soil types (Figure 10 and Table 2). In the minerogenic horizons sorption of TCAA is clearly
of no importance and even in the organic horizon, sorption is not significant enough to cause the
complete loss of water extractable TCAA after its presumed formation. These results are in good
agreement with the only previous soil sorption experiment carried out with TCAA, where sorption
was found to be of no importance in nine agricultural soils and only detectable in the two soils with
605 high concentrations of SOM and clay (Streibig, 1980).

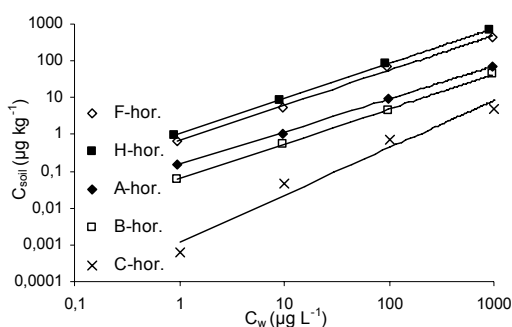


Figure 10. Sorption study with $[2\text{-}^{14}\text{C}]\text{-TCAA}$ and five different soil horizons. Each data point represents the average of duplicate measurements. The fit of the Freundlich model is shown. The Freundlich parameters are given in Table 2.

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The sorption of TCAA was not much dependent on concentration in the investigated interval of 1–1000 $\mu\text{g/L}$ ($0.9 < n < 1.0$) indicating either that the sorption type was a simple partitioning between two phases (soil/SOM and water) or that specific sorption sites were in surplus even at the highest concentration. Since the sorption experiment was conducted at a $\text{pH} \sim 5$, TCAA will exist as its trichloroacetate anion, and a simple partitioning between SOM and water would be expected to be
615 clearly in the favour of water and the sorption should then not be correlated to SOM-content. A more plausible explanation of the positive relationship with SOM would be sorptive interactions between TCAA and positive moieties in either SOM itself or in complexes of SOM and cations. The latter could perhaps also explain the high sorption in the H-horizon compared to the F-horizon, despite the higher SOM-content of this horizon; since the F-horizon contains fewer carboxylic acids as well as less inorganic material (Supplementary Table S1) it possibly also contains fewer SOM-cation complexes.
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Table 2. Results of the sorption study with [2-¹⁴C]-TCAA and 5 different soil horizons (Figure 10). TH is the Tisvilde Hegn site, NF is the Nordre Feldborg site. Note, that the sorption in the C-horizon is so low that the exact values of both K_F and n are highly uncertain. For comparison, K_F -values for the sorption of CHCl_3 to the same or similar soils are also shown (Albers et al., Submitted b).

Soil sample (site)	SOM (%)	pH	K_F	n	$K_F (\text{CHCl}_3)^*$
F-horizon (TH)	92	4.8	0.68	0.95	21.6**
H-horizon (TH)	45	4.6	1.07	0.95	
A-horizon (NF)	11	4.7	0.15	0.89	1.5***
B-horizon (TH)	1.9	5.3	0.065	0.94	0.62
C-horizon (TH)	0.56	5.7	0.001	1.28	0.27

*Data from Albers et al. (Submitted b). **Determined in a mixture of the F- and H-horizon. ***This NF A-hor. contained only 6.4% SOM.

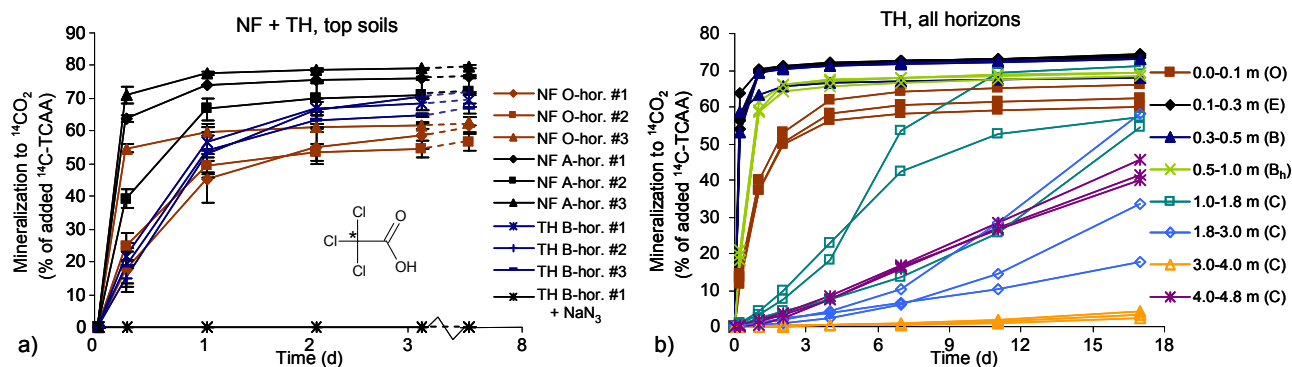
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3.6.2. Mineralization

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Experiments to determine the mineralization of TCAA were carried out for a number of natural forest soils (Figure 11). The variation between different soil samples collected in the same horizon in the same area, was minor (Figure 11a), and also the mineralization rate was somewhat similar in top soil from different areas (NF and TH). Much more variation was found down the soil profile in TH (Figure 11b). In soil sampled from 0–1 m depth, mineralization is fast ($T_{1/2} < 24$ h) without a lagphase. Just below 1 m depth, a small lagphase is seen, but mineralization is still relatively fast and almost complete within the 17 d experimental period. In even deeper horizons, the lagphase seems to be longer, except for the soil sampled around the groundwater table (4.0-4.8 m depth), where the lag phase is less pronounced and ~40% of the [2-¹⁴C]-TCAA is released as ¹⁴CO₂ within the 17 days.

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Figure 11. a) Accumulated ¹⁴CO₂-release from [2-¹⁴C]-TCAA in soil from NF (O- and A-hor.) and TH (B-hor.). Error bars are standard deviation on three replicates for each of the 9 soils. TH B-hor. #1 + NaN₃ is the average of three replicates with the same soil as TH B-hor. #1, but added 0.5% sodium azide (w/w) to inhibit microbial activity. * marks the ¹⁴C-atom. b) Accumulated ¹⁴CO₂-release from [2-¹⁴C]-TCAA in 8 horizons of a soil profile in TH. All replicates are shown to indicate differences between replicates in some of the C-horizon samples, where a lag-phase exists.

650 All samples were extracted with water at the end of incubation to determine any non-mineralized
 ^{14}C -TCAA. For the sterilized samples and for the TH sample with very low mineralization (3.0–4.0
m depth), the recovery of ^{14}C in the aqueous extraction was >99%. In the TH samples with
slow/partial degradation, the total recovery was 72–91%, while in all samples displaying fast
655 recovery was equal to the ^{14}C released as $^{14}\text{CO}_2$ (60–80%). The remaining 20–40% ^{14}C not
accounted for in the soils with fast degradation, is most likely incorporated into the degrading
biomass. The slow but continuous $^{14}\text{CO}_2$ -release of 0.1–0.3% of the applied radioactivity per day
during the last part of the incubation period supports this, as its likely origin is the slow turnover of
microbial biomass.

660 CO_2 was the single degradation product analyzed in this experiment, since degradation of TCAA
seems always to follow a pathway leading to complete mineralization to CO_2 and Cl^- (Smith, 1974;
Matucha et al., 2003). One study has suggested that small quantities of CHCl_3 might be formed
during microbial degradation of TCAA (Haselmann et al., 2000) and Weightman et al. (1992)
suggested that CO might be a degradation product of TCAA, but neither of these products could be
665 confirmed in studies using $[1,2-^{14}\text{C}]$ -TCAA (Forczek et al. 2001; Matucha et al., 2003, 2006) and as
also found by Smith (1974), CO_2 seems to be the only degradation product.

The lag phase, for the mineralization of TCAA in deeper horizons, is somewhat similar to what has
previously been found for agricultural soils, where a potential for the mineralization of TCAA
670 always exists, but only after a certain lag phase (Jensen, 1957; Smith, 1974; McGrath, 1976).
McGrath (1976) performed a second application with TCAA, which was quickly mineralized
without a lagphase. This adaptation was retained in the soil for almost three years. Somewhat
similar to what is seen in Figure 11, previous studies with forest top soils, have shown that no lag
phase exists and that TCAA is degraded fast (Forczek et al., 2001; Matucha et al., 2003, 2006). One
675 could then speculate that TCAA is not normally present in sufficient amounts to sustain a degrading
population in agricultural soils and in forest sub soils, while in forest top soil, there is a clear
microbial tolerance, indicating a regular input of TCAA. The most obvious regular input is natural
formation in the soil, and the fast mineralization could then explain why it is not possible to detect
the TCAA during analyses. Continuous uptake of soil water and accumulation of TCAA in the
680 needles may make the vegetation a more suitable compartment for the detection and even semi-
quantification of TCAA formation in the soil.

3.7. A conceptual model for trichloromethyl compounds in forests

Based on the present as well as various previous studies, a conceptual model for the cycling of trichloromethyl compounds in coniferous (and possibly other) forests is suggested (Figure 12).

685 For CHCl_3 and trichloroacetyl containing compounds, the input is suggested only to be natural formation in the top soil. Furthermore, trichloroacetyl containing compounds may be converted to CHCl_3 in deeper horizons, especially if the pH is increasing down the soil profile. The formation of CHCl_3 in soil is well established (Hoekstra et al., 1998), although the spatial variation has been shown to be very large (Albers et al., Submitted a). For TCAA several possible inputs to the system

690 are known. We (Table 1 and Figure 2) and others have shown that TCAA is present in rain, although at a rather low concentration (Kohlert et al., 1993; Berg et al., 2000; Scott et al., 2005). This TCAA may end up in the soil or in vegetation, the latter either indirectly through roots or directly through the needles (Cape et al., 2003; Dickey et al., 2004; Matucha et al., 2006). The formation of TCAA in soil has been suggested (Haiber et al., 1996; Hoekstra et al., 1999) and even

695 shown in a laboratory study (Matucha et al., 2007) and furthermore TCAA may be formed in needles after the uptake of certain volatile organochlorines like TCE and PCE from the air (Cape et al., 2006; Weissflog et al., 2007; Strycharz and Newman, 2009).

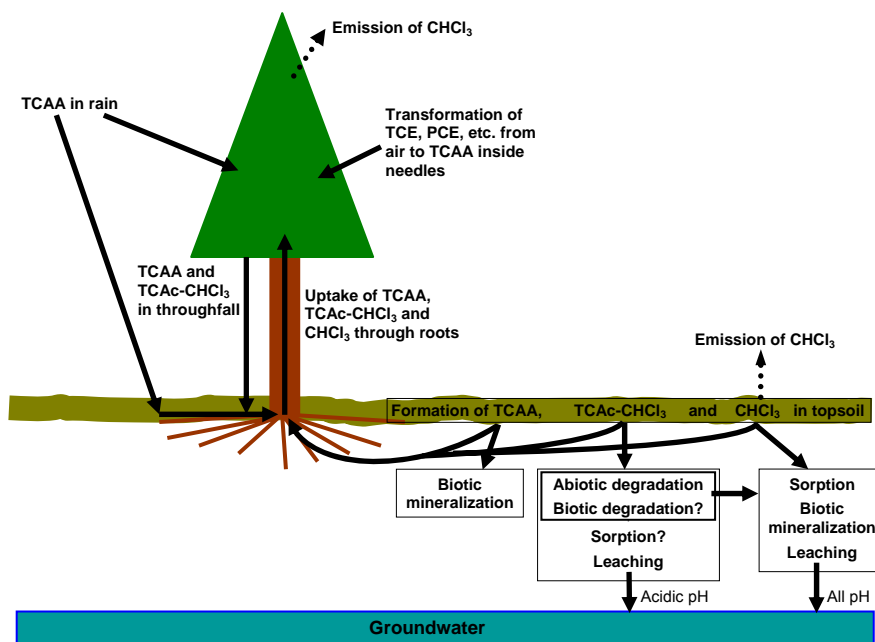
TCAA formed in the soil is likely to be exposed to fast mineralization by microbes as we (Figure 11) and others (Forczek et al., 2001; Matucha et al., 2003; Matucha et al., 2006) have shown.

700 Although at much lower rates, CHCl_3 has also been shown to be mineralized by forest soil microbes (Albers et al., Submitted a, b). Leaching of CHCl_3 to the groundwater has been shown (Laternus et al., 2000; Albers et al., 2008a; Albers et al., Submitted b) and we found trichloroacetyl containing compounds to leach to groundwater in cases where pH remains acidic throughout the unsaturated zone (Figure 3 & 5). TCAA, on the other hand, seems to be degraded before significant leaching

705 may occur and even in deeper horizons a potential for the mineralization of TCAA exists. CHCl_3 is the only trichloromethyl compound likely to be emitted to the atmosphere, a process which has been shown in previous studies (Hoekstra et al., 2001; Dimmer et al., 2001; Hellén et al., 2006; Albers et al., Submitted a). As suggested here, all the trichloromethyl compounds are likely to be taken up by roots and translocated to the needles from where CHCl_3 will most likely be emitted

710 to the atmosphere, while TCAA and trichloroacetyl containing compounds will probably accumulate and either be degraded as has been shown for TCAA with approximate half lives of 10 days (Frank, 1991) or somewhat longer (Matucha et al., 2006) or excreted and leached with precipitation, leading to the elevated concentration in throughfall (Figure 2).

For CHCl_3 , sorption processes are likely to slow down leaching and emission processes in the upper soil horizons (Albers et al., Submitted b), while sorption seems to be of minor influence for TCAA (Table 2). Trichloroacetyl containing compounds in macromolecular structures will of course leach slowly, among others due to sorption processes, while the effect of sorption on the fate of smaller sized trichloroacetyl containing compounds is hard to predict as long as their full structures remain unknown.



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Figure 12. Principal model for the major fluxes and processes concerning trichloromethyl compounds in a temperate coniferous forest environment, based on this and previous papers (see the text for bibliographic sources). TCAC- CHCl_3 is an abbreviation for trichloroacetyl containing compounds. TCAA in rain and volatile TCAA-precursors (trichloroethene (TCE), tetrachloroethene (PCE) etc.) in the atmosphere are expected to be mainly caused by human activity. All other processes are expected to be mainly natural. Processes, for which no data are reported, are followed by a ?.

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Although some of the processes in the conceptual model like e.g. emission of CHCl_3 from the soil have been dealt with in several publications and are somewhat well established, several processes concerning both the formation and fate of both TCAA and CHCl_3 remain on a rather hypothetical level. The formation, fate and even speciation of trichloroacetyl containing compounds needs further attention, if we want to understand better the importance of this recently discovered group of natural organochlorines. If we want to insert general values on the various natural background concentrations and fluxes, more studies are therefore needed concerning all parts of the model, not the least taking the great spatial variation, which has been shown for the formation of CHCl_3 , into account.

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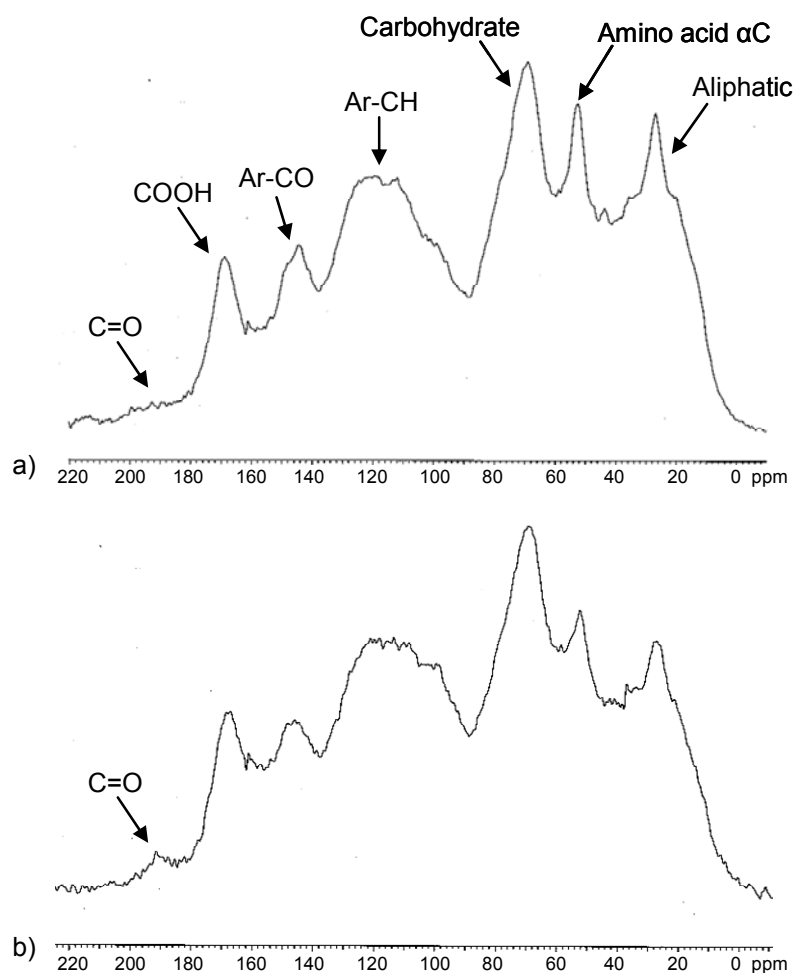
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Supplementary Material



955 Figure S1. Solid State ^{13}C -spectra of a) F-horizon HA and b) H-horizon HA from the TH site. The carbonyl- ($\text{C}=\text{O}$ -) peak was integrated to 1.7% of the total carbon for the H-horizon, while it was too small to integrate in the F-horizon. For details on peak assignment, see Albers et al. (2008b).

960 Table S1. Elemental analysis in mass% of F- and H-horizon HA and powdered whole soil F- and H-horizon from the TH site, corrected for ash-content. $\text{O} = (100\% - (\text{C} + \text{H} + \text{N}))$. The carbonyl and carboxylic acid carbon content (in % of total carbon) determined by integration of solid state ^{13}C -NMR spectra is also shown. Low means too low to integrate.

	C	H	N	O	C/H	C/N	Ash /%	C=O	COOH
F-horizon HA	55.3	5.6	2.7	36.4	9.9	20.2	0.0	Low	7.1
H-horizon HA	53.9	4.8	2.2	39.0	11.2	24.2	0.4	1.7	8.0
F-horizon	53.4	5.9	2.0	38.7	9.1	26.9	7.3	Low	4.9
H-horizon	54.9	5.8	2.0	37.4	9.5	27.5	41	Low	7.8

