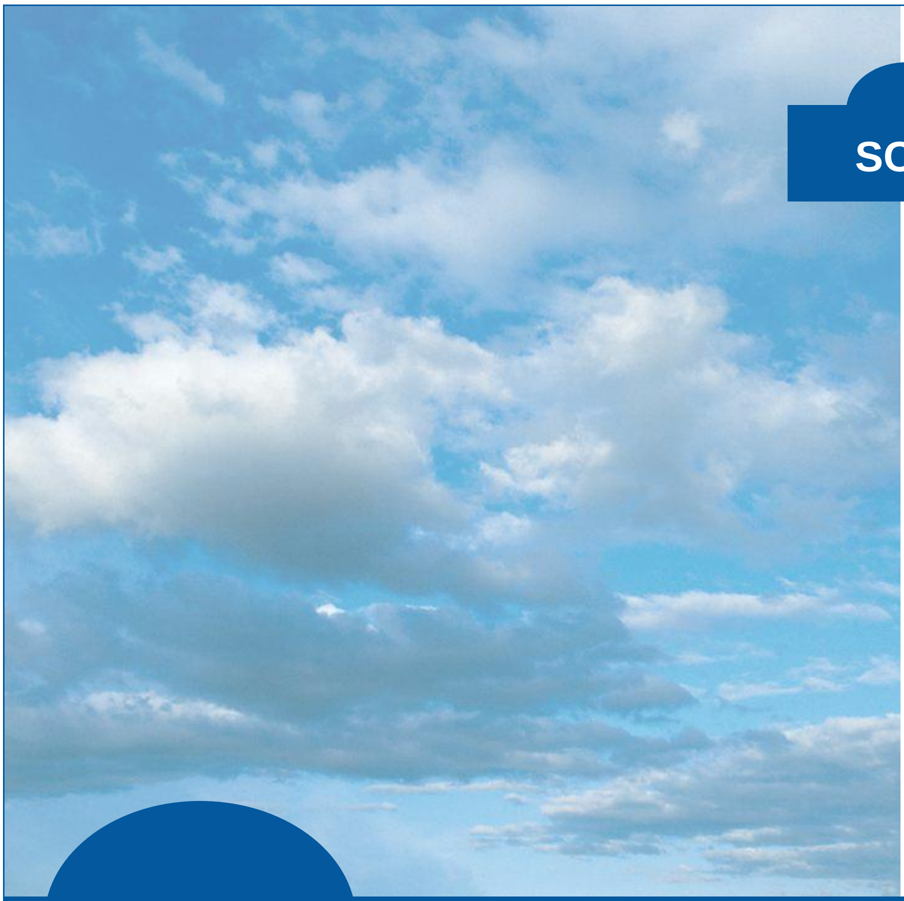

SCIENCE DOSSIER

Euro Chlor workshop on soil chlorine chemistry: Workshop proceedings

November 2004

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November 2004

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Foreword

Euro Chlor continuously works with scientists to acquire a greater understanding of chlorine, its derivatives and how they behave in nature. At this soil chlorine chemistry workshop Euro Chlor expressed its particular interest in the fate and persistence of industrial organochlorines in the environment.

The issue of the persistence of some chlorinated chemicals in the environment continues to be debated by scientists, NGOs and the media alike. Taking their environmental responsibilities seriously, the behaviour of chlorinated substances in nature is a matter that also concerns chlor-alkali producers.

Scientists at the workshop wanted to gain a better understanding of how long chlorinated substances remain in soil and how they degrade. Also examined were the sources and distribution of organochlorines in the environment – whether they originate from natural sources or human activity.

Specific subjects discussed were the biogeochemical cycling of chlorine in soil, abiotic formation of organohalogens in the terrestrial environment, the role of humic substances in the anaerobic degradation of chlorinated solvents, vanadium haloperoxidases and their role in the formation of chlorinated compounds and the chloro-oxo acid cycle in soil.

In the final discussion, there were calls for field experiments to verify mechanisms, for example, to distinguish between the relative contributions of microbial and “diagenetic” processes to the formation of chloroform. Also raised was the need for more work to be done on identifying the soil enzymes responsible for chlorination.

These are the proceedings to the Euro Chlor workshop on soil chlorine chemistry which took place on 3 December 2003.

1 Introduction

Jean-Marie Libre, chairman of Euro Chlor's "Monitoring & Environmental Chemistry Working Group" – the sponsor and organiser of the current workshop – outlined the aims of this committee, founded in 1994.

In brief, its main objectives are to:

- Collect (and, in some cases, fund the acquisition of) monitoring data on levels of organochlorines in the environment, develop statistical methods for assessing the data and perform time-trend analyses;
- Determine what fractions of these organochlorines arise from natural sources and human activities, respectively, bearing in mind that, in some cases (e.g. chloroform) the natural contribution is predominant;
- Understand the fate of organochlorines in the environment: i.e., determine their persistence, their emission and degradation fluxes and the nature of their degradation products;
- Produce "Science Dossiers" that are subsequently published in the open literature, in order to provide an up-to-date review of the best existing knowledge for specific topics of interest;
- Organise workshops on targeted subject areas where data gaps exist.

The data generated by these activities is used primarily:

- In risk assessments, carried out in the framework of various regulatory instruments (OSPAR Commission, E.U. Water Framework Directive, etc.);
- For model evaluation, in order to compare predictions with actual measurements.

J.-M. Libre stated that the objective of the current Workshop on Soil Chlorine Chemistry was to review our existing knowledge on the natural chlorine cycle in soil, i.e. primarily to discuss the fluxes and mechanisms of formation and degradation of organochlorines in this compartment.

It was recognised that the subject could not be covered completely in a single day. However, the conclusions of the workshop would enable Euro Chlor to decide if further activities in this area might warrant sponsoring in the future.

2 The biogeochemical cycling of chlorine in soil

Dr. Gunilla Öberg, Linköping University, Sweden

2.1 Introduction

G. Öberg stated that, despite extensive research in the area, the existence of natural organochlorines is not a generally accepted fact. The scientific community is still divided on this issue. For example, on the one hand, the Swedish Research Council is reluctant to provide funding on natural organochlorines, considering that the subject is no longer sufficiently novel. On the other hand, a paper published in *Science* on this subject by Myneni and co-workers (2002) was commented on as if something completely new had been disclosed.

2.2 Chlorine content of soil

Chlorine is a natural constituent of soil organic matter. Its concentration in soil varies from 5 to 1500 µg/g dw. The soil organic matter contains 40-50% C, 30-40% O, 3-6% H, 1-5% N, 0.1-1.5% S, 0.03-0.2% P and 0.01-0.5% organic chlorine.

From these values one can calculate that there is about one chlorine atom per 5600 carbon atoms. While this may seem like a low proportion, chlorine is still the 6th or 7th most abundant element. In top soils in Scandinavia, chloride levels are 2-5 times lower than organic chlorine, but lower down the relative amount of chloride increases. This is mainly due to the fact that the organic matter content decreases with depth, hence the ratio of organic chlorine to chloride also decreases.

2.3 Chlorine mass balances in forested catchment studies – 1

A PhD student from G. Öberg's department is studying the chlorine cycle in the small uninhabited Stubbetorp catchment near Norrköping, 200 km south of Stockholm. At this location, the hydrology is well known, since the Swedish Meteorological and Hydrological Institute has been conducting studies for a decade. Although work is being performed on chloroform, it is currently premature to present the results, the 2-year project being now only about half completed. Therefore, only results on total inorganic and organic chlorine will be discussed.

The wet deposition of chloride is about 0.4 g/m^2yr, typical of the general area. In fact, there is a sharp gradient across Sweden, with values of about 5 g/m^2yr on the west coast and < 0.01 g/m^2yr east of the mountains in Northern Sweden.

Through fall contributes about 0.3 g/m^2yr to chloride deposition, so chloride in runoff from the catchment is 0.4 + 0.3 = 0.7 g/m^2yr.

Deposition of organic chlorine in precipitation is negligible (< 0.01 g/m^2yr) compared to chloride.

Organic chlorine in through fall has not been measured in Stubbetorp, but can be inferred to be about 0.04 g/m^2yr from studies carried out in a similar catchment at Klosterhede in Denmark (Öberg and Grön, 1998). One might have expected a gradient, with the chloride through fall deposition falling off from the forest edge inwards, since this coastal area is subject to a strong westerly wind with a high salt content. However, the observed organic chlorine deposition is found to decline with distance from the nearest tree, as depicted in Figure 1 (after Öberg *et al*, 1998).

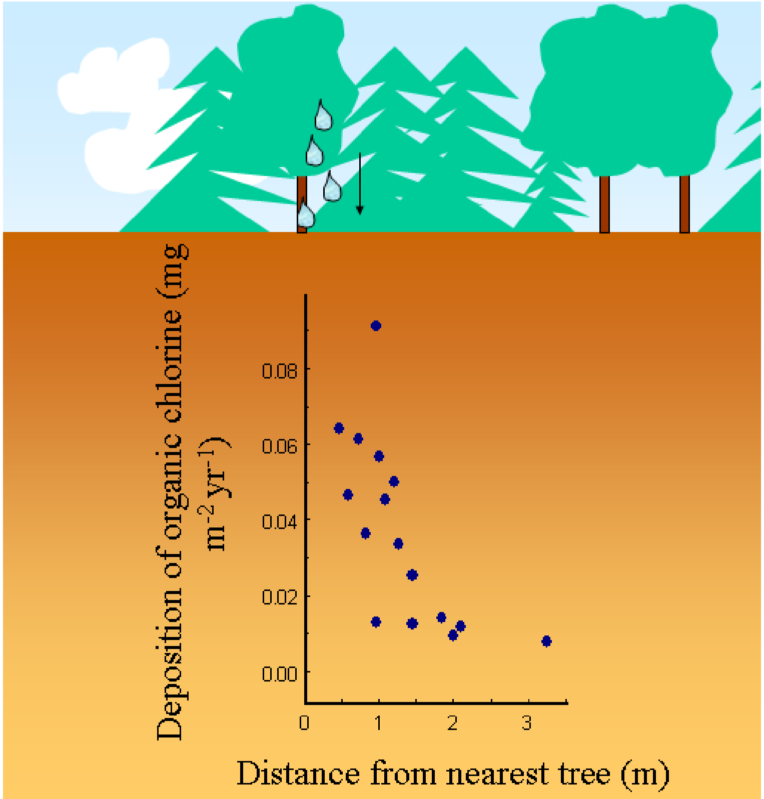


Figure 1

From other studies on through fall, this is the pattern that one sees for compounds that originate from within the forest itself, i.e. for substances produced by trees or by organisms living on them. The element potassium shows this type of pattern (study by Lennart Rasmussen, Denmark), while sulphur levels decline from the forest edge inwards.

Organic chlorine deposition in litter fall was also measured in the Klosterhede catchment and was found to be 0.04 g/m^{2}yr, i.e. comparable to through fall. This result contrasts with observations for carbon, for which litter fall is the major contributor to deposition, through fall being negligible.

Having outlined the deposition fluxes, G. Öberg turned to the “storage” of chloride and organic chlorine, in principle in the top 40 cm layer of soil, although sometimes the bedrock was shallower and did not allow sampling down to this depth.

At Stubbetorp, the organic chlorine amounted to 18 g/m^2 in this top layer, while chloride was only 7 g/m^2. In the Klosterhede study, organic chlorine was 50 g/m^2 in the top 60 cm layer. In a more extensive study conducted all over southern Sweden, there was found to be 2-5 times more organic chlorine than chloride in the top soil.

To summarise:

- Wet deposition is not a major source of organic chlorine in soil;
- Organic chlorine in through fall originates from sources “internal” to the forest;
- The contribution from through fall and litter fall is comparable;
- The storage of organic chlorine in top soil is considerably greater than the storage of chloride.

Question from J. Field: How about temporal changes?

Response from G. Öberg: The spatial variation in the same plot is so large; one would need to take thousands of samples in the same area to determine possible temporal changes.

Question from J. Franklin: Is organic chlorine contained in through fall a true input, or just an internal recycle? That is to say, if it is organic chlorine formed in soil and transported with the transpiration flow and out through the stomata, it would go back into soil, thus constituting an internal loop.

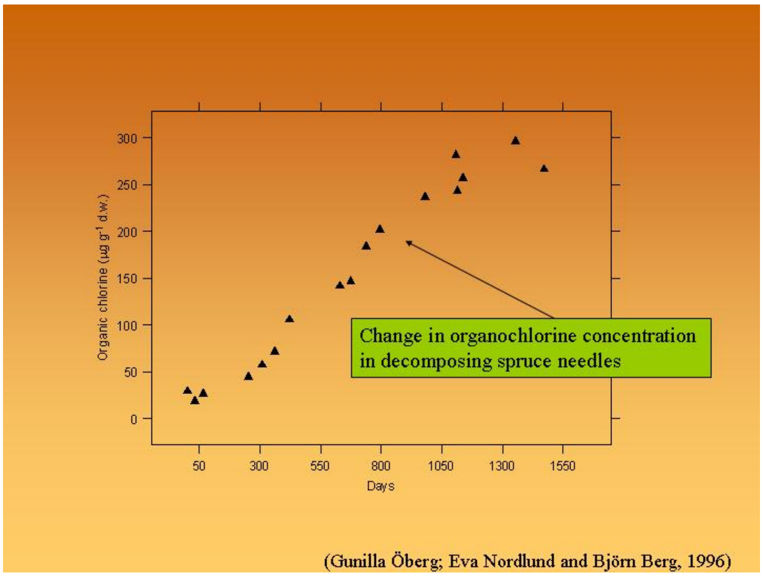
Response from G. Öberg: The organic chlorine in soil is present largely as humic-like molecules, considered by plant physiologists to be probably too large to be taken up by trees. On the other hand, studies by Myneni and his group at Princeton suggest that water-leachable chlorine compounds are formed on leaves, so this could be the origin of the organic chlorine in through fall.

Question from J. Franklin: Another explanation might be the uptake of atmospheric aerosols or gases by leaves acting as a sort of "sponge", with leaching occurring during precipitation events.

Response from G. Öberg: Then one would see a decline in concentration from the edge of the forest inwards, not from the nearest tree. This point needs however to be looked into more closely before we can exclude dry deposition as the origin of organic chlorine in through fall. Use of ^{36}Cl-labelling would be helpful in this respect.

2.4 Origin of organic chlorine in soil

The Swedish researcher Björn Berg has been collecting "litterbags" for 40 years. In these studies, litter is put into small bags, deposited in soil, and recovered for analysis after a period of up to 7 years. The change in lignin composition, N and P content, etc., is monitored. G. Öberg's group was given access to certain samples and determined their organic chlorine content, which was found to rise steadily over the duration examined (1500 days), as shown in Figure 2.



(Gunilla Öberg; Eva Nordlund and Björn Berg, 1996)

Figure 2

These studies (Öberg *et al*, 1996) suggest that chloride is incorporated into the organic matter during decomposition. This conclusion is now supported by X-ray studies (Myneni, 2002).

It has long been known that numerous soil micro organisms are reported to:

- Contain organochlorine compounds (Curd, 1933; Doering, 1945; Raistrick, 1951; McMaster, 1960; Ando, 1970). This was first described for marine organisms in the late 19th century, then for lichens in the 1930s and later for fungi (during research on pharmacologically active substances);
- Contain endo-enzymes within the cells that are able to convert chloride to organic chlorine and form specific compounds (Morrison, Schonbaum, 1976; Hunter *et al*, 1987; van Pee, 1996);
- Exude specific chlorinated compounds, the so-called "chlorometabolites" (Harper *et al*, 1988; de Jong, 1993);
- Be able to convert chloride to organic chlorine (Clutterbuck *et al*, 1940; Verhagen *et al*, 1996; Öberg *et al*, 1997). Clutterbuck *et al* found conversions of up to 97% of the chloride, irrespective of the chloride concentration;

- Excrete exo-enzymes that catalyse chlorination (Clutterbuck, 1941; Hunter and Sotos, 1987). *[Comment from R. Wever: In the Hunter and Sotos study, most of the species contain extra-cellular vanadium enzymes]*. The Öberg *et al* (1993) study was conducted with an enzyme from *Caldariomyces fumago*, a mould living in greenhouses (considered to be a "lab rat", not necessarily representative of what is happening in soil, but commercially available).

There are 3 biotic processes:

- Endo-enzymatic formation of specific compounds;
- Formation and excretion of specific compounds. A Linköping PhD student working with a "fairy ring" found that 20% of organic chlorine was made up of low molecular weight phenolic substances. These compounds disappear later. Either they are incorporated into organic matter or they decompose. However, the organic matter does not resemble the structure that one would find in humic acids. *[Comment from J. Field: I believe you are referring to chlorinated anisyl metabolites. We have done studies on forest soil spiked with such compounds and they are metabolised immediately under aerobic conditions]*;
- Exo-enzymatic formation of reactive chlorine (HOCl).

There is also endo(exo?)-enzymatic formation of unspecific compounds, e.g. cardiovascular plaque, with an enzyme that produces active chlorine when infected and intra-cellularly forms organic chlorine as a by-product.
The intracellular compounds are often (but not always) produced in low amounts within organisms. It seems as if the specific compounds that are excreted have a rapid turnover rate. The major part of the organically bound chlorine is incorporated into high molecular weight structures. Soils contain catalysts with chloroperoxidase activity.
All this suggests that exo-enzymatic formation of reactive chlorine is a more likely source of chlorinated organic matter than the other two biotic processes.
G. Öberg stated that she was not aware of any researchers doing soil extraction procedures to see what exactly the soil contains.

> *Comment from R. Bruyndonckx: If formation of organic chlorine occurs outside micro organisms, this "takes away their purpose".*

> *Response from G. Öberg: Micro organisms may have difficulty in degrading organic matter, but production of HOCl may help (as in pulp bleaching) by oxidising organic matter into smaller fragments and making it more amenable to biodegradation. HOCl may also be used by organisms living on leaves in a "suicidal" defence mechanism.*

> *Comment from R. Wever: There is a large group of fungal pathogens growing on leaves and capable of killing plants that contain vanadium haloperoxidases and form HOCl. They probably use it to degrade lignocellulose in the plant cell wall. Fungi have to penetrate in some way into the cell and HOCl is used to open it up. A side reaction is formation of all kinds of organochlorines, including chloroform.*

The following question then arises: Is exo-enzymatic formation of reactive chlorine a major source of naturally formed organochlorines in soil?

2.5 Chlorine mass balances in forested catchment studies – 2

As stated previously, there is an organic chlorine flux of 0.04 g/m^{2}yr deposited in through fall and the same amount coming from litter fall. This total of 0.08 g/m^{2}yr does not come from outside the forest system, since the flux of organic chlorine in precipitation is negligible. So there must be a conversion of inorganic chlorine to organic chlorine, in the plants or on the plants. In addition, litterbag studies show an organic chlorine formation in soil of about 0.3 g/m^{2}yr.
Water coming out of the top soil contains organic chlorine. In the catchment studies, organic chlorine represented 20-60% of total leached chlorine out of the top layer (0.7 g/m^{2}yr), so roughly 0.2 g/m^{2}yr was organic and 0.5 g/m^{2}yr was inorganic.
To maintain a steady state, the amount of inorganic chlorine converted to organic chlorine in soil would however need to be reduced (arbitrarily) from 0.3 to 0.1 g/m^{2}yr, since only 0.2 g/m^{2}yr of organic chlorine is leached out of soil. This latter flux is therefore made up of 0.1

g/m^2yr from through fall + litter fall, and 0.1 g/m^2yr from conversion of inorganic chlorine in soil.

2.6 The fate of organic chlorine in soil

Runoff of organic chlorine from the lower layers of soil is about 0.01 g/m^2yr, much smaller than from the top layer (0.2 g/m^2yr). In fact, the concentration of organic chlorine decreases with depth as shown in Figure 3 for Skullebo (south of Linköping, S) and Klosterhede (DK).

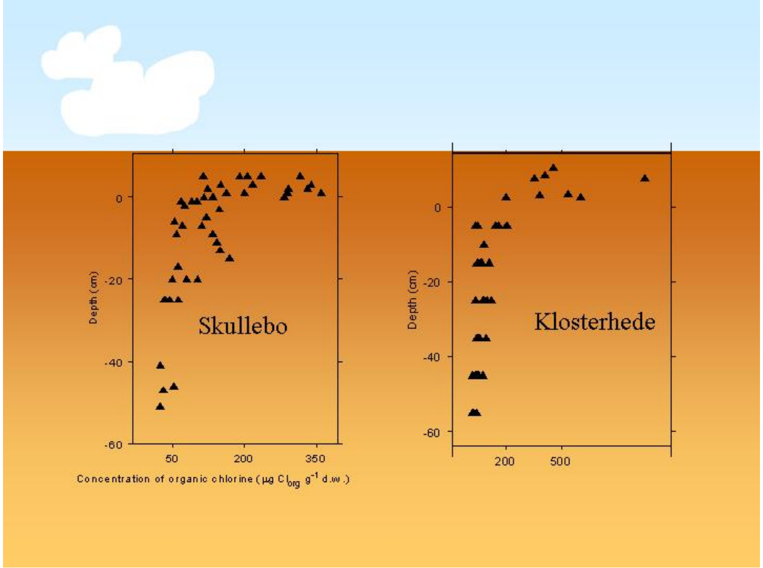


Figure 3

This decline follows to some extent the organic matter content, but the latter decreases faster than the organic chlorine, so the ratio of organic chlorine to carbon actually increases with depth over the first 15-20 cm, before levelling off.
The figure below compares the org Cl/C ratio (R) in the various soil horizons (listed in order of depth), leachate, litter, through fall and surface water (the later being a compilation from 150 lakes). It can be seen that R is much greater in through fall than in litter or in the F-horizon, but comparable to the value observed in the A-horizon. Furthermore, the leachate from the A-horizon has an even greater R value, comparable to that for the B-horizon.

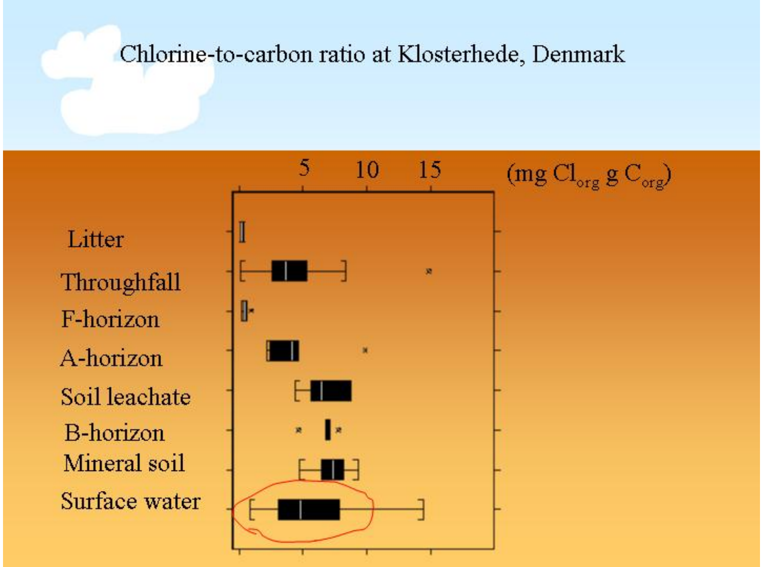


Figure 4

G. Öberg's suggested that there are two types of organic chlorine:

- A large storage of not very highly chlorinated and rather immobile material in litter and top soil;
- More highly chlorinated mobile material in through fall, perhaps formed on the leaves, that is precipitated when the leachate reaches the deeper soil horizons.

In conclusion, G.Öberg proposed the following theoretical framework for the chlorine cycle in soil:

- A considerable part of the chloride brought to the system through deposition is converted into organic chlorine, as a consequence of exo-enzymatic formation of reactive chlorine species;
- The reactive chlorine oxidises, depolymerises and chlorinates the organic matter;
- As a result, the chlorinated organic matter more easily follows the movement of water;
- Eventually, the chlorinated organic matter precipitates at lower levels in soil;
- The precipitated organic matter is slowly mineralised, thus releasing chloride;
- Hence, a considerable part of chloride in surface water originates from decomposing organic matter (contrary to the conventional wisdom, which has traditionally assumed that it all originates as chloride).

Since the decomposition of part of the organic matter can occur on time scales of thousands of years, the chloride released may come from quite old organic chlorine.

Question from J. Field: Can isotope ratio techniques be used to resolve this question? Have these ratios been constant over geological time?

Response from G. Öberg: This should be looked into.

Comment from J. Field: In the degradation of perchloroethylene, it has been observed that there is a slight "fractionation" of the chlorine isotopes.

Question from R. Wever: How about the formation of chloroform during degradation of the organochlorines?

Response from G. Öberg: Chloroform is definitely produced. A soil remediation company in Princeton found 100 µg/L of this compound (in soil leachate). If HOCl is formed and if the same chemistry is involved as in pulp bleaching, then one would expect $CHCl_3$ *to be a product.*

Question from J. Franklin: What is the mechanism of "precipitation" of organic chlorine in the lower soil horizons?

Response from G. Öberg: It is a combination of physical processes ("filtration") and chemical ones (redox reactions). The literature shows contrasting effects of chloride on precipitation: in some cases salt enhances precipitation of organic matter; in others the reverse effect is observed.

2.7 Chlorine mass balances in forested catchment studies – 3

It is difficult to estimate the fluxes of volatile organochlorines released from the forest or soil, but a value of 0.1 g/m^2yr has been estimated by Claudia Dimmer from a study in an Irish forest. Results from a salt marsh study tend to indicate higher fluxes. Nothing is known about temporal and spatial trends.

Question from J.-M. Libre: Are there not good studies on fluxes of individual compounds, such as chloroform?

Response from G. Öberg: Most studies merely give concentrations, and it is not possible to estimate fluxes from them.

Comment from J. Franklin: Both Khalil and Hoekstra have published results on chloroform emission fluxes.

Response from G. Öberg: But nothing is known about the effect of environmental variables on the fluxes.

The overall mass balance for the chlorine cycle in the forest system, as deduced in the preceding discussion, is shown in Figure 5.

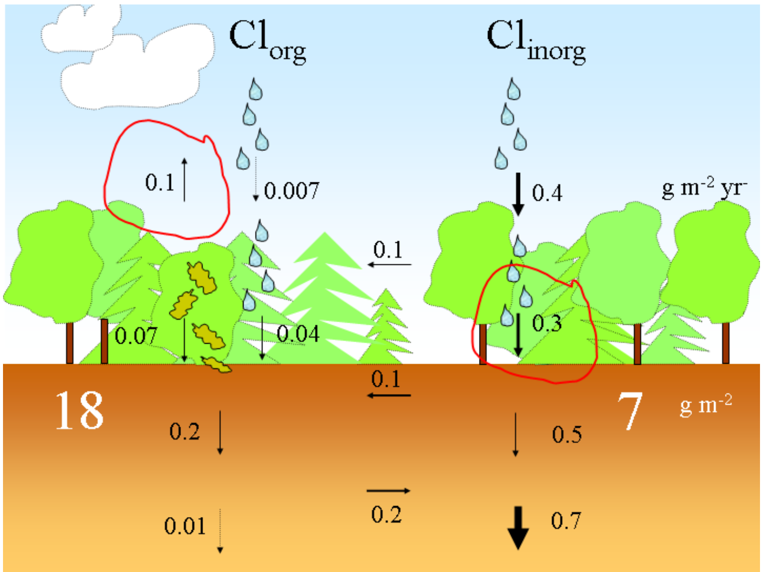


Figure 5

2.8 Influence of environmental variables

In a study of 21 Swedish lakes, there was found to be a clear statistical correlation between the AOX and the TOC values.

However, a study of 200 soils in southern Sweden (Johansson *et al*, 2003) showed a much clearer spatial correlation between organic chlorine and chloride, than between organic chlorine and organic matter, although a statistically significant correlation exists in both cases.

We know that the occurrence of organic chlorine in soil is influenced by a number of environmental variables, such as organic matter content, pH, nitrogen, chloride and redox potential. However, the relative importance of the variables seems to vary with the scale and we do not know if the influence is due to changes in the formation rate (consumption of chloride) or changes in the degradation rate (release of chloride), or both. And we do not know if the influence is caused by changes in (bio)chemical reaction rates, or in microbial activity, or the production of enzymes, etc.

2.9 Open questions

- Is exo-enzymatic formation of reactive chlorine a major source of naturally formed organochlorines in soil? It would seem so, but far too little is known;
- How large is the relative contribution of endo-enzymatically produced organochlorines? Probably small, but we need quantitative experimental data;
- Are chlorinated (high molecular weight) organic matter and chlorinated volatiles formed by the same processes? Probably for $CHCl_3$ but not for CH_3Cl;
- How large is the emission flux of volatile organochlorines?
- Which environmental variables are important and on what temporal and spatial scales and for which compounds?
- How large is the natural variation on various scales? Huge, as shown for $CHCl_3$ in the Stubbetorp catchment study. But this variability is very important to establish, before any reliable conclusions can be drawn.

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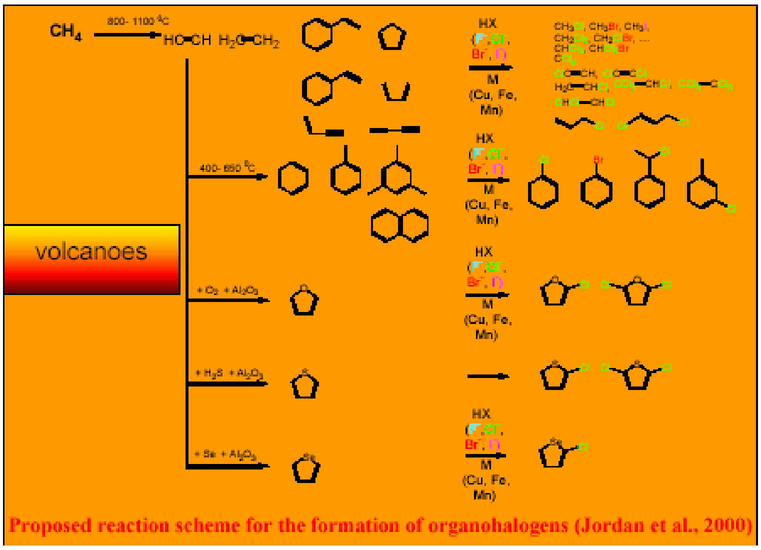
3 Abiotic formation of organohalogens in the terrestrial environment

Dr. Heinfried Schöler, University of Heidelberg, Germany

3.1 Introduction

H.F. Schöler stated that he would discuss the abiotic formation of organohalogens during “diagenetic processes”. These were defined to mean chemical reactions occurring when organic matter comes into contact with the mineral phase of soil.
Volcanoes are known to produce a great variety of aliphatic and aromatic volatile organofluorines, organochlorines, organobromines and organoiodines, as well as mixed organohalogens (Jordan *et al*, 2000; Harnisch *et al*, 2000; Schwandner *et al*, 2002). Some of these compounds contain double or triple bonds. The mechanism proposed by Jordan *et al* (2000), starting from methane and involving Cu, Fe and Mn catalysts, is shown in Figure 6.



Proposed reaction scheme for the formation of organohalogens (Jordan et al., 2000)

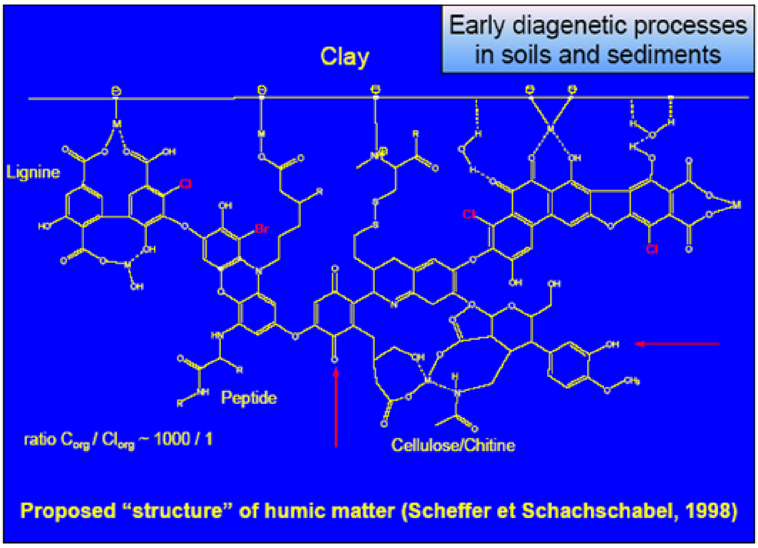
Figure 6

Biomass burning is also known to produce volatile organohalogens, in particular methyl halides (Crutzen *et al*, 1990; Andreae *et al*, 1993, 1996). High-temperature free-radical chemistry, or “fire chemistry”, is involved. According to Butler (2000), biomass burning accounts for a significant fraction of the atmospheric CH_3Cl flux (1 Mt/yr out of a total of ~ 4-5 Mt/yr, determined from the known atmospheric concentrations and lifetimes) and a smaller fraction for CH_3Br (20 kt/yr out of ~ 300 kt/yr). However, in both cases, there are “missing” or unknown sources that are very important: about 1.2 Mt/yr for CH_3Cl and 70 kt/yr for CH_3Br. These fluxes may well be terrestrial.
For biomass burning, the mechanism is not yet understood, but halides are present in the plant material, so reactions catalysed by available metals (Fe, Mn, Cu) may play a role, alongside high-temperature free-radical chemistry.

3.2 Formation of organochlorines in soil

In addition to formation of organohalogens in volcanoes and during biomass burning, early diagenetic (abiotic) processes in the terrestrial environment need to be considered (Keppler *et al*, 2000, 2001; Pracht *et al*, 2001; Fahimi *et al*, 2002). These purely geochemical processes consist of the decay and transformation of organic material in contact with soil ingredients, and lead to the formation of a multitude of organohalogens: alkyl halides (C_1-C_4), reactive unsaturated compounds (vinyl chloride, chloroethyne), halogenated carbonic acids (acetic, benzoic acids, etc.), halogenated humic acids, halogenated phenols (?) and possibly halogenated dioxins in clay pits.

Soil results from the degradation of primary rocks and contains dead organic material resulting from plants. Halide ions present arise from the weathering of the rocks, their concentrations in humic soils amounting to 100-300 mg/kg for Cl^-, 5-50 mg/kg for Br^- and 1-10 mg/kg for I^-. The bromide and iodide contents, relative to chloride, are much higher than in the oceans, possibly due to reactions leading to storage of these compounds in soils.
The organic content of the uppermost “O”-horizon can be as great as 100%, e.g. in peat and moor land. The first soil layer (Ah-horizon) comprises 0.5-15% of organic material (the former value referring to sandy soils and the latter one being typical of the “black soil” of the Ukraine).
Iron oxides are also present, arising from primary rocks (olivine, biotite and magnesite). Fe(II) is released by weathering processes. It comes into contact with the oxygen of the atmosphere and is oxidised to Fe(III). Secondary rocks are formed in the soil. Because of the oxic environment, all of the iron is deposited in the soil as ferrihydrite, goethite, lepidocrocite and hematite.
All the prerequisites are present in soil for the production of organohalogens. Organic material is stored in a reduced form in plant material. On contact with the Fe(III) present in soil, there is a slow oxidation. Mineralisation leads ultimately to CO_2, but intermediate compounds are formed, with concurrent slow conversion of Fe(III) to Fe(II). If halide is present, organohalogens can be formed.
A proposed structure of humic material is shown in Figure 7. Quinone-like, hydroquinone-like, and cathechol-like structures are present.


Early diagenetic processes in soils and sediments

Proposed “structure” of humic matter (Scheffer et Schachschabel, 1998)

Figure 7

It is now known that chlorine is present, at a ratio C_{org}/Cl_{org} ~ 1000/1. Most of the soil compounds arise from rather recalcitrant lignin, peptide, cellulose and chitin, via anaerobic and aerobic pathways (see proposed pathway in Figure 8). Sugars, carbohydrates, short-chain carboxylic acids and amino acids degrade easily and are reduced by organisms for their purposes.

Polyphenols are essential intermediates. They can be oxidised by phenoloxidase to quinone-like structures, but also by Fe(III) if vicinal hydroxyl groups are present. In the presence of amino compounds, humic and fulvic acids are formed.
Some of the polyphenols are called “siderophores” (iron carriers for plants and micro-organisms). The most important ones are catechol, 2,3-dihydroxybenzoic acid and 2,3-dihydroxyphenyl acetic acid. The main feature of these compounds is that they can dissolve iron from minerals, since their complexing energy exceeds the lattice energy. The siderophores are redox-sensitive, i.e. Fe(II) can be reduced to Fe(II) which is the required form for transport through cell membranes. In natural environments, iron is one of the limiting factors and the siderophores enable the micro-organisms to extract sufficient iron from the soil minerals. During this process, the siderophores are partially degraded by the Fe(III), leading to oxidation products.

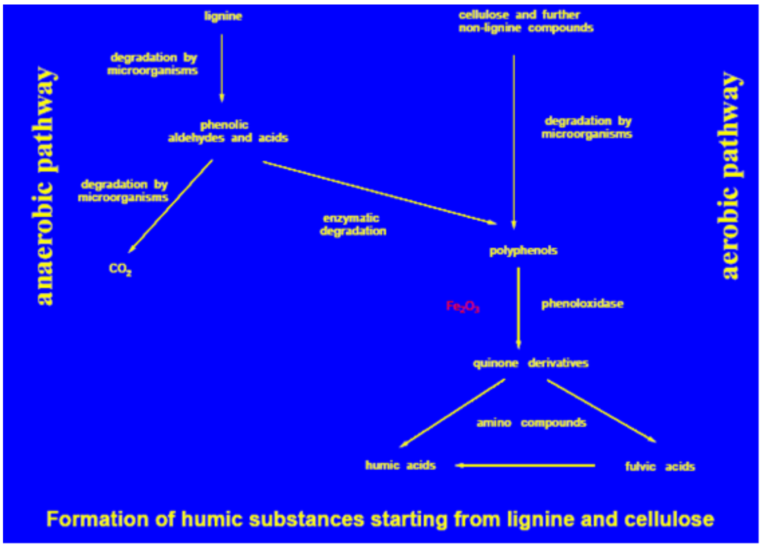


Figure 8

Thus, for example, 2,3-dihydroxybenzoic acid forms a blue intermediate complex with Fe(III), but after a period of time, the colour turns to yellow, due to the formation of a quinone, together with Fe(II) (Xu and Jordan, 1988). Two Fe(III) ions are necessary to oxidise the catechol-like structure to the quinone-like product.

The incorporation of chlorine into organic structures can be done at ambient temperature in two “low-energy” pathways involving nucleophilic addition of chloride:

(a) To an epoxide ring to form a chlorohydrin, as in the formation of chlorogentisylol (Nabeta *et al*, 1975);

(b) To “vinylogous ketones” such as 1,4-benzoquinones, to give chlorohydroquinones (Dargelos *et al*, 1971). This type of reaction is exploited industrially in the manufacture of tetrachloro-1,4-benzoquinone (chloranil). In the case of humic acid, a similar reaction is assumed to occur, with iodide as the halogen source, the iodine being in any case integrated into the organic structure (Rädlinger and Heumann, 1997).

Schöler and co-workers have shown that guaiacol can be oxidised by Fe(III) in aqueous solution to 1,2-benzoquinone, in the presence of halide, with concomitant formation of small quantities of methyl halides (CH_3Cl, CH_3Br, CH_3I). This reaction works not only with dissolved Fe(III), but also with iron-bearing minerals such as ferrihydrite, 5 $Fe_2O_3.9H_2O$ (Keppler *et al*, 2000).

If oxygen is not excluded and Fe(III) and chloride are present, a range of products is formed. Thus, catechol leads to CO_2, together with CH_3Cl, C_2H_5Cl, C_3H_7Cl, $CH_2{=}CHCl$ and $HC{\equiv}CCl$. These compounds can be found in soil, if appropriate analytical procedures are used.

Question from J. Field: Can you say anything about the product yield?

Response from H.F. Schöler: About 1 out of 30000 C atoms is converted into alkyl halides, under controlled laboratory conditions.

Question from R. Wever: Is this a free-radical, Fenton-type mechanism?

Response from H.F. Schöler: Yes, as I will explain later.

It is believed that an oxygen atom is inserted into the bond between the carbonyl-bearing carbon atoms of the 1,2-benzoquinone, to form a cyclic anhydride, which is further hydrolysed to a diacid (muconic acid when catechol is used as the starting material). The latter is further mineralised to CO_2.

Question from J. Field: You have referred so far to iron, but is Mn not a more powerful oxidant?

Response from H.F. Schöler: The problem is that you need to work with Mn(IV), but it is hardly soluble, so you have to use it as a crystalline solid and the reaction rate is very slow.

Comment from J. Field: In that case, I would suggest using Mn(III) lactate.

Response from H.F. Schöler: We would like to use only compounds that are stable in nature. We are working currently with various forms of Mn, Cu and Hg.

When the reaction is monitored by measuring the CO_2 evolved, it is seen that formation of this product is particularly rapid with catechol, several times slower with guaiacol and nil with hydroquinone. When ferrihydrite is used as the oxidant, a similar conclusion is reached, but the reactions are slower than with completely dissolved Fe(III).

Vinyl chloride, chloroethyne and other organochlorines were demonstrated to be formed in field measurements on soil air, laboratory studies using freeze-dried soil samples and experiments with model compounds.

Thus, monitoring was carried out on air from the top soil layers of a coastal salt marsh, a deciduous forest and a peat land. The ratios of concentrations in top soil air to those in ambient air are given in Table 1 for $CH_2{=}CHCl$ and CH_3Cl:

	Vinyl chloride	Chloromethane
Coastal marsh	853	43
Deciduous forest	64	3.6
Peat land	111	3.2

Table 1

These ratios show that both vinyl chloride and chloromethane are produced in soil. On the other hand, for 1,1,1-trichloroethane and trichloroethylene, the corresponding ratios were found to be close to (or lower than) unity, indicating that there is no formation in soil, and there may be some destruction. Chloroethyne is detected in soil air, but not in ambient air, so ratios cannot be given (it also degrades in the sample during transport to the laboratory).

When freeze-dried samples were dissolved in water, chlorinated hydrocarbons (including vinyl chloride) were evolved spontaneously at ambient temperature. This was demonstrated with soil from Patagonian grassland, peat from Germany and Fluka humic acid. When Fe(III) was added to the Patagonian soil, formation of vinyl chloride and chloromethane (but not 1,1,1-trichloroethane) was enhanced. The effect was much greater when H_2O_2 was added in addition to Fe(III).

Even with autoclaved soil small amounts of alkyl chlorides were observed.

Similar observations were made when working with catechol as a model compound. The formation of vinyl chloride, chloromethane and chloroethyne (but not trichloroethane) underwent increasing enhancement in the presence of

(a) chloride + Fe(III); and
(b) chloride + Fe(III) + H_2O_2.

The marked effect of H_2O_2 suggests that OH radicals play a role in the reaction.

Question from G. Öberg: Did you add enzymes, to observe the impact?

Response from H.F. Schöler: No.

Putting together all the evidence from the Heidelberg group’s work and other studies, one can propose the reaction pathways shown in Figure 9.

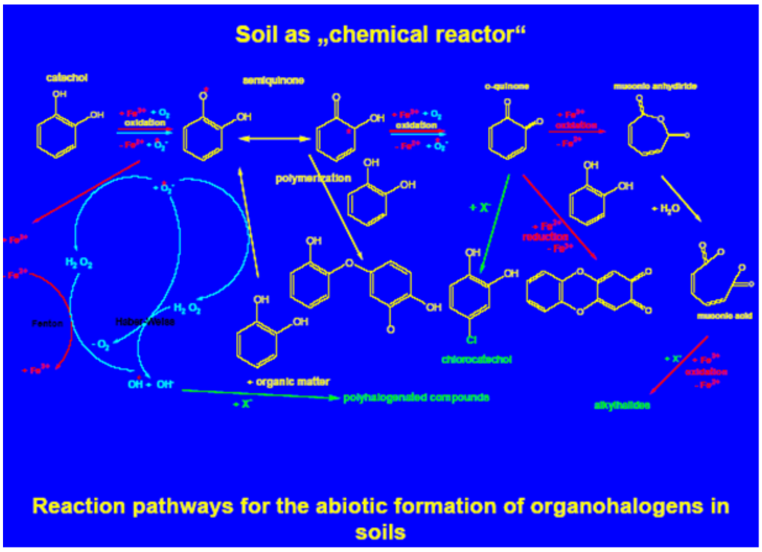
Either Fe(III) or O_2 can cause oxidation of catechol to the corresponding semi-quinone, which is then oxidised further to the o-quinone and to muconic anhydride, the latter being hydrolysed to muconic acid. Research with HPLC-MS is under way to identify further intermediates from this reaction. Alkyl halides might be formed from these intermediates.

Reactions involving the o-quinone can lead to dioxin-like structures, and also to chlorocatechol.

Polymerisation of the semi-quinone intermediate may give a “building block” that could lead to humic-like structures.

The left-hand side of the figure shows production of H_2O_2 from the superoxide radical anion and the Haber-Weiss reaction of superoxide radical with H_2O_2 to give OH radical and OH^-. Fenton chemistry involving H_2O_2 and Fe(II) also gives OH radicals.

But the existence of OH radicals in soil would not be easy to demonstrate. If present, as hypothesised here, they could oxidise chloride to chlorine atoms and the latter might be responsible for the formation of organochlorines.


Soil as „chemical reactor“

Reaction pathways for the abiotic formation of organohalogens in soils

Figure 9

3.3 Formation of chloromethane in leaves

H.F. Schöler then reported on recent work (Hamilton *et al*, *Science*, 2003) on the formation of chloromethane in leaves.
Senescent leaves or grass produce chloromethane. As the water content decreases, the production of CH_3Cl increases. Methanol is believed to be a precursor. At very high temperatures (300°C), up to 70-100% of chloride is converted into CH_3Cl.

> *Comment from R. Wever: A different explanation for these observations may be that as plants become dried they become stressed and hence more susceptible to fungal attack. HOCl formation by the fungi may thus occur, explaining the formation of organochlorines*
>
> *Response from J. Field and H.F. Schöler: But the phenomenon also occurred with a control sterilised using gamma radiation.*

Hamilton *et al* believe that the starting material for this chemistry is pectin, a polymer consisting of around 100 galacturonic acid molecules and a major component of the cell wall in plants.
If chloride is added to commercial pectin and the mixture is heated to temperatures above 250°C, then all the chloride can be converted to CH_3Cl. This may explain what happens during biomass burning.

3.4 Conclusions

- Abiotic halogenating (or “early diagenetic”) processes at ambient temperature exist in the aerobic layer of soil. The extent of such processes is currently far from being realistically assessed;
- One pathway produces monohalogenated alkyl halides (C_1-C_4) and reactive intermediates (e.g. vinyl chloride and chloroethyne). Radical chemistry, induced by Fe(III) and Mn(IV) seems to be involved, as well as possibly H_2O_2;
- Another, recently discovered, pathway produces methyl halides starting from pectin and inorganic halides;
- Yet another process produces polyhalogenated volatile and polar compounds (chloroform and trichloroacetic acid. There is some evidence for involvement of a Fenton-like reaction producing OH radicals.

3.5 Significance

It is known that:

- The reduction of insoluble Fe(III) oxides is one of the most significant geochemical processes taking place in the sedimentary environment;
- Many soils exhibit high salinity (up to 1% halide content);
- Worldwide, 1500-2200 Gt of organic carbon is stored as humic matter;
- In the Heidelberg group's experiments, up to 1 out of 30000 carbon atoms is liberated from soil as alkyl halides;
- There are large "missing sources" of methyl chloride and methyl bromide in their atmospheric budgets. The terrestrial environment may well contribute to these "unknown" emissions.

3.6 Discussion

Question from J.-M. Libre: How difficult would it be to estimate emissions fluxes from soils, e.g. for vinyl chloride?

Response from H.F. Schöler: It is not so difficult in principle, but requires funding, since flux chambers are expensive.

Comment from G. Öberg: It takes a long time to develop the required expertise and programmes with the necessary equipment are already running in various countries (for compounds other than those discussed at the workshop).

Comment from J. Franklin: For vinyl chloride, which is very reactive in the atmosphere, we do not have reliable measurements of the background concentrations, and we do not know the overall emission flux (anthropogenic + natural). It would only be of interest to determine the natural emissions if we had an estimate of the overall flux to the atmosphere.

Response from H.F. Schöler: The release of vinyl chloride from soil is probably small compared to its "polymerisation".

Question from R. Wever. Is not vinyl chloride rapidly metabolised by bacterial systems?

Response from H.F. Schöler: But is radical chemistry faster than microbial degradation?

Comment from J. Field: Microbes degrade only down to a threshold concentration (often several hundred μg/kg). Can you translate your observation into a concentration, which we could compare to the threshold?

Response from H.F. Schöler: Yes, these values have been published in ES&T.

Comment from J. Field: Mn is very important in soil and organic matter and is a much more powerful oxidant than ferrihydrite. Mn oxide ordered from chemical suppliers is much too crystalline and insoluble. It needs to be prepared fresh and can be reduced by bacteria. Mn is central to the degradation of lignocellulose involving white rot fungi, Mn(III) oxalate participating as the chelator.

Comment from H.F. Schöler: If one wants to prepare freshly precipitated compounds in the laboratory, one needs to be careful to avoid the presence of halides and to not allow the product to dry (which leads to crystallisation of an insoluble product). The Heidelberg group uses commercial birnessite as a source of Mn.

Comment from H.F. Schöler: An interesting review on lignin degradation was published by Goodall (from the paper industry, New Hampshire). All the siderophores studied were related to chlorinated products: anisoles, benzoic acids, phenyl acetic acids. This inspired the Heidelberg group to pursue research on the production of organochlorines in the presence of iron.

Question from K. van Ginkel: Inaudible.

Response from H.F. Schöler: There about 10-12 well characterised siderophores, including hydroxamates. But the Heidelberg group tested only catechols, and benzoic and phenylacetic acids.

Question from K. van Ginkel: The reactions with Fe and Mn proceed much faster in the slightly acidic range. Do you see a difference between acidic and alkaline soils?

Response from H.F. Schöler: Most of the experiments were done with soils at pHs of 4-6, typical of woods.

Comment from G. Öberg: In a Swedish study on 200 soil samples, there was no discernible correlation between pH and total organochlorine content. Water content may play a role, in addition to pH.

Question by J. Franklin: How significant is volatilisation of organochlorines (such as methyl chloride) from the terrestrial environment, compared to runoff of chloride back to the oceans via rivers, in the overall chlorine budget? That is which process is more important in maintaining a steady state chloride concentration in soil?

Response from G. Öberg: In the catchment study, the total chlorine deposition was about 0.7 g/m^{2}yr, while only 0.1 g/m^{2}yr, or less, was emitted as volatiles.

Question from J.-M. Libre: Are there no measurements of fluxes of CH_3Cl *from the terrestrial environment?*

Response from H.F. Schöler: There are no field measurements.

Response from G. Öberg: Harper and co-workers have made some estimates from lab studies. But these cannot explain the "missing" CH_3Cl *flux.*

Comment from several participants: The mechanisms for formation of chloroform and methyl chloride are presumably very different.

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4 The role of humic substances in the anaerobic degradation of chlorinated solvents

Dr. Jim Field, University of Arizona, USA

4.1 Introduction to humic substances

Humic substances are poorly degradable organic matter accumulating in the biosphere. They are made up of highly condensed aromatic structures, rich in quinones, and have half-lives ranging up to 250-1900 yr.
The mechanism for the genesis of humus is illustrated in Figure 10.

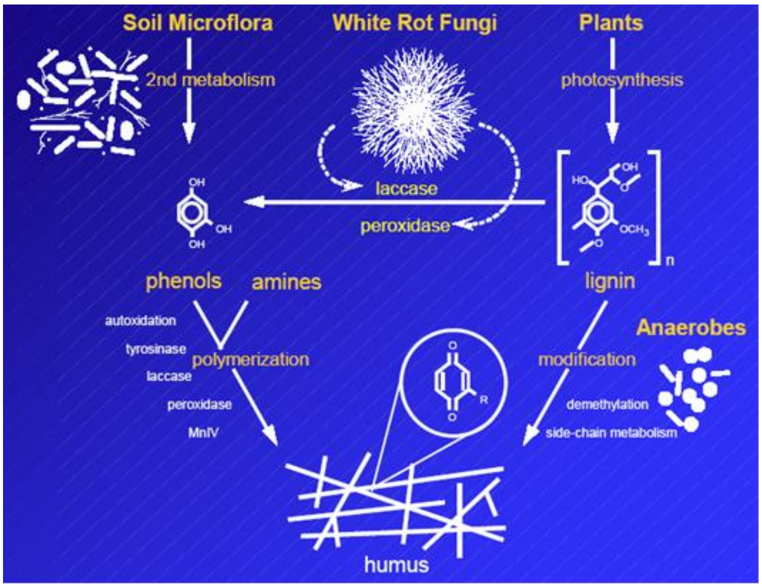


Figure 10

Lignin is probably an important source of polyphenolic substances. Humus was originally considered to result from the modification of lignin mainly by anaerobic micro-organisms that remove a methyl group or metabolise a side chain. Later, it was recognised that lignin can be broken down, e.g. by white rot fungi, to give low molecular weight polyphenols that then form polymeric products via a number of pathways involving uncatalysed autoxidation, or catalysis by enzymes, Mn(IV), etc.
Secondary metabolites produced by micro-organisms are also involved in humus formation.
Important sub-structures for the degradation of organochlorines are those that are redox-active, such as quinones. There is 2-electron cycling between quinones and hydroquinones, and 1-electron cycling between semi-quinones and hydroquinones (see Figure 11). EPR measurements can be used to quantify the quinones present in humus.
Popular model compounds for laboratory studies are:

- anthraquinone disulphonate, or AQDS (soluble in water up to about 300 mg/L, which is sufficient for the purposes of investigation)
- juglone.

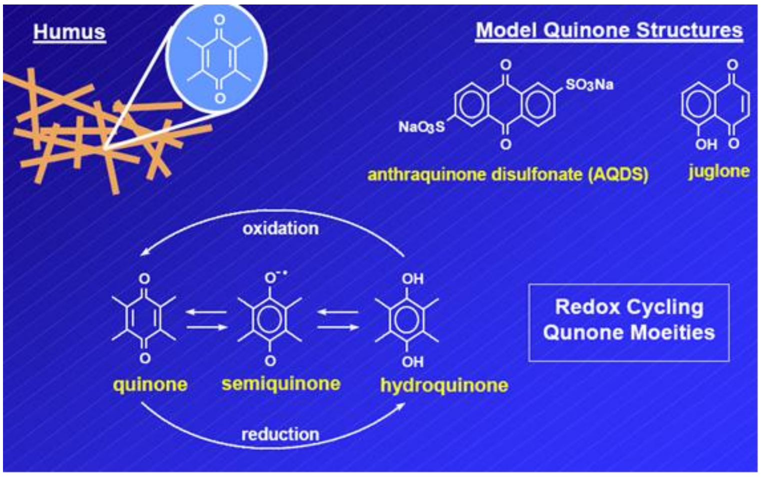


Figure 11

4.2 Anaerobic conditions

Anaerobic conditions can exist in waterlogged soil, where the diffusion of elemental oxygen is hindered. Also, if there is a high organic load, even in aerobic soils, the consumption of O_2 may be so high that anaerobic micro-niches exist.
There are many different types of anaerobic metabolism, depending on the dominant electron-accepting species. If none are present other than carbon itself, then methanogenesis will probably occur. In the presence of $SO4^{2-}$, sulphate-reducing bacteria are likely to play a role. Some organochlorines can themselves act as electron acceptors and induce a process known as halorespiration. Iron is also a good electron acceptor; it exists in many mineral forms. The various electron-accepting processes and their redox potentials are illustrated in Figure 12.

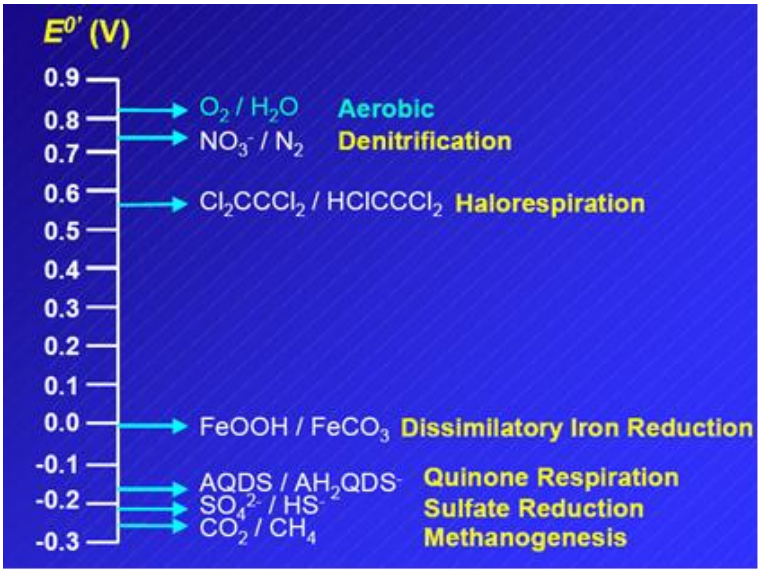


Figure 12

A methanogenic consortium will reduce acetate to methane. However, when a “humic-like” substance, such as the model compound AQDS, is added in large excess so as to accept all the electrons from the acetate, then hardly any methane is formed but there is a switch to a “quinone-respiring system”, AQDS being reduced to the corresponding hydroquinone.

4.3 Formation of organochlorines in soil

The recent paper by Myneni (2002) shows that aliphatic organochlorines are produced during the earlier stages of the humification process (fresh leaves → senescent leaves on plant → senescent leaves on soil → humified leaves on soil → powdered top soil → isolated soil humics) with aromatic structures appearing later on in the process.

The mechanisms for formation of the aliphatic organochlorines were not described in detail, since they had been discussed earlier in the workshop.

4.4 Degradation of organochlorines in soil - The role of humus

Humus plays a role not only in the formation of organochlorines, but also in their degradation. There are two major modes for the involvement of humus:

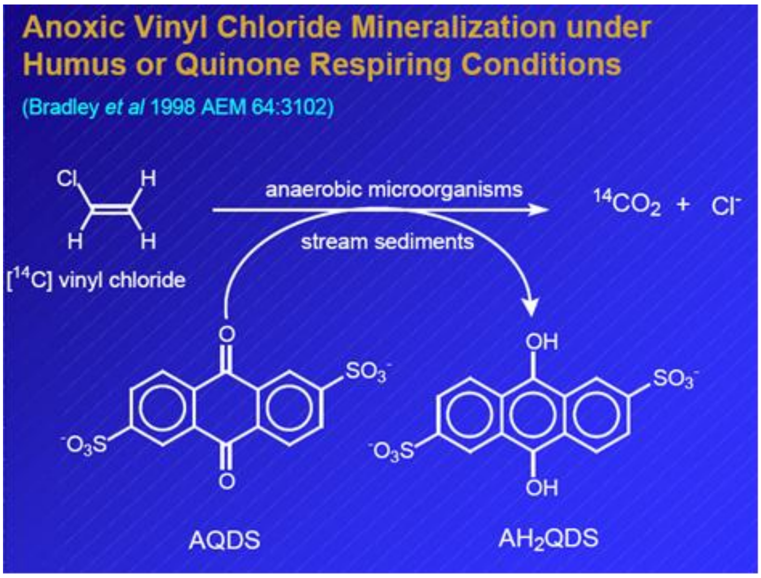
- An oxidative mode in which it accepts electrons from the organochlorines, which thus behave as a food source. They are used for obtaining energy to support growth, being oxidised to CO_2, e.g.:

$$CH_2{=}CHCl + e^- \rightarrow Cl^- + CO_2;$$

- A reductive mode in which humic substances serve as “redox mediators”, taking electrons from the micro-organisms and “dumping” them on the organochlorines, e.g.:

$$CCl_4 + e^- \rightarrow CHCl_3 + Cl^-.$$

The first mode is illustrated by work by Bradley *et al* (1998), as shown in Figure 13:



Anoxic Vinyl Chloride Mineralization under Humus or Quinone Respiring Conditions

(Bradley *et al* 1998 AEM 64:3102)

Figure 13

Vinyl chloride is oxidised to CO_2 and chloride is released, at the expense of using a quinone as an electron acceptor. In much the same way as humans use oxygen and reduce it to water, these anaerobic micro-organisms take quinones and reduce them to hydroquinones. Bradley *et al* showed that this reaction occurs not only with the model compound AQDS, but also with humus.

4.5 Degradation of organochlorines in soil – Humus as a redox mediator

4.5.1 Overview

The rest of the presentation focussed on the role of humus as a redox mediator supporting the reductive dechlorination of organochlorines.

Such processes may be both abiotic (as discussed in the presentation by H.F. Schöler) and biotic:

In the abiotic pathway, the hydroquinone reduces the organochlorine, being itself oxidised to a quinone. The latter is reduced back to the hydroquinone form by Fe(II), which is oxidised in the process to Fe(III). A similar process exists in which the Fe(II) is replaced by elemental sulphur or H_2S.

In the biotic pathway, illustrated in Figure 14, a bacterium oxidises a substrate and dumps its electrons on to a sub-stoichimetric amount of humus, which then reduces the organochlorine. in this process, the quinone and hydroquinone present in the humus play the same role as in the abiotic mode.

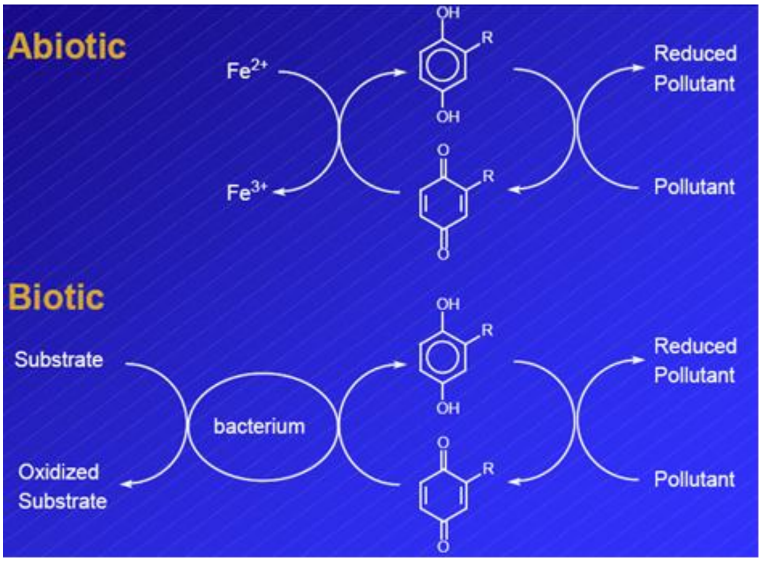


Figure 14

4.5.2 Abiotic pathway

Some examples of humus as a redox mediator in the abiotic mode are given in Table 2 (Curtis and Reinhard (1994); Perlinger *et al* (1996)):

Electron donor	**Mediator**	**Reductive reaction***
Fe(II) or HS^-	humic acid	HCA → PCE
HS^- or S^0	Juglone	HCA → PCE
Fe(II) or HS^-	humic acid	CT → CF
Fe(III) or HS^-	humic acid	$CHBr_3$ → ?

* HCA = hexachloroethane; PCE = perchloroethylene;
CT = carbon tetrachloride; CF = chloroform

Table 2

4.5.3 Biotic reduction of carbon tetrachloride - University of Arizona studies

The investigations of the biotic reduction of CCl_4 carried out by the University of Arizona researchers are performed in small serum bottles containing:

- 50 mL of a chloride-free mineral medium (so that the formation of Cl^- can be monitored), with a phosphate buffer system (pH 7.0);
- Granules of methanogenic consortia present in sludge that separates readily from the water. The sludge levels is 0.25 g/L;
- A mixture of volatile fatty acids (acetate, propionate and butyrate);
- CCl_4 at a concentration of 100 μM;
- AQDS at a variable level, e.g. 20 μM;
- An inert-gas head-space (N_2).

Comparative experiments show the following results:

- If CCl_4 is incubated in the medium alone, no decrease in CCl_4 concentration is observed;
- Adding AQDS under the previous conditions does not enhance the reaction;

- In the presence of “killed” (autoclaved) sludge, either with or without AQDS, there is some decline in CCl_4 concentration, possibly on account of abiotic reactions induced by sulphide;
- With “live” sludge, but in the absence of AQDS, the reaction is significantly enhanced;
- Finally, with live sludge and AQDS, there is marked further enhancement of the reaction rate.

The first-order rate constant (k) for degradation of CCl_4 increases with the concentration of AQDS, but levels off as this parameter approaches 50 µM.

The chlorine balances after 5 days of incubation show that chloroform is not the only reaction product: indeed, the number of moles of Cl^- formed greatly exceeds the number of moles of $CHCl_3$.

The effect of adding electron-donating substances (methanol, acetate, glucose and hydrogen) was also studied. In each case, a considerable enhancement of the first-order rate constant was observed when AQDS was added. In the “endogenous” situation, in which the only electron donor was the sludge itself, adding AQDS had relatively little effect. If an antibiotic - such as neomycin - was added, in addition to AQDS, then the rate was similar to that found in the absence of AQDS. This clearly shows that there are certain specific organisms responsible for the rate enhancement, and they are not the same as those responsible for the “basal” activity without AQDS.

After 27 days of incubation in the presence of AQDS and the various electron-donating substances, chloroform was a relatively minor product. The high levels of chloride observed showed that it had been transformed further. Indeed, perchloroethylene was identified as a product (up to 9% of the chlorine initially present in the CCl_4). Dichloromethane was also found to be a trace product.

From the original methanogenic consortium, an “enrichment culture” was prepared, capable of respiring quinones, using acetate as an electron donor. This culture was dominated by a single organism, shown by sequencing to be *Geobacter*. Experiments with this culture showed CCl_4 to be degraded in the presence of either AQDS or humus, but not in their absence. The products were similar to those found with the methanogenic consortium.

Experiments were also carried out in a totally abiotic system, in which AQDS reduced chemically to the corresponding hydroquinone (AH_2QDS) was shown to degrade CCl_4 and produce chloroform, while AQDS itself did not lead to any significant reaction.

4.6 Mechanisms

In the anaerobic degradation of CCl_4, it is generally recognised that there are two possible pathways (see Figure 15):

- Reductive hydrogenolysis, in which the chlorine atoms are replaced sequentially by H atoms, to give chloroform, dichloromethane, etc.;
- Reductive hydrolysis, in which a dichlorocarbene radical reacts with water to give formic acid or CO, and ultimately CO_2. This might involve the CCl_3 radical as an intermediate, formed by one-electron oxidation of CCl_4.

Perchloroethylene could then be produced:

- Either by recombination of two CCl_3 radicals to give hexachloroethane, that is subsequently dechlorinated to C_2Cl_4 (as is well-known in the literature);
- Or by reaction of dichlorocarbene with CCl_4, also giving C_2Cl_6 as an intermediate.

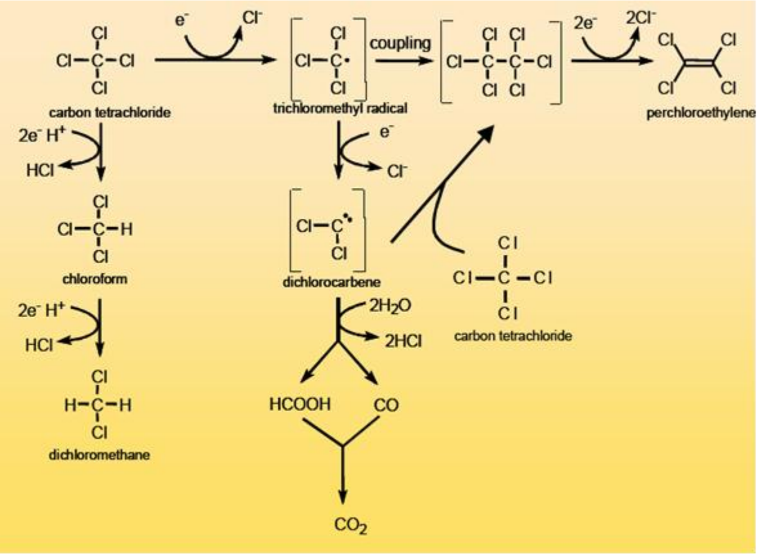


Figure 15

The proposed role of humus is illustrated in Figure 16:

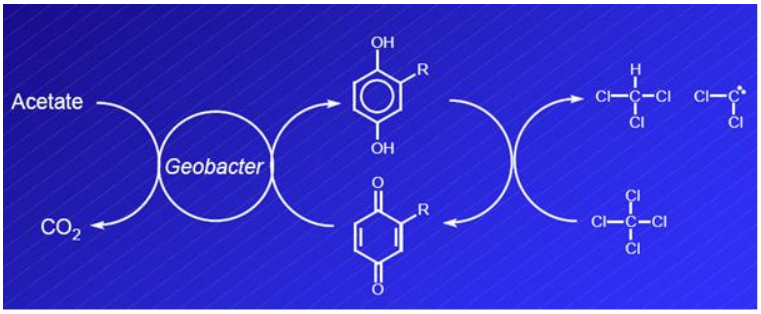


Figure 16

Only sub-stoichiometric amounts of quinones (present in humus) are required, provided that an organochlorine such as CCl_4 is available to recycle the hydroquinone back to the quinone.

4.7 Conclusions

The evidence that redox mediation humus is involved in the reductive biotransformation of carbon tetrachloride is as follows:

- Humus or its model compound AQDS is required for biotransformation of CCl_4 in the quinone-respiring enrichment culture (*Geobacter*) and they both enable a large rate enhancement in the case of the mixed-consortium anaerobic sludge;
- Antibiotics (such as neomycin) that kill the iron-reducing (or “humus-respiring”) *Geobacter* also cancel any rate enhancement induced by AQDS in anaerobic sludge;

Question by R. Wever: Is it not true that antibiotics in general do not kill directly? That is, the bio-system will still function, only the reproduction will stop.

Response by J. Field: When we start with a methanogenic consortium, we hardly have any iron-reducing bacteria, but depend on their growth during incubation.

- An electron-donating substrate is required, so the process is clearly reductive;
- AH_2QDS directly reduces CCl_4.

The broad conclusions of the presentation are therefore:

- Humic substances greatly increase the rate of dechlorination of CCl_4 by anaerobic micro-organisms, with anaerobic sludge (this study) or the iron-reducing *Shewanella putrefaciens* (Collins and Picardal (1999));

- One pathway is reductive hydrogenolysis to chloroform and dichloromethane, but the hydrolysis of the intermediate dichlorocarbene radical is probably more important;
- This is the first time that perchloroethylene has been reported as a product of the biotransformation of CCl_4. It may arise from a reaction of dichlorocarbene with CCl_4, giving hexachloroethane, which is subsequently dechlorinated.

Question from R. Wever: What is the comparative bioenergetics of the two degradation pathways?

Response from J. Field: The pathway going to CO_2 should be more favourable than the one going to methane.

Comment from J. Franklin: The formation of C_2Cl_4 from CCl_4 involves a second-order process with respect to the substrate, while the formation of chloroform should be first-order. So, as the concentration of the substrate decreases, the latter process should become predominant. Formation of C_2Cl_4 may be significant in grossly polluted soils, but is probably less so in situations closer to "background".

Response from J. Field: It would be interesting to study the product distribution as a function of the concentration of CCl_4.

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5 Vanadium haloperoxidases and their role in the formation of chlorinated compounds

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5.1 Introduction to haloperoxidases

Haloperoxidases are enzymes capable of oxidising halides to the corresponding hypohalous acids:

$$H_2O_2 + H^+ + X^- + \text{enzyme} \rightarrow H_2O + HOX \ (X = Cl, Br \text{ or } I)$$

A chlorperoxidase can oxidise chloride or bromide, while a bromoperoxidase is essentially only able to oxidise bromide. The hypohalous acids produced may halogenate organic compounds:

$$HOX + RH \rightarrow RX + H_2O$$

There are essentially two types of haloperoxidases:

- Heme-containing haloperoxidases. One of these, myeloperoxidase, is present in the white blood cells of mammals and plays a role in the body's defence system. Another is a fungal system, the chloroperoxidase from *Caldariomyces fumago*, which is commercially available;
- Vanadium-containing haloperoxidases.

Of the latter group, vanadium bromoperoxidases were discovered in 1985 in macro-algae and vanadium chloroperoxidases were first found in fungi, in 1992.
The prosthetic group is vanadate, VO_4^{3-}, in which the vanadium is present in the +5 oxidation state, and which has a structure analogous to that of the phosphate anion. Indeed, if these enzymes are dialysed at low pH against phosphate, or incubated in phosphate buffers, then vanadium is lost and the enzymes are inactivated. Nevertheless, they can be reactivated by addition of vanadate at neutral pH.

5.2 Vanadium bromoperoxidases

Vanadium bromoperoxidases (VBPO), which have been extensively studied, can be found in a broad range of brown, red and green seaweeds, some of which have been shown to form bromoform and other volatile organohalogens. VBPOs have also been identified in the lichen *Xantoria parietina*.
Heme haloperoxidases have been found in marine diatoms and green seaweeds.
VBPOs have been shown by histochemical staining to reside mainly in the outer cuticle and external cortex of the seaweed.
The VBPO from the brown seaweed *Ascophyllum nodosum* has been demonstrated to have the following properties:

- A homodimeric structure (over 1100 amino acids);
- Vanadate in the active site;
- High stability towards denaturation (for structural reasons, namely high helical content, S-S bridges and dimeric interactions);
- Reaction mechanism:

$$E + H_2O_2 \rightarrow E.H_2O_2$$
$$E.H_2O_2 + Br^- + H^+ \rightarrow E + HOBr + H_2O$$

- Optimum pH: 6.0-7.0.

5.3 Production of organohalogens by seaweeds

The purified enzyme is able to form HOBr. In *Ascophyllum nodosum* and other seaweeds, the enzyme is extra-cellular or located on the outer cellular surface. The following question then arises: Is whole seaweed able to produce HOBr?

This can indeed be shown to be the case by taking fresh seaweeds and incubating them in seawater (1 mM Br^-), with phenol red as a scavenger for HOBr. This indicator is converted by bromination to bromophenol blue. However, no reaction occurs, unless H_2O_2 (2 mM) is added, when rapid conversion of the phenol red is observed for a variety of plants within a reaction time of about 1 hour. Since the usual concentration of H_2O_2 in seawater is only 1 μM, the reaction would be much slower under natural conditions.

The natural trigger for formation of HOBr in seaweeds is sunlight and stress, according to Wever *et al* (1991) and Abrahamsson (2003). This phenomenon is thus related to photochemical activity.

A rough calculation of the amount of HOBr produced is given below:

- From the laboratory investigations of Wever and co-workers, the production of HOBr in full sunlight is 112 nmol/g.h;
- The biomass on a stretch of the “Afsluitdijk” in The Netherlands is about 10^5 kg;
- The production of HOBr at this location would therefore be about 1.1 kg/hour, or about 1000 kg/year (assuming 900 hours of sunshine per year?);
- Assuming that all brown seaweeds (10^{13} g) produce HOBr at the same rate, this would yield an extrapolated global annual production of about 500,000 tons.

In addition to HOBr, it is however known that seaweeds produce specific organobromines. Thus, Gschwend *et al* (*Science*, 1985) demonstrated the formation of $CHBr_3$, $CHBr_2Cl$ and CH_2Br_2. Assuming the global biomass of macro-algae to be 10^{13} g, these authors derived an estimate for the annual global production of organobromines equal to 10,000 tons/year. The production of halocarbons by macro-algae has now been investigated for around 70 species. Thus, Carpenter and Liss (2000) estimated a global annual production of $CHBr_3$ by these species equal to 200,000 tons/year.

Two mechanisms have been proposed to explain the formation of $CHBr_3$ from HOBr:

- Reaction of HOBr with 3-keto-octanoic acid, present in seaweed (Theiler *et al*, *Science*, 1978). The reaction is postulated to proceed via the successive formation of 1-bromo-2-heptanone, 1,1-dibromo-2-heptanone and 1,1,1-tribromo-2-heptanone (“haloform reacton”). Dibromomethane and 1-bromopentane are by-products;
- Reaction of HOBr with “dissolved organic matter” (DOM). It is known from water treatment chemistry that the reaction of oxidised bromine species with DOM is extremely rapid.

Two possible roles for the formation of HOBr by seaweeds have been hypothesised:

- A major defence against colonisation or a response to pathogenic attack;
- Conversion of H_2O_2 produced photosynthetically (Manley). However, the HOBr produced is much more reactive than H_2O_2.

Returning to the more likely hypothesis of defence against pathogenic attack, it was pointed out that inter-cellular signalling in many different types of bacteria occurs through the use of N-acetylated homoserine lactones (AHLs). These compounds:

- Facilitate interaction between the organism and the surrounding environment;
- Play a role in invasion of higher organisms;
- Are involved in biofilm formation and the production of surface-active compounds by bacteria.

HOCl and HOBr disturb cell-to-cell communication in bacteria by reacting rapidly with the AHLs, leading to their selective deactivation (Borchardt *et al*, 2001). Thus, biofilm formation and fouling of the surface of the seaweed is inhibited. In other words, the peroxidases act as a natural defence mechanism to prevent biofouling on the surface of marine macro-algae.

Ekdal *et al* (1998) studied release of bromoform and other halocarbons ($CHClBr_2$, CH_2Br_2, C_4H_9I, etc.) from macro-algae in a rock pool, as a function of the time of day. This release was found to present a sharp maximum around mid-day. Interestingly, the concentration of H_2O_2 in the seawater also reached a maximum as the same time as $CHBr_3$, suggesting a relationship between photosynthesis, H_2O_2 and halocarbons. However, it is not known why a macro-alga should excrete H_2O_2 to the environment.

Dyrssen and Fogelqvist (1981) measured bromoform concentrations in seawater along a northerly transect starting from the north coast of Spitzbergen and proceeding over about

1.5 degrees latitude. These authors found that the surface concentration of $CHBr_3$ fell off from about 90 ng/L to 10 ng/L along the transect, providing support for the postulate that $CHBr_3$ is produced mainly in the coastal algal belt. The bromoform concentration was also shown to fall off sharply with water depth, the surface waters being supersaturated and hence providing a net flux of $CHBr_3$ to the atmosphere. Most of the various species of macro-algae growing in the studied region are known to contain bromoperoxidases and to release volatile organohalogens.

Certain vanadium haloperoxidases are also able to oxidise chloride, albeit at a slow rate (Soedak and Butler, 1991). Furthermore, some seaweeds may contain chloroperoxidases. However, any HOCl formed in seawater will rapidly react with bromide to give HOBr. Table 3 gives some rates of formation of chlorinated species by seaweeds, expressed as percentages of the rate of formation of bromoform.

Seaweed species	**Organochlorines**	**% of rate of $CHBr_3$ formation**	**Study**
Laminaria saccharina	$CHBr_2Cl$ $CHBrCl_2$ CH_2ICl	10 1 5	Laturnus (1996)
Laminaria digitata	$CHCl_3$	?	Nightingale (1995)
Mixed (field) population in Gran Canaria	$CHCl_3$ $CHBrCl_2$ CH_2ICl $CHBr_2Cl$	5 5 2 5	Ekdal *et al* (1998)
Meristellia gelidium	$CHBrCl_2$ $CHBr_2Cl$	5 10	Collen (1994)
Enteromorpha ahlneriana	$CHCl_3$	100% or more	Abrahamsson (2003)

Table 3

5.4 Halomethane formation and atmospheric processes

It has been shown by Barrie *et al* (1988) that there is a correlation between tropospheric ozone depletion at polar sunrise in the Arctic and the levels of certain halomethanes. In this context, Wever (1988) postulated that these halomethanes were produced by (macro)-algae in the Arctic Ocean at polar sunrise and that vanadium haloperoxidases were involved.

The ozone depletion is caused by catalytic cycles involving bromine atoms, produced by tropospheric photolysis of the organobromines.

5.5 Vanadium chloroperoxidases

Wever and co-workers discovered in 1992 the existence of vanadium chloroperoxidases, capable of oxidising chloride to hypochlorous acid:

$$H_2O_2 + H^+ + Cl^- + \text{enzyme} \rightarrow H_2O + HOCl$$

These enzymes were detected in a broad variety of fungi, the so-called “dematiaceous hyphomycetes”. They also have vanadate in the active site.

The pH optimum is around 5. The turnover is rather low (22 s^{-1}) and the K_m value for Cl^- at pH 5 is about 1 mM. However, the affinity for bromide is higher (K_m ~ 10 μM).

The mechanism of action involves reaction with hydrogen peroxide to given an enzyme peroxo intermediate which then picks up a chloride ion and a proton to give an enzyme-HOCl complex that subsequently liberates HOCl:

$$E + H_2O_2 \rightarrow E.H_2O_2$$
$$E.H_2O_2 + Cl^- + H^+ \rightarrow E.HOCl + H_2O$$
$$E.HOCl \rightarrow E + HOCl$$

X-ray studies demonstrate the formation of the E.H_2O_2 complex, in which the peroxide is bound side-on in the active site. This makes the peroxide oxygen more susceptible to nucleophilic attack by chloride.

Dematiaceous hyphomycetes known to contain vanadium chloroperoxidases are: *Curvularia inaequalis*, *Curvularia verrucolosa*, *Drechlera biseptata*, *Drechlera subpapendorfii*, *Ulocladium chartarum* and *Embellisia didymospora*. These are very common fungi and they belong to a family that are plant pathogens and/or are saprophytes. Other members of the family (which are known to have exhibit haloperoxidase activity, but have not necessarily been shown to contain vanadium haloperoxidases) are listed in Table 4.

Cladosporum fulvum	Causes serious plant disease in tomatoes
Helmintosporium	Parasite on leaves of grass
Alternaria solani	Causes destructive disease ("early blight") in tomatoes and potatoes
Curvularia blight	Causes thinning out and decline of grass - Primarily a stress pathogen that attacks plants stressed by drought or heat

Table 4

Question from D. van Wijk: Do any of these fungi occur in the marine environment?

Response from R. Wever: The sample of Embellisia comes from the Adriatic Sea. The others are from the terrestrial environment, or from salt marshes.

Some hypotheses may be proposed regarding the biological function of the chloroperoxidases in these pathogenic fungi, based on the following observations:

- The enzyme is secreted in the medium and/or found on the surface of the growing hyphal tips;
- In fact, it can be readily extracted in an active form by treatment of the fungus with detergent;
- These enzymes produce HOCl;
- HOCl by itself is a strong oxidising agent able to damage the lignocellulose of the cell wall;
- When nutrients are available and the enzyme is not needed, the transcription is toned down.

Wever and co-workers therefore propose that the function of the enzymes is to oxidise and degrade the cell walls of the host plants, in order to allow penetration of the fungus into the plant cell.

Question from G. Öberg: In the Swedish "litterbag" studies, when the plant is fertilised, there is a smaller increase in net organic chlorine content than for the unfertilised plant. One may wonder if this is because mineralisation is increased, or formation decreased. You are suggesting that it is the latter.

Response from R. Wever: Yes.

Comment from J. Field: In lignin degradation, it is known that as you increase the nutrient supply, smaller amounts of oxidative enzymes are produced. So, by analogy, one might postulate that the extra-cellular vanadium peroxidase is also induced only under nutrient-limiting conditions. However, in general, biodegradation is more rapid when nutrients are increased, so there are two conflicting trends.

Comment from R. Wever: In this particular case, we are certain that when the fungi are grown without nutrient limitation, they produce the enzymes only at the very end of their growth. This is one reason why it took so long before these enzymes were detected, particularly so because the fungi grow slowly.

Comment from J. Field: Although you have referred to lignocellulose, the pathogenic fungi attack the leaves where there is little lignin, but mainly chitin and pectin. There may be lignin in the stem, but not on the upper cuticle of the leaf.

Response from R. Wever: The main point is that HOCl is a strong oxidising agent that will help to degrade whatever is there, enabling the fungus to penetrate into the cell.

Comment from K. van Ginkel: If you add urea as a nutrient, it destroys hypochlorite easily, so it would act as a scavenger for HOCl.

5.6 Fate of the HOCl formed in the terrestrial environment

So far, there has only been a single study on the interaction of fungal vanadium chloroperoxidases, as well as the heme chloroperoxidase from *Caldariomyces fumago*, with lignin structures (Hammel and co-workers, 2003). These researchers showed that a model lignin dimer was chlorinated in the aromatic rings. It should however be noted that the heme peroxidase has a pH optimum of about 3, compared to 5-6 for the vanadium enzyme. Furthermore, heme peroxidases are easily inactivated by excess H_2O_2 (> 0.2 mM), in a similar way to lignin peroxidases and contrary to the vanadium enzymes that can be exposed to 0.5 M H_2O_2.

Question from H.F. Schöler: How does the heme peroxidase function, if it is destroyed by H_2O_2, which is necessary for the chlorination reaction?

Response from R. Wever: The conditions need to be adjusted carefully, to maintain low concentrations of H_2O_2.

Comment from G. Öberg: The purchased chloroperoxidase exists as a "conglomerate", before purification, consisting of peroxidase, catalase and glucosidase. There is internal production of H_2O_2 at very low concentrations, in the natural system. If experiments are performed even at 0.1 mM H_2O_2, the enzymes are denatured.

Question from G. Öberg: Do the vanadium peroxidases exist as conglomerates, in the organism?

Response from R. Wever: As far as we can tell, production is extra-cellular and there is only one enzyme.

Question from G. Öberg: So where does the H_2O_2 come from?

Response from R. Wever: If a plant is attacked by fungi, the host responds by producing H_2O_2, together with superoxide radicals, so the plant kills itself.

Question from G. Öberg: Does a stressed root also excrete H_2O_2?

Response from J. Field: When white-rot fungi are attacked by a colony of bacteria, they start producing H_2O_2.

The work of Hammel and co-workers (2003) also shows that synthetic lignins are depolymerised in the presence of H_2O_2, Cl^- and the enzymes studied.
This supports the conclusion of Wever and co-workers that fungal chloroperoxidases are potential chlorinators of lignin in plant debris and soil and thus may account for some of the high molecular weight organochlorines that occur naturally.

These chloroperoxidases have indeed been shown to be present in soil:

- Asplund *et al* (1993) reported the presence of a peroxidase in soil with properties similar to heme chloroperoxidase, and capable of chlorinating organic compounds;
- Laturnus *et al* (1995) observed high chlorinating/brominating activity in several spruce forest soil extracts (Klosterhede, Denmark). The reported pH optimum was 2.5-4, suggesting the presence of a heme chloroperoxidase (but other chloroperoxidases may be present).

The production of organochlorines in soil has been demonstrated in a number of studies, including those of:

- Flodin *et al* (1997), who showed that chlorinated aromatic structures (apparently "humic-like") are present in organic matter derived from different types of decaying plants and soil;
- Niedan *et al* (2000), who demonstrated that when fulvic acid can be chlorinated by heme chloroperoxidase. Apart from chlorination of the fulvic structures, production of di- and trichloroacetic acid was also observed;
- Hoekstra *et al* (1995), who showed that humic acids reacted in the presence of heme chloroperoxidase, H_2O_2 and chloride, to give chloroform as the main product, together with trichloroacetic acid and other chlorinated acids as minor products;
- Hoekstra *et al* (1998), who demonstrated the production of trihalomethanes by soil top layers.

Therefore, one may conclude that haloperoxidases are a major cause of natural halogenation processes in soils.

Comment from H.F. Schöler: Trichloroacetic acid (TCA) and chloroform are "co-products". To convert TCA to $CHCl_3$ requires heating (to 60°C) or an alkaline medium.

Response from R. Wever: Some soils are alkaline, and temperatures of 60°C are not to be excluded in nature.

Comment from R. Peters: Both TCA and chloroform are formed from a common precursor, with $CHCl_3$ being the major product.

5.7 Release of chloroform from soils

There have been various estimates of chloroform release from natural systems, shown in Table 5.

Source	**Amount (tons/year)**	**Study**
Northern temperature forests	4900 (400-24000)	Haselman *et al* (2000)
Global oceans	340000	Khalil *et al* (1999)
Macro-algae	800	Nightingale *et al* (1995)
Micro-algae	23000	Scarret and Moore (1999)
Peat land	4700	Dimmer *et al* (2001)
Rice paddies	23000	Khalil *et al* (1998)
Anthropogenic (pulp and paper, water treatment)	71000	Laturnus *et al* (2002)

Table 5

The release of biogenic chloroform can be seen to be larger than anthropogenic sources, and the terrestrial environment is an important contributor to the global atmospheric input.

Comment by G. Öberg: We have studied rice paddies and do not find any non-volatile organochlorines (other than the pesticides used). This seems to be contradictory to the observation of $CHCl_3$ formation by Khalil et al.

5.8 Conclusions

- The role of vanadium bromoperoxidases in the formation of bromoform in the marine environment is obvious and relatively well documented;
- However, despite this, hardly any research has been done that establishes the physiological function of the vanadium chloroperoxidases and their possible involvement in the formation of halomethanes;

- There are many terrestrial and marine fungi in the world and it is likely that the terrestrial hyphomycetes, in particular, contain chloroperoxidases with interesting properties;
- Also, seaweeds may contain chloroperoxidases that are responsible for the formation of chloroform in the marine environment (however, this is difficult to understand, since HOCl will immediately be converted to HOBr in seawater).

5.9 Addendum - Formation of methyl chloride

Methyl chloride formation was not considered in the main body of the presentation, since it is generally agreed that it is not produced by a peroxidase-mediated reaction; cf. work by Harper (1985) on wood-rotting fungi which produce CH_3Cl as a secondary metabolite, and studies on plants by Wuosmaa and Hager (1990).
A methylating enzyme, methyl transferase, is involved. It transfers a methyl group from S-adenosyl methionine to chloride (White, 1982).
Keppler *et al* (2000) showed that it is also produced abiotically from organic matter in soil by oxidation involving Fe(III) as an electron acceptor.
In the oceans it has been suggested (Zafiriou, 1975) that methyl chloride is produced by nucleophilic attack of Cl^- on CH_3I, but this mechanism was not supported by Singh *et al* (1983). It is known that in the oceans there is a close correlation between CH_3Cl and CH_3Br concentrations, suggesting a common source (micro-algae?). However, there is no correlation between CH_3I and either CH_3Br or CH_3Cl.

5.10 Discussion

Comment from J. Field: You have an interesting model in which plants are attacked by pathogens, via a model involving chloroperoxidases. However, a better model would involve attack not of lignin, but of phenolics such as cathechins or tannins. It would be interesting to do experimental work on this.

Comment from R. Wever: Note that these vanadium enzymes are very specific for halides, but they do not oxidise organic compounds themselves, unlike heme chloroperoxidases which oxidise many kinds of organics in a 1-electron step. So there is a clear difference between the different classes of compounds.

Comment from G. Öberg: Work by the Myneni group seems to show that there is formation of organohalogens on the leaves before they become litter, and our Klosterhede studies show that there is as much organochlorine in through fall as in the litter itself. This might be a quite mobile fraction that moves rapidly through soil.

Comment from R. Wever: When the leaves fall to the ground, the vanadium enzyme will still persist and not be easily inactivated. So, in the presence of H_2O_2, it will be able to cause chlorination of lignolytic compounds.

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6 The chloro-oxo acid cycle in soil?

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6.1 Introduction

K. van Ginkel stated that the objective of the presentation was to review current knowledge concerning the microbial reduction of (per)chlorate, focussing on the possible existence of a chloro-oxo acid cycle in terrestrial ecosystems. The question mark in the title meant that the speaker was not sure if such a cycle really exists in soil.

The work to be presented was performed in the Akzo-Nobel laboratories about 7 years ago. The conclusions from more recent literature would however also be included in the talk.

The work to be presented on the purification of enzymes was performed in the microbiology laboratory at the University of Wageningen.

The terminology “chloro-oxo acids” was meant to denote oxidised forms of chloride, as listed in Table 6.

Formula	Name	Oxidation state	Stability in water	Primary applications
Cl^-	chloride	-1	stable	
ClO^-	hypochlorite	+1	unstable*	biocide (bleaching of pulp and paper in past)
ClO^{2-}	chlorite	+3	unstable*	
ClO^{3-}	chlorate	+5	stable	bleaching of pulp and paper (as ClO_2)
ClO^{4-}	perchlorate	+7	stable	rocket fuel (US)

Table 6

* Chlorite and especially hypochlorite react with many reducing species and disproportionate under environmental conditions

6.2 Microbial reduction

The preliminary experiments consisted of incubating a mixed microbial culture (activated sludge) in the presence of chlorate, together with a limiting amount of sodium acetate, under anaerobic conditions.

It is important to point out that the system is anaerobic, since the bacteria involved prefer oxygen as an electron acceptor.

With a very minor amount of bacteria, the reduction of chlorate to a stoichiometric amount of chloride was observed after about a week, but the reaction stopped when all the acetate (reducing agent) had been consumed.

This type of experiment can be performed with a broad range of naturally occurring substances, acting as energy substrates for (per)chlorate-respiring micro-organisms (van Ginkel *et al*, 1995; Coates *et al*, 2001). These substrates may be either inorganic or organic, as listed in Table 7.

Inorganic compounds	hydrogen gas, hydrogen sulphide
Carboxylates	acetate, butyrate, lactate, palmitate, propionate, succinate, malate
Alcohols	ethanol, 1-propanol, 2-propanol, 1-butanol
Amino acids	proline, alanine, leucine, aspartate
Aromatic compounds	benzene, phenol
Polymers	starch, cellulose

Table 7

The first papers on microbial chlorate reduction appeared in the literature in the 1950s. Then, in the 1960s, Russian authors isolated a bacterium on perchlorate. Still later, a bacterium was isolated by Swedish scientists during studies on pulp bleaching.

The Akzo-Nobel researchers isolated a strain they called “GR-1”, a Gram-negative, oxidase-positive motile rod, belonging to the β-subdivision of the *Proteobacteria*, as demonstrated by rDNA sequencing This isolate was able to respire not only chlorate and perchlorate, but also oxygen, nitrate and Mn(IV). GR-1 grows on acetate, propionate, caprionate, malate, succinate and lactate.

Related *Proteobacteria* present minor differences: one strain can only use chlorate and not perchlorate, while another cannot use nitrate.

Experiments were performed on the formation of chloride from either perchlorate or chlorate, using a washed whole-cell suspension of GR-1. In both cases, the reduction was found to proceed much more rapidly in the presence of acetate than in its absence, showing that the bacterium derives energy from the acetate.

In the case of chlorite, the activity was very great compared to that observed with (per)chlorate, but there was no increase in activity when acetate was added. When the oxygen concentration was monitored in the head-space above a medium containing GR-1, a consumption of O_2 was observed when acetate was added, and an immediate evolution of O_2 was noted when chlorite was injected. The release of O_2 was proportional to the amount of chlorite added. Once all the chlorite had been consumed, the endogenous respiration or substrate respiration continued.

If the activities in the whole cells are compared, then the slowest (rate-limiting) reaction is the perchlorate reduction. Chlorite dismutation is by far the fastest, followed by O_2 consumption. Although these bacteria produce oxygen, the system remains anaerobic. The reaction sequence is illustrated in Figure 17.

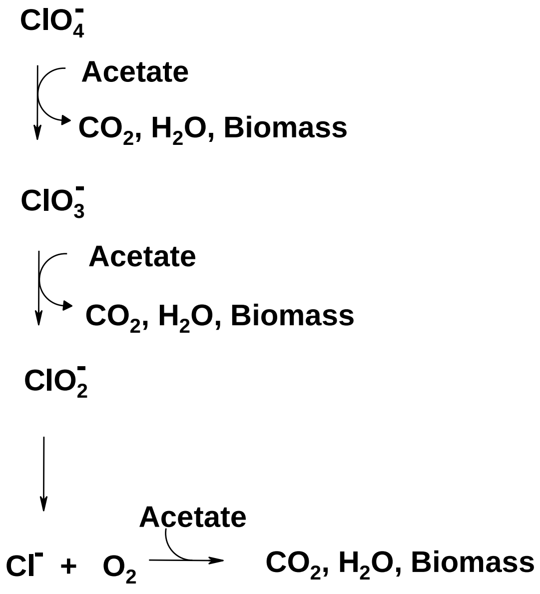

Figure 17

The production of toxic hypochlorite is prevented by the dismutation of chlorite. The O_2 formed is reduced very rapidly by acetate, so the whole system is kept anaerobic. This is essential, since the enzymes involved are inactivated by oxygen. The enzymes involved in the various steps are shown in Figure 18.

$\mathbf{ClO_4^-}$

$\downarrow$ **(per)chlorate reductase**

$\mathbf{ClO_3^-}$

$\downarrow$ **(per)chlorate reductase**

$\mathbf{ClO_2^-}$

$\downarrow$ **chlorite dismutase**

$\mathbf{Cl^- + O_2}$

Figure 18

6.3 Characteristics of the enzymes

A single enzyme in GR-1 catalyses the reduction of both perchlorate and chlorate (despite their structural difference). Some characteristics of this (per)chlorate reductase are:

- It is located in the periplasm;
- It has a molecular weight of 420 kDa, with sub-units of 95 and 40 kDa in an $\alpha_3\beta_3$ composition;
- It contains Fe-S clusters, together with Se and Mo.

The maximum specific activities of the reductase for perchlorate and chlorate are 3.7 and 13.2 U/mg, respectively.

More recently, a Swedish group has isolated and purified a chlorate reductase from *Ideonella dechloratans*, and the genes encoding the enzyme have been sequenced. The enzyme is composed of three different sub-units and contains Mo, Fe (probably in Fe-S clusters) and heme *b* (Danielsson-Thorell *et al*, 2003).

The second enzyme isolated by the Akzo-Nobel researchers in GR-1 is capable of catalysing the dismutation of chlorite into chloride and oxygen. This chlorite dismutase is a homo-tetramer composed of 32-kDa sub-units containing protoheme and iron. The activity is huge (V_{max} = 2200 U/mg, K_m = 170 µM). The maximum activity is found at pH 6.0 and 30°C.

The homo-tetrameric composition of chlorite dismutase is similar to that reported for enzymes capable of catalysing the disproportionation of hydrogen peroxide (catalases). However, most of the characteristics of chlorite dismutase distinguish it from other heme enzymes such as catalases and peroxidases. It is very specific to chlorite as a substrate.

6.4 Reduction of (per)chlorate in soils

(Per)chlorate-reducing micro-organisms are practically ubiquitous in rivers, ditches, soils, activated sludge plants and digesters (van Ginkel *et al*, 1995; Coates *et al*, 1999; Wu *et al*, 2001; Hunter, 2001).

Most probable number counts of acetate-oxidising (per)chlorate-reducing bacteria in soils have been published by Coates *et al* (1999) and Wu *et al* (2001), and are shown in Table 8.

Soil type	**Counts** (cells per g)
Pristine	$2.3 \pm 1.3 \times 10^3$
Petroleum-contaminated	$9.3 \pm 4.2 \times 10^3$
Perchlorate-contaminated	22-130

Table 8

The reduction of (per)chlorate:

- Will occur only if O_2 is practically absent: even low concentrations of dissolved oxygen (5% of saturation) will prevent induction of the reaction;

- Is also negligible until nitrate has been consumed. The mechanism involved is not clear;
- Is inhibited when molybdate is limiting. In acidic soils, molybdate is often the limiting nutrient (Chaudhuri *et al*, 2002).

Results were presented for experiments on reduction of chlorate in pristine clay and loam at ~ 50% of the maximum moisture capacity, in bottles open to the atmosphere. These experiments showed that the concentration of ClO_3^- fell by half within about 3 weeks.

Question from G. Öberg: If the moisture capacity is only 50%, then O_2 will be present, so will not the enzymes be inactivated?

Response from K. van Ginkel: There will still be anaerobic niches in the soil, where the reaction will occur. Denitrification in soil would be very minor if there were no such niches. The wetter the soil, the faster the reaction will be.

6.5 Analogy with denitrification

Denitrification consists of four sequential reactions. They are:

- Reduction of NO_3^- to NO_2^-, catalysed by the enzyme nitrate reductase:
- Reduction of NO_2^- to NO, catalysed by nitrite reductase;
- Reduction of NO to N_2O, catalysed by nitric oxide reductase;
- Reduction of N_2O to N_2, catalysed by nitrous oxide reductase.

Field measurements of denitrification losses to the atmosphere lead to values in the range 0.2-1.4 x 10^3 mol N/ha.year (Ottow and Benckiser, 1994). The potential of soils to reduce chlorate is 10^3 (10^4?) mol ClO_3^-/ha.year. This estimate is based on rates derived from the Akzo-Nobel experiments discussed above, assuming a soil top layer of 10 cm. Such high reaction rates do not actually occur, of course, because the chlorate is not there, generally speaking.

6.6 Anions of chloro-oxo acids in soil

It is generally assumed that anions of chloro-oxo aids are not formed naturally, apart from hypochlorite, which originates from the action of chloroperoxidases, as discussed above. However:

- The disproportionation of hypochlorite can lead to chlorate and chlorite;
- Deposition of oxidised halogen species from the troposphere may be a source;
- Perchlorate is present in Chile saltpetre and in a large number of wells (apparently not from pollution).

Comment from R. Wever: Although HOCl is produced in nature, it reacts so rapidly with any organic matter present that it is unlikely to lead to other chlorinated oxyacid species.

Response from K; van Ginkel: But what then is the purpose of the (per)chlorate-reducing bacteria in nature?

Comment from J. Field: Is it possible that there is another substrate that they can use, besides the chloro-oxo acids?

Response from K. van Ginkel: They can survive with oxygen and nitrate. But they have developed special enzymes for chloro-oxo acids.

Question from J. Field: But how many of these bacteria can only use chlorate and do not have any of the alternatives (nitrate, oxygen…)?

Response from K. van Ginkel: As far as I know, they always have the alternative of oxygen, because they have to work under anaerobic conditions and all the isolates studied contain chlorite dismutase.

Question from R. Wever: Is it not possible that the enzyme is using chloro-oxo acids “by coincidence”, that is normally another substrate is present, such as sulphate, so these are sulphate reducers?

Response from K. van Ginkel: I do not think so, since, while the activity of the perchlorate reductase is not as narrow as that of the chlorite dismutase, it is relatively narrow. However, it has been known for decades that chlorate can also be reduced by nitrite reductase.

The proposed chlorine cycle in the terrestrial environment is summarised in Figure 19, where the red arrows represent reactions catalysed by micro-organisms and the black arrows represent chemical disproportionation and reduction reactions.

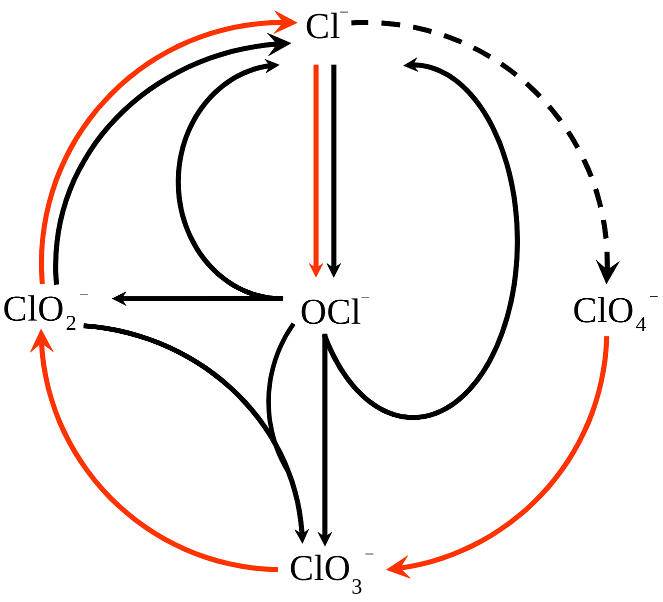

Figure 19

Comment from G. Öberg: There is a red arrow missing, for the conversion of chloride to hypochlorite by chloroperoxidases, which is probably a major pathway.

Response from K. van Ginkel: Yes, I agree.

Question from H.F. Schöler: Do you have any evidence for oxidised forms of chlorine in precipitation?

Response from K. van Ginkel: All I know is the data from Texas, on perchlorate in water wells, and in breast milk.

Comment from J. Franklin: Keene and co-workers have found some forms of oxidised chlorine (thought to be Cl_2 + HOCl) in precipitation, at levels of tens to hundreds of ppt.

6.7 Conclusions

- The reduction of perchlorate and chlorate to chloride is coupled to the oxidation of many naturally occurring compounds by (per)chlorate reducing bacteria;
- Chlorite, an intermediate, disproportionates into molecular oxygen and chloride;
- The (per)chlorate-reducing strain GR-1 utilises the oxygen generated by the dismutation reaction to prevent inhibition of the (per)chlorate reduction;
- Absence of oxygen and “nitrate” in the immediate vicinity of the bacteria are prerequisites for microbial reduction of (per)chlorate;
- (Per)chlorate-reducing micro-organisms are widespread in nature. Rapid growth-linked biodegradation is usually found with naturally occurring substances;
- The potential of terrestrial ecosystems to reduce (per)chlorate indicates a high turnover of chloro-oxo acids;
- An open question is: Are oxidised forms of chlorine formed naturally at high rates?
- Knowledge of a biogeochemical cycle is essential to evaluate the importance of anthropogenic versus natural sources of chloro-oxo acids.

6.8 References

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7 Final discussion

7.1 Introduction

J.-M. Libre suggested three major topics for discussion:

- How to assess source strengths for natural organochlorines. For atmospheric emission fluxes, it was noted that during the workshop a recommendation had been made to “piggy back” on existing E.U. or national programmes, which had already invested in expensive flux chambers and had the required expertise. A related item was how laboratory data could be extrapolated to environmental conditions;
- Specific data-gaps on the mechanisms of processes leading to natural organochlorines;
- Possible identification of “new” natural organochlorines (CCl_4 …?).

7.2 Discussion

D. van Wijk: The natural variability is an important issue to be taken into account when estimating fluxes, since it can lead to huge errors. However, I assume this is also true, for example, for the nitrogen cycle, which is now supposed to be well known and is given in all the textbooks. How much worse is our knowledge of the chlorine cycle?

G. Öberg: All depends on the scale of interest. I believe that a catchment of at least 1 km^2 *is of sufficient size to extrapolate to larger scales and to provide useful data for policy decisions, such as for the Water Framework Directive. When you look at the variation you should settle for flux measurements which enable flux and budget determinations on that scale. We can learn from the nitrogen and carbon cycles, for which investigations have been made over a range of scales, so we are not as bad off as researchers were for these cycles over 50 years ago, but we need some basic variation data.*

J. Field: What I would like to see is field experiments to verify mechanisms, such as adding isotopically-enriched chloride to the soil and observing in situ the products formed. Since abiotic mechanisms have now been shown to be important, one could also add an inhibitor of microbial activity to a small plot, in order to distinguish between the relative contributions of microbial and “diagenetic” processes to the formation of, say, chloroform.

G. Öberg: The catchment scale is appropriate for budget studies, but totally wrong if one wishes to focus on processes. Detailed studies both on specific organisms or on soil or litter are called for, and the use of radio-isotopes such as ^{36}Cl *would also be useful. A group at Imperial College London claims to have overcome problems with quenching of the* ^{36}Cl *signal in soil.*

H.F. Schöler: Studies in a small catchment might not be sufficient, since different climatic zones need to be investigated, to take into account the impact of environmental variables, such as humidity.

J. Field: In some locations, such as Hawaii, many different types of climate can be found within a very small area.

G. Öberg: One catchment study could be combined with a number of plot studies.

J.-M. Libre: What are the ongoing studies?

G. Öberg: We are doing a catchment study, on chloroform, TOC, AOX and chloride, in soil and water. This will be continuing until February 2005. We are not however measuring fluxes of chloroform and we are not monitoring changes in the soil. Nor are we studying through fall, litter fall or precipitation. I would like to extend the programme to other specific compounds, such as vinyl chloride in soil. Flux measurements might be best carried out in collaboration with an ongoing large

Swedish study (LUSTRA?) on the nitrogen cycle, at another catchment, where the equipment already exists. Patrick Crill, a newly appointed professor at Stockholm University, has been measuring bromoform and is starting on chloroform, in New Hampshire.

G. Öberg: More work is needed to characterise the soil enzymes responsible for chlorination.

R. Wever: Indeed. But one needs additionally to identify the organisms that excrete the enzymes and elucidate the physiological function of the enzymes. Also, the production of chloroform by seaweeds is still not understood and needs to be explored.

K. van Ginkel: Returning to the chlorine cycle, do we understand all the major reactions sufficiently? Even the chemistry of the nitrogen cycle has been revised recently, with the addition of the anaerobic ammonium oxidisers.

J. Field: The nitrogen cycle is basically simpler. There are more reactions involved in the chlorine cycle. We have to determine which ones are major processes, and which ones are only minor contributors to the global budget.

D. van Wijk: What is being done in this direction?

G. Öberg: We are attempting to produce a global chlorine budget for the terrestrial environment (see chapter entitled "The Biogeochemistry of Chlorine in Soil", in the Springer Handbook of Environmental Chemistry, Volume 3, Part P, pp. 43-62, 2003). Graedel and Keene (1995) have produced a global budget of reactive chlorine in the troposphere, which includes both terrestrial and marine contributions.

J. Field: For other cycles, we know which groups of organisms are associated with major parts of the cycles (nitrification, denitrification), but this type of information is basically lacking for the chlorine cycle. Also, it is possible that the abiotic processes are equally important or more important.

H.F. Schöler: In this respect, we observed that even commercial humic acid can produce trichloroacetic acid in the presence of H_2O_2.

G. Öberg: But one needs to be careful to use environmentally relevant conditions. High concentrations of H_2O_2 that do not exist in nature can destroy enzymes and hence mask the contribution of biotic processes.

J. Field (addressing H.F. Schöler): In your abiotic experiments, you only looked at certain specific products, rather than determining AOX, so there may have been at lot of other chlorinated products, particularly partially polymerised ones, that may well have escaped detection with the methods used.

H.F. Schöler: We intend to use HPLC-MS to look more closely into the chlorinated acids formed as intermediates.

J. Field: But what about the high molecular weight material?

H.F. Schöler: We can at least look at the beginning of the polymerisation process.

G. Öberg: You may have depolymerisation simultaneously with the chlorination process, since the organic matter becomes more mobile and moves with the water more readily than the non-chlorinated part.

J. Field: So we should look at molecular weight distributions.

H.F. Schöler: What is the concentration of hydrogen peroxide in soil?

G. Öberg: H_2O_2 is constantly being produced and consumed. It is very reactive and its concentration, though highly variable, is "close to zero".

J. Field: When two colonies of fungi are attacking each other, there may be high concentrations of H_2O_2 at the interface.

R. Wever: Seawater concentrations of H_2O_2 are well known. They are about 1-2 µM, and in areas of high biological activity about 2-10 µM. In aerobic systems, H_2O_2 is produced whenever there is biological activity.

J. Field: Forest soils are very heterogeneous. It does not make much sense to determine "average" concentrations. One should try to find out where H_2O_2 is concentrated locally.

H.F. Schöler: Does the OH radical play a role in soil?

R. Wever: The superoxide radical leads to OH in the presence of HOCl.

G. Öberg: Myneni has shown that there are "hotspots" of organic chlorine on leaves, which are heterogeneous. It would be interesting to look more closely at these, to determine which organisms or enzymes are concentrated there.

J. Franklin: On a more general level, the diversity of organochlorines in nature has been amply demonstrated (see, for example, the regular reviews on this subject by G. Gribble). What is now of greatest interest to Euro Chlor is the determination of production fluxes of those organochlorines that have both natural and anthropogenic sources, to put the latter into perspective when compared to the former.

J.-M. Libre: That is true, but it is nevertheless important to elucidate the mechanisms involved, if one wants to estimate global fluxes by extrapolating results from catchment studies to areas where environmental conditions are different.

J. Field: Which compounds are the ones Euro Chlor is most interested in, by order of priority?

D. van Wijk: Euro Chlor has recently published, for the first time, actual emissions of industrial organochlorines from production sites in Europe. Although this list of compounds is incomplete and is not prioritised, it gives a good indication of the compounds of interest.

J.-M. Libre: Euro Chlor is also interested in fate and persistence of these compounds in the environment and part of the work on mechanisms might be useful in this context.

R. Wever: What has not been discussed today is degradation by natural enzyme systems. A lot of work is being performed in this area.

G. Oberg: Another area of interest might be the relation between the chlorine cycle and other biogeochemical cycles (such as the carbon cycle).

D. van Wijk: Can one give an indication of the importance of the chlorine cycle, compared to the other cycles?

G. Öberg: I think that the chlorine cycle is related to the carbon cycle, and since the latter is related to other cycles, then the chlorine cycle is related to them all. But its role is not fully understood.

H.F. Schöler: An additional topic of interest could be the production of trichloroacetic acid on particles in the troposphere. This might seem far removed from what we have discussed at this workshop, but such particles contain organic carbon (30-35%), including humic acid (3-5%), so the chemistry might be similar to the reactions occurring in soil.

G. Öberg: Over the last 15 years, there has been a "paradigm shift" in thinking regarding the existence of natural organochlorines. Risk assessment of organic compounds is based on the notion that naturally produced ones are good, whereas anthropogenic ones are bad. This thinking is impregnating the whole legislation and risk assessment policy. The situation is different for radon or heavy metals, because we recognise that we have to cope with them. I would like to see research that is truly inter-disciplinary - involving good social scientists and good natural scientists that are used to working with each other - on the risk assessment of organic compounds, in particular organochlorines.

J.-M. Libre: That is good idea, but it goes beyond the Euro Chlor remit.

D. van Wijk: It would be useful for such thinking to be developed within the scientific community, but the question is, how could Euro Chlor use it with the regulatory community? It would not be appropriate for industry to invoke such arguments in the risk assessment of specific compounds. It is usually wrongly interpreted if industry argues that certain industrial compounds also have natural sources that contribute to the environmental burden.

G. Öberg: It is a question of communication between experts and policymakers. The environmental field is one where the influence of experts is the strongest. There are numerous studies on how the information can be provided to the policy system. Two-way communication is required.

7.3 Future meetings on natural organochlorines

In 1995, R. Wever organised a workshop in Amsterdam on halogenating and dehalogenating enzyme systems.

In 2001, H.F. Schöler organised a conference in Heidelberg on naturally produced organohalogens.

Both of these meetings received funding from Euro Chlor.

It would seem timely to envisage a follow-up conference. G. Öberg stated that she is considering organising such a meeting in 2005.

D. van Wijk pointed out that Euro Chlor is now a sustaining member of SETAC and is therefore entitled to organise a “special session” at SETAC meetings. Since SETAC is “risk assessment oriented”, this would be a good opportunity to facilitate such a session on natural organochlorines.

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Euro Chlor

The voice of the European chlorine industry, Euro Chlor plays a key communication and representation role on behalf of its members, listening and responding to society's concerns about the sustainability of chlorine chemistry.

Euro Chlor helps members improve safety standards whilst conducting science, advocacy and communications programmes. The Brussels-based federation was founded in its current form in 1989 and speaks on behalf of 97% of chlorine production in the EU-25 and EFTA regions.

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