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NATURAL PRODUCTION OF CHLOROFORM BY FUNGI

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Key Word Index—*Mycena metata*; *Peniophora pseudopini*; *Caldariomyces fumago*; basidiomycetes; deuteromycetes; natural production; chloroform.

Abstract—Chloroform production was detected in the headspace of pure cultures of the basidiomycetes *Mycena metata* and *Peniophora pseudopini* and the deuteromycete *Caldariomyces fumago*. The average production rates were in the range of 0.07–70 μg/l culture fluid/day for *Caldariomyces fumago* and 0.7–40 ng/l culture fluid/day for the basidiomycetes; they depended on the composition of the medium, pH and the initial concentration of oxygen. In incidental cases, chloroform was identified in the headspace of pure cultures of the basidiomycetes *Agaricus arvensis*, *Bjerkandera* sp. BOS55, and *Phellinus pini*. It is suggested that fungi are important sources of elevated concentrations of chloroform in soil air.

INTRODUCTION

The presence of elevated concentrations of chloroform in soil air compared to those in atmospheric air has been demonstrated several times. Frank *et al.* [1], Frank and Frank [2] and Hoekstra and De Leer [3] observed this phenomenon in soil air from coniferous and deciduous forests in Germany and The Netherlands and in wood chip piles and peat areas from The Netherlands. Kahlil *et al.* [4] found the same in soil air at a distance of 1–10 m from termite mounds in Australia.

There is one known pathway which can be important for the natural formation of chloroform in soil. It involves the natural formation of reactive chlorine species, such as hypochlorous acid, from chloride and hydrogen peroxide by a chloroperoxidase (CPO)-mediated reaction [5]. Next, the reactive chlorine species chlorinate the organic material in soil in a non-specific way. Walter and Ballschmiter [6] found the formation of chloroform from simple organic compounds such as acetone, propionic acid and citric acid but not from D(+)glucose. Hoekstra and de Leer [7] demonstrated the formation of chloroform from humic acids, which was also found in water disinfection by chlorine [8]. In this study, CPO originated from *Caldariomyces fumago*.

De Jong *et al.* [9, 10] demonstrated the *de novo* production of specific chlorinated aromatic compounds from glucose and chloride by basidiomycetous fungi. It is still unknown which enzyme(s) is (are) responsible for the specific chlorination reactions involved.

The natural biosynthesis of chlorinated metabolites as observed by De Jong *et al.* [9, 10] and the mechanism involving the natural formation of reactive chlorine species seem to be different. Some chlorinating enzymes may not be able to form reactive chlorine species but produce an activated enzyme-containing complex instead, which can accelerate specific chlorination reactions inside or outside the cell [5].

CPO activity has been observed in several soil extracts [11, 12]. Hoekstra *et al.* [13] showed that ^{37}Cl-chloroform was formed in different soils which were spiked with an aqueous solution of $Na^{37}Cl$. The question remains as to which organisms were responsible for the chloroform production in soil. Up to now, only Pyysalo [14] mentioned the identification of chloroform in the pressed juice from the fruiting bodies of *Cantharellus cibarius*. However, in this study it does not become clear whether chloroform was produced or taken up by *Cantharellus cibarius*.

In the present study, we looked at the formation of

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chloroform by several basidiomycetes and the deuteromycete *Caldariomyces fumago*. The basidiomycetes were chosen because of their known production of significant amounts of organohalogens [15, 16] and *Caldariomyces fumago* was chosen because of its known production of CPO.

RESULTS

The fungi were grown on glucose, soil-glucose mixtures and soil. The soil originated from the litter layer of a forest and had a pH of 3.0–5.5. The initial concentration of oxygen in the cultures was 21% or 75% vol.

Caldariomyces fumago

Figure 1 and Table 1 show the average cumulative amounts of chloroform in the headspace of glucose cultures. The average production rate was about 70 μg/l culture fluid/day during the first 10 days, while it decreased to about 20 μg/l of culture/day after 10 days. In 40 days 5000–8000 ng chloroform per culture was produced.

Figure 2 shows the average cumulative production of chloroform in the headspace of soil cultures. The average production rate was about 0.07 μg/l culture fluid/day or 0.2 ng/g soil/day, which was significantly lower than with the glucose cultures. In 40 days 8–20 ng chloroform per culture was produced.

Peniophora pseudopini

Figure 3 and Table 1 show the average cumulative amounts of chloroform in the glucose and the soil-glucose cultures. The average production rate of the glucose cultures with an initial concentration of oxygen of 75% was about 40 ng/l culture fluid/day, while those of both glucose and soil-glucose cultures with an initial concentration of oxygen of 21% was about 8 ng/l culture fluid/day.

It appears from Fig. 3 that the total amount of chloroform produced in glucose and soil-glucose cultures with an initial concentration of oxygen of 21% were in the same range of about 0.7–4 ng/culture and 1–2 ng/culture, respectively. The total amount of chloroform produced by *Peniophora pseudopini* in the glucose medium with an initial concentration of oxygen of 75% cannot be read from Fig. 3, because the production was not yet finished after 28 days of incubation. It is clear from Fig. 3 that the concentration of oxygen distinctly influenced the total produced amount of chloroform.

Peniophora pseudopini grown on soil only, also produced chloroform but less than in the case of the glucose medium. The total produced amount of chloroform in the soil cultures was about 0.5 ng/culture after 35 days of incubation. This was somewhat lower than the total amount of chloroform than was produced in the soil-glucose cultures, i.e. 1–2 ng/culture.

Mycena metata

The average cumulative amounts of chloroform produced by *Mycena metata* in cultures with an initial concentration of oxygen of 75% are shown in Fig. 4 and Table 1. The average production rate of chloroform was about 0.02 μg/l culture fluid/day. Or, in other words, under the same conditions, *Mycena metata* produced lower amounts of chloroform than *Peniophora pseudopini*.

In incidental cases, chloroform was detected in the headspace of glucose and soil-glucose cultures of *Mycena metata* with an initial concentration of oxygen of 21% in amounts of 0.2–1 ng/culture after 20 days of incubation.

Other fungi

Agaricus arvensis produced 0.11–0.38 ng of chloroform in the headspace of glucose cultures with an initial concentration of oxygen of 75% after the first 3 days and 0.14–0.19 ng in the headspace of soil cultures with an initial concentration of oxygen of 21%. In some glucose and soil-glucose cultures with an initial concentration of oxygen of 21% *Bjerkandera* sp. BOS55 and *Phellinus pini* produced 0.67 ng and 0.44–9.5 ng of chloroform, respectively, after the first 14 days of incubation. *Hypholoma fasciculare* did not produce detectable amounts of chloroform in the headspace of glucose and soil-glucose cultures with an initial concentration of oxygen of 21% although it is known to produce significant amounts of organohalogens (AOX) [16].

DISCUSSION

The results of this study demonstrate that chloroform is produced *de novo* by *Caldariomyces fumago*, *Peniophora pseudopini* and *Mycena metata*. Addition of soil to the glucose medium did not enhance the chloroform production by *Peniophora pseudopini* and the total cumulative amount of chloroform produced in the soil culture was lower than that in the soil-glucose culture. These observations suggest that *Peniophora pseudopini* produced chloroform mainly from *de novo* biosynthesis. Chloroform by *de novo* production is possibly a minor side product of the chlorinating enzymes of the basidiomycetes because the amounts of undefined AOX and chlorinated anisyl metabolites (CAM) reported by Field *et al.* [15] and Verhagen *et al.* [16] are produced in 45–660 fold higher amounts. However, the ratio of the concentration of AOX and chloroform and the ratio of the concentration of CAM and chloroform in Dutch forest soils are much higher and are estimated to be in the range of 10^6–10^8 [3] and 2000–$20*10^6$ [3, 9], respectively. The growing medium and environmental con-

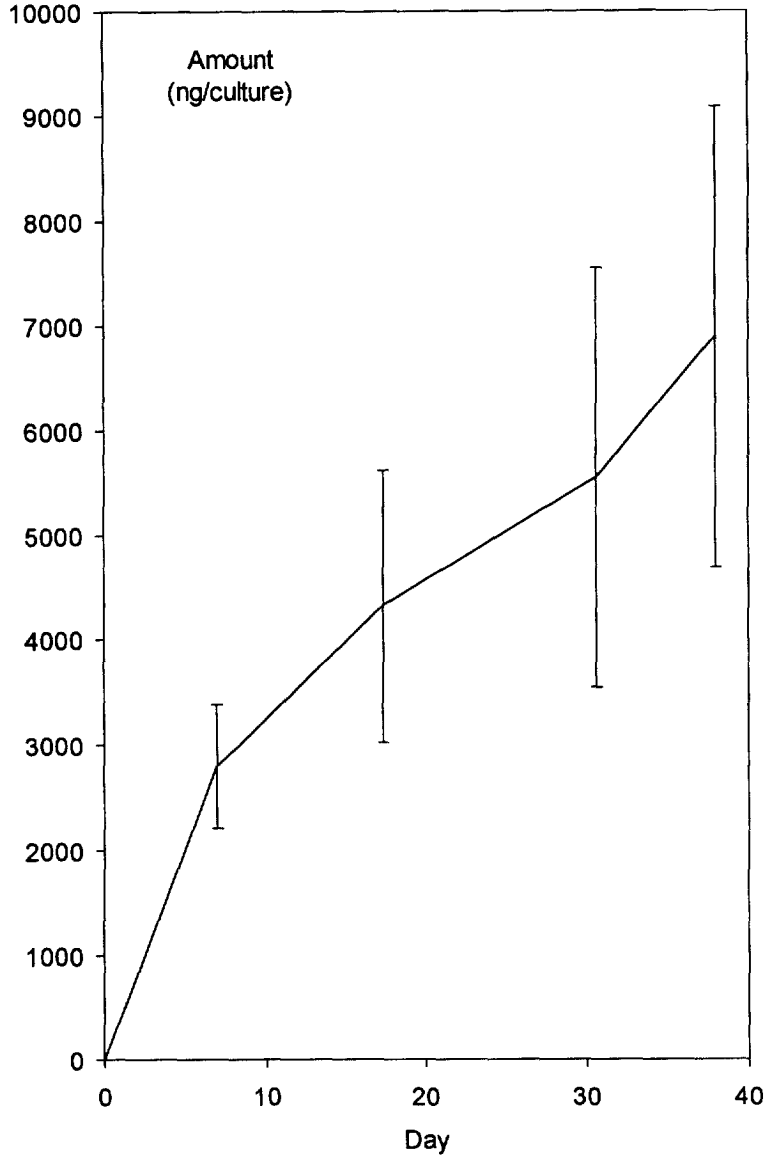

Fig. 1. Average cumulative amounts of chloroform in the headspace of glucose cultures of *Caldariomyces fumago* and their standard deviations.

Table 1. Average cumulative amounts of chloroform (ng/culture) after 4–5 weeks of incubation

Substrate: Initial O_2 (%):	Glucose 75	Glucose 21	Soil-glucose 21	Soil 21
Caldariomyces fumago Woron.	n.t.*	7000 (0.5)	n.t.	10 (0.5)
Peniophora pseudopini Wer. & Gibs.	7 (0.4)†	2 (0.3)	2 (0.1)	0.5 (0.08)
Mycena metata (Fr.) Kummer	2 (0.4)	0.7 (0.1)‡	0.7 (0.05)§	n.d.
Agaricus arvensis Schaeff.: Fr.	0.3 (0.05)§	n.t.	n.t.	0.2 (0.04)§
Bjerkandera sp. BOS55 (Willd.: Fr.) Karst.	n.t.	n.d.*	0.7 (0.05)‡	n.t.
Phellinus pini Br.: Fr.	n.t.	0.3 (0.1)‡	5 (0.05)§	n.t.

* n.d. not detected; n.t. not tested
† number between brackets, the cumulative amount of chloroform in blank
‡ result obtained from one culture
§ result obtained from two cultures

Table 2. Average production rates of chloroform (ng/l culture fluid/day)

Substrate: Initial O_2 (%):	Glucose 75	Glucose 21	Soil-glucose 21	Soil 21
Caldariomyces fumago Woron.		70,000		70
Peniophora pseudopini Wer. & Gibs.	40	8	8	3
Mycena metata (Fr.) Kummer	20	6*	6*	
Agaricus arvensis Schaeff.: Fr.	20†			0.7†
Bjerkandera sp. BOS55 (Willd.: Fr.) Karst.			6*	
Phellinus pini Br.: Fr.		5*	40†	

* result obtained from one culture
† result obtained from two cultures

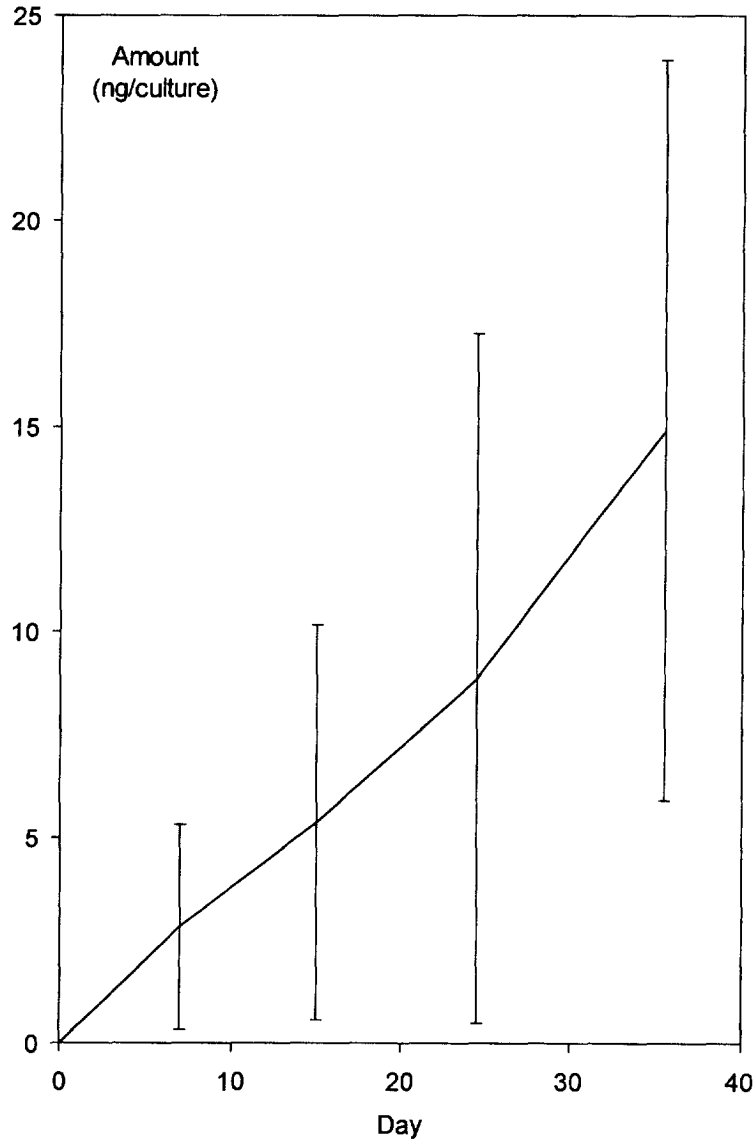

Fig. 2. Average cumulative amounts of chloroform in the headspace of soil cultures of *Caldariomyces fumago* and their standard deviations.

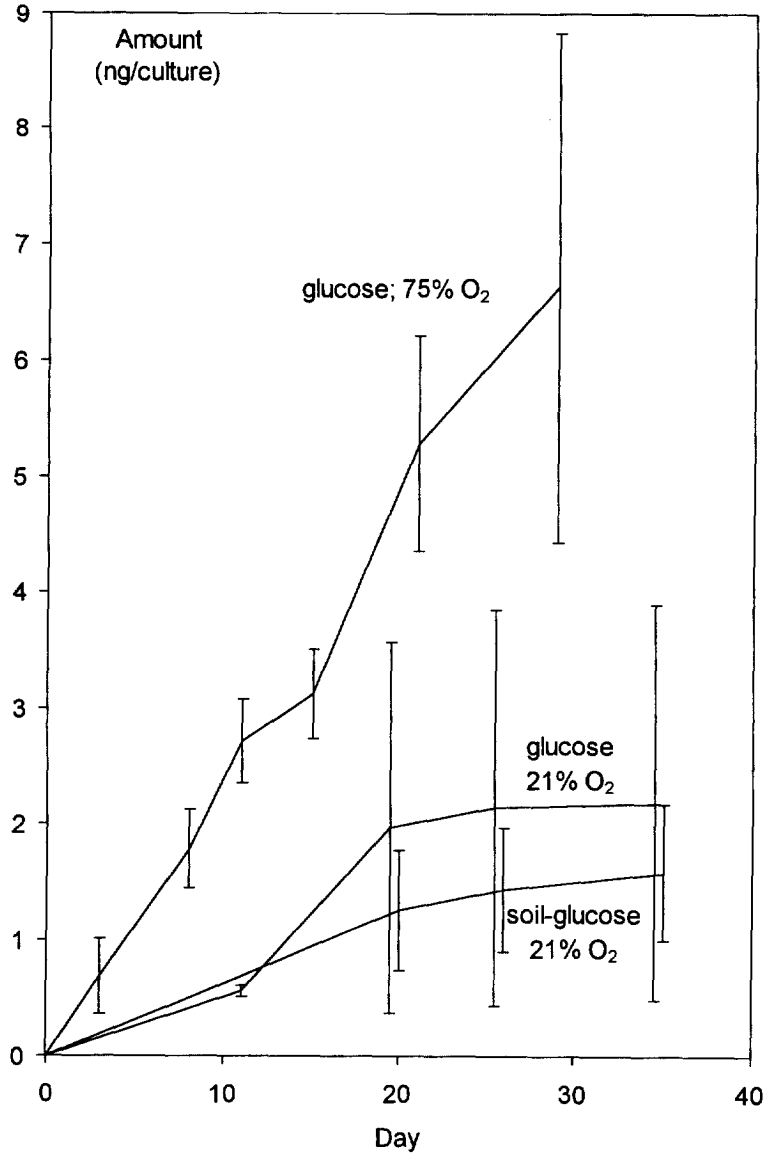

Fig. 3. Average cumulative amounts of chloroform in the headspace of glucose and soil-glucose cultures of *Peniophora pseudopini* and their standard deviations.

ditions will mainly contribute to the large difference of the ratio of AOX and chloroform and the ratio of CAM and chloroform observed in the laboratory and the field.

Caldariomyces fumago produced significantly more chloroform in the soil cultures than the basidiomycetes which suggests the influence of CPO. However, the higher product rate of chloroform in glucose cultures compared to that in soil cultures shows that we only have clear evidence that *Caldariomyces fumago* produces chloroform via *de novo* biosynthesis. The role of extra-cellular CPO in the formation of chloroform is not clear yet.

Fungi are probably responsible for the presence of chloroform in soil air, although they are perhaps not the only natural source in soil, since the production of chloroform by other (micro)organisms cannot be excluded. However, if we extrapolate the average production rates of chloroform in the soil cultures by the basidiomycetes (2–6 ng/kg soil/day) and *Caldariomyces fumago* (200 ng/kg soil/day) and if we assume production rates of the tested fungi to be representative for all the fungi that produce chloroform in soil, a soil density of 1 kg/l and the fungi to be present in the upper 10 cm of the soil layer, we can calculate an emission rate of chloroform from soil in the range of 8–25 ng/m^2/h for the basidiomycetes and 800 ng/m^2/h for *Caldariomyces fumago*. The calculated emission range is in the same order of magnitude as we measured in a grass area, a Douglas and

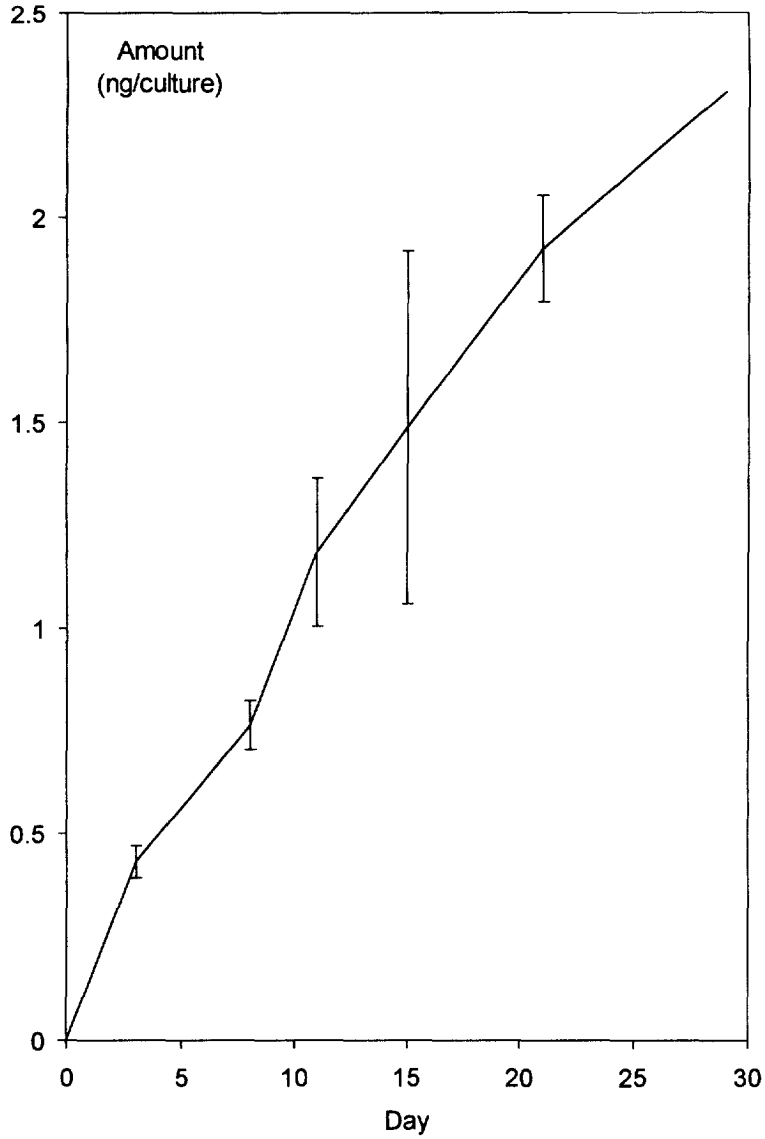

Fig. 4. Average cumulative amounts of chloroform in the headspace of glucose cultures of *Mycena metata* with an initial concentration of oxygen of 75% and their standard deviations.

beech forest and wood chip piles in The Netherlands, i.e. 10–1300 ng/m^2/h [13]. The presence of chloroform in soil air is therefore probably due to the presence of *Caldariomyces fumago*-like fungi rather than basidiomycetes. The heterogeneity of the soil air concentrations and emissions from one type of soil which was found to be more than 10-fold, can probably be explained by the uneven distribution of different fungi species and/or their biomass in the soil.

EXPERIMENTAL

Cultures

The glucose medium consisted of D(+)glucose (20 g/l), mycological peptone (5 g/l), yeast extract (2 g/l), KH_2PO_4 (1 g/l), $MgSO_4 \cdot 7H_2O$ (0.5 g/l) and NaCl (0.06 g/l). The pH of the glucose medium was 5.5. The mycological peptone solution was sterilised separately. Sterilised medium (6 ml) was put into a sterilised erlenmeyer (100 ml) and the erlenmeyers were closed with teflon-lined rubber septa.

The humic layer which was obtained from a soil of a beech forest in Speulderbos (Speuld, The Netherlands) was dried (105°; 24 h) and ground in a mortar. The organic matter content was 95% and the pH was 3. The soil-glucose cultures consisted of 2–3 g of soil which was put into an erlenmeyer and was sterilised two times for 30 min with an interval of 24 h. Glucose medium (2 ml) as described above but without mycological peptone was added to the soil. The soil cultures consisted of 5 g of soil and 5 ml of water.

The glucose medium, the soil-glucose and the soil cultures were inoculated with 5 mm^2 plugs which were taken from an agar medium overgrown with mycelium of *Agaricus arvensis* (Wag93.1), *Bjerkandera* sp. BOS55, *Mycena metata* (Rhen93.1), *Hypholoma fasciculare* (Rhen93.5), *Peniophora pseudopini* (CBS 162.5) and *Phellinus pini* (628(B13)). The blank experiments were inoculated with sterile agar plugs. All cultures were incubated in triplicate and the blank experiments in duplicate at 20° in the dark.

Caldariomyces fumago (IMI 089362, new name *Leptoxyphium fumago*) grew on the glucose medium as described above, but at a pH of about 4.5, and on soil of the first 3 cm below the humic layer which was obtained from a Douglas forest in Speulderbos (Speuld, The Netherlands). The organic matter content was 10% and the pH was 3. Soil (5 g) was put into an erlenmeyer and was sterilised with 7 ml of water twice for 30 min with an interval of 24 h.

Sampling and analysis

The cultures and the blanks were sampled every 6 to 10 days. The headspace of a bottle was flushed by 300 ml of sterile air (50 ml/min) and trapped on an adsorbent tube which was filled with CarboPack B. The adsorbent tube was analysed by on-line automatic thermal desorption and GC-MS. The compounds in the adsorbent tube were de-sorbed by He gas at 300° for 10 min and they were trapped at −100° in a cold trap containing Tenax-GR. The compounds were injected into the GC by heating the cold trap to 300° for 5 min. The temperatures of the valve and the transfer line to the GC were 180° and 200°, respectively. The pressure on the capillary column was 23 psi.

The capillary column (DB-5 or CP-Cil 8 CB; l = 60 m; ϕ_{out} = 0.25 mm; film thickness = 0.25 μm) was programmed as follows: 0° (3 min)–90° (3°/min; 0 min)–280° (10°/min; 2 min). The transfer line to the ion-trap MS was kept at 210°. The ion-trap MS used a mass range of m/z 46–280 which was scanned at a scan rate of 1 scan/sec.

Before sampling the concentration of oxygen was measured by GC-TCD. The capillary column (carboplot P7; l = 25 m; ϕ_{out} = 0.53 mm; film thickness = 25 μm) was held at 40° and the column flow was 5 ml He/min. The injector was held at 50° and the detector at 80°.

Quality assurance

After thermal conditioning, one of every series of 20 adsorbent tubes has been tested to be clean, which meant that the amount of chloroform was below the detection limit which was taken to be three times the background noise, i.e. 0.01 ng.

Formation of chloroform was detected positive when the amount in the headspace of a culture exceeded the determination limit which was taken to be three times the amount of chloroform in the corresponding blank, i.e. 0.02–0.40 ng. The amount of chloroform in the headspace of a culture was calculated from the calibration curve and was corrected for the amounts in the blank experiments. The calibration curve was linear ($R^2 \geqslant 0.99$) in the range of 0.5–50 ng of chloroform at m/z 83 of its characteristic mass fragment $CHCl_2$. Chloroform was identified by retention time and the isotope ratio of m/z 83 with m/z 85 and 87 of the same mass fragment.

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