Global Change Biology (2004) **10**, 1009–1016, doi: 10.1111/j.1365-2486.2004.00782.x

Ectomycorrhizal fungi: A new source of atmospheric methyl halides?

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Abstract

Incomplete source budgets for methyl halides – compounds that release inorganic chlorine and bromine radicals which, in turn, catalyze atmospheric ozone depletion – limit our ability to predict the fate of the stratospheric ozone layer. We report here the first measured emissions of methyl chloride, methyl bromide, and methyl iodide from ectomycorrhizal fungi. We grew nine fungal isolates on growth media containing halide concentrations similar to those found in soils and plant tissues. The observed range of emissions was 0.003–65 μg methyl chloride, 0.001–3 μg methyl bromide, and 0.02–12 μg methyl iodide g^{-1} dry weight fungi day^{-1}. Species varied in production rates of methyl chloride vs. methyl bromide vs. methyl iodide. *Cenococcum geophilum*, a widespread ectomycorrhizal fungus, was further tested to investigate the effects of halide substrate concentration in growth media. Emissions from this species increased linearly with increasing concentrations of both bromide and iodide. In addition, a subset of four fungi was studied with two media concentrations each of chloride, bromide, and iodide (0.2 or 20 mM). These fungi had similar responses to halide concentration, despite 1000-fold differences in baseline emission rates between isolates. Finally, high chloride concentrations (20 mM) in media did not appear to inhibit emissions of methyl bromide or methyl iodide. Overall, ectomycorrhizal fungi might be an important source of methyl halides to the atmosphere, and substrate concentrations and community composition may influence production levels in ecosystems.

Key words: ecosystem-atmosphere exchange, ectomycorrhizal fungi, methyl bromide, methyl chloride, methyl iodide

Received 28 October 2003; revised version received and accepted 26 November 2003

Introduction

A significant portion of halogen radicals in the troposphere and stratosphere are delivered by methyl chloride, methyl bromide, and methyl iodide (Weisenstein *et al*., 1992; Schauffler *et al*., 1993), with consequences for ozone depletion (Solomon *et al*., 1994; Olsen *et al*., 2000; Schauffler *et al*., 2000), cycling of atmospheric HO_x and NO_x species (Yung *et al*., 1980; Anderson *et al*., 1991; Vogt *et al*., 1999; McFiggans *et al*., 2000), and regional aerosol formation (O'Dowd *et al*., 2002). However, we can currently describe only 60% of atmospheric sources of methyl chloride, 75% of those for methyl bromide, and 40% of sources for methyl iodide (Kurylo & Rodriguez, 1998). Moreover, since many anthropogenic sources of halocarbons are currently regulated through the Montreal Protocol, natural sources are expected to dominate methyl halide production in the near future. In order to predict potential shifts in methyl halide production under human-induced global change, we must identify the 'missing' natural sources. Fungi may be a significant component of these sources.

Emissions of methyl chloride from hymenochaetaceous white rot fungi have been extensively studied (Harper & Kennedy, 1986; Harper *et al*., 1990; Watling & Harper, 1998; Harper, 2000). Methyl chloride production in white rot fungi appears to fulfill a methyl donor role within the fungal metabolism (Harper & Kennedy, 1986; Harper *et al*., 1990). On a global scale, methyl

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halide production by these fungi are already included in the methyl chloride budget (Watling & Harper, 1998) and are not likely to account for the entire 'missing source' of methyl bromide (Lee-Taylor & Holland, 2000). An estimate of the impact of white rot fungi on the methyl iodide budget has not yet been attempted.

The possibility that ectomycorrhizal fungi (root symbionts that access nutrients in the soil) may emit methyl halides has not been previously investigated. Ectomycorrhizal fungi are widespread globally and are especially common in temperate forests (Allen *et al*., 1995), where they can constitute an estimated 15% of soil organic matter (Treseder & Allen, 2000). Since global change factors such as elevated atmospheric CO_2 and anthropogenic nitrogen deposition can alter ectomycorrhizal growth (Treseder & Allen, 2000), these perturbations may feed back to affect methyl halide production rates from ectomycorrhizal fungi.

We examined several common ectomycorrhizal species to determine their capacity to affect the atmosphere through emissions of methyl halides under natural substrate concentrations. We addressed the questions: Are ectomycorrhizal isolates comparable in rates of methyl halide production? If so, are closely related ectomycorrhizal fungi more likely to display similar emission rates than distantly related ectomycorrhizal fungi? Also, does increasing availability of bromine, iodine, or chlorine in growth media elicit higher methyl halide production? Finally, if increasing media halide concentrations affect methyl halide production, what kinetics are associated with this increase?

Methods

Preparation of fungal cultures

For methyl halide analyses, we selected nine isolates that represented broadly to closely related ectomycorrhizal fungi from the western United States (Table 1). *Cenococcum geophilum* was chosen for comprehensive analysis because this species is one of the most widespread and common ectomycorrhizal fungi in the world (Trappe, 1977; Massicotte *et al*., 1992). Cultures were originally obtained by L. Egerton-Warburton and R. Molina from fresh fungal reproductive bodies (e.g. mushrooms) or colonized root tips collected in Oregon and southern California. From the time of collection until the start of the experiment, fungi were maintained and propagated aseptically on modified Melin Norkans (MMN) medium (Marx, 1969) in Petri dishes. As ectomycorrhizal cultures grow very slowly and show distinct growth patterns, any sample that showed contamination could be identified and eliminated.

To prepare fungal cultures for methyl halide analysis, 120 mL airtight glass flasks were filled with 20 mL of halogen-free MMN media to which potassium chloride, potassium bromide, or potassium iodide was added as detailed below. Flasks were inoculated with ~3 mg plugs cut from active fungal cultures and capped with sterile aluminum foil. Cultures were grown at 22 °C in the dark for 3–12 weeks until colonies were ~1.5 cm diameter. 'Blanks' received the same treatment but were not inoculated with fungi.

Three separate tests were conducted: (1) a comparison among ectomycorrhizal isolates, in which fungal cultures were grown on media supplemented with all three halides (chloride, bromide, and iodide); (2) a determination of linear relationships between halide availability and methyl halide emission, in which *Ce. geophilum* was grown on media to which a single halide was added (either chloride, bromide, or iodide) at four different concentrations; and (3) effects of high vs. low halide availability in several isolates, in which a subset of isolates were grown in media containing one halide (chloride, bromide, or iodide) at concentrations of either 0.20 or 20 mM. In each case, concentrations of halides in the media were selected to fall within the natural range of halide concentrations in soils and plants (see Appendix A).

For comparisons among isolates (Test 1), potassium halide concentrations equaled 0.02, 0.2, and 20.0 mM iodide, bromide and chloride, respectively, for all

Table 1 Isolates of ectomycorrhizal fungi screened for methyl halide production

Species	Family	Collection location
Phylum Ascomycota		
Cenococcum geophilum	Elaphomycetaceae	Oregon
Phylum Basidiomycota		
Boletus satanus	Boletaceae	Southern CA
Cortinarius fulmineous	Cortinariaceae	Southern CA
Hebeloma crustuliniforme	Cortinariaceae	Southern CA
Inocybe maculata (two Oregon morphotypes)	Cortinariaceae	Southern CA OR (white)
Laccaria amethyst-occidentalis	Tricholomataceae	Southern CA
Laccaria laccata	Tricholomataceae	Southern CA

samples. These amounts are equivalent to concentrations of 3.5, 16, and 700 ppm (parts per million) iodide, bromide, and chloride, respectively. Three to four replicates per isolate were used, with the exception of *Inocybe maculata* (CA), in which all but one replicate was lost due to contamination with other microbes.

To determine methyl halide emissions from *Ce. geophilum* grown on a gradient of halide availability (Test 2) a 4×3 factorial design was used, with four concentrations (0.02, 0.2, 2.0, or 20.0 mM) of three potassium halides (iodine, bromide, and chloride). Each treatment was represented by 5–10 replicates.

The effects of high and low halide concentrations on methyl halide production in several isolates (Test 3) were tested by addition of potassium bromide, potassium iodide, or potassium chloride at one of two levels: 0.20 or 20.0 mM, with two to four replicates per treatment. The isolates used in Test 3 included *Ce. geophilum*, *Hebeloma crustuliniforme*, *Laccaria laccata*, and both morphotypes of *I. maculata* from Oregon. These fungi were selected to encompass low, moderate, and high producers of methyl halides, as observed during Test 1. Also within Test 3, two sets of *Ce. geophilum* cultures were initiated, on different dates, to determine total variability of fungal emissions, including natural variability as well as variations due to preparation and sampling methodology (culture collection location on fungal bodies, culture age, media preparation).

Collection of gas samples

Once fungal colonies had been sufficiently established in the glass flasks, we began incubations for methyl halide production. The first step was to flush the samples in order to remove methyl halides that may have accumulated in the headspace or media during the growth phase. For 24 h, the headspace of each flask was continuously flushed with methyl halide-free air at a rate of 10–20 mL min^{-1}. Preliminary tests indicated that this process sufficiently reduced pre-existing methyl halides in the samples to levels below the detection limit. After flushing, each vial was capped with a sterile silicon stopper. At this point, the flask was airtight and any methyl halides produced by the fungi would accumulate in the headspace. At specific intervals, 5 mL of headspace air was then drawn from the vial by using a gas-tight glass syringe inserted through the silicon stopper. This gas sample was then analyzed for methyl halide content (see below).

To determine appropriate sampling times, we conducted preliminary measurements of emission rates from *Ce. geophilum*, in which air samples were collected at regular intervals over 48 h. We found that the highest emission rates from the fungi occurred near the beginning of the sampling period, before O_2 loss or CO_2 buildup in the headspace (data not shown). We selected sampling times of 0 and 4 h because methyl halides accumulated linearly with time over this period.

Once gas sampling was complete, we determined the dry weight of fungi in each flask so that we could report results in units of fungal biomass. Fungi-containing flasks were heated in a 60 °C oven until the growth media was liquified. The melted media was slowly poured from each flask, taking care to ensure that the fungal colony remained intact inside the flask. Approximately 20 mL of 0.5% (w/w) sodium metaphosphate solution was added to each flask, and the flask was sonicated for one minute to separate the fungal material from any residual media. The flask contents were poured into a plastic dish, and fine forceps were used to transfer all fungal material to a pre-weighed aluminum weigh tin. The tin was heated in a 100 °C oven for 3 days, and then weighed to determine dry fungal biomass.

Analysis of gas samples for methyl halide content

Once collected, gas samples were placed immediately (<2 min) in a pre-concentration system connected to a gas chromatograph with an electron capture detector (GC/ECD). GC/ECD analysis follows methods described in Redeker *et al*. (2000). This method is capable of detecting concentrations as low as 75, 5, and 0.5 ppt (parts per trillion) of methyl chloride, methyl bromide and methyl iodide, respectively, in gas samples. Detection limits for fluxes of each compound are smaller than 1.5 ng methyl iodide g^{-1} dry weight fungi day^{-1} and 0.2 ng methyl halide g^{-1} dry weight fungi day^{-1} for methyl chloride and methyl bromide.

Method blanks and precision

Uninoculated blanks for methyl bromide and methyl chloride were consistently below the detection limit. While we detected low (10–100 ppt) levels of methyl iodide in blanks, they were well below the range observed in flasks that contained fungi (1 parts per billion to 3 ppm). Background levels of methyl halides – whether from the media or from the air used for flushing the samples – were unlikely to influence our assessments of fungal emissions.

Instrument precision for methyl chloride, methyl bromide and methyl iodide was better than 3, 3% and 10%, respectively. Variability among *Ce. geophilum* replicates in Test 1 was 60%, 40%, and 55% for methyl chloride, methyl bromide and methyl iodide, correspondingly (Fig. 1). Reproducibility between the

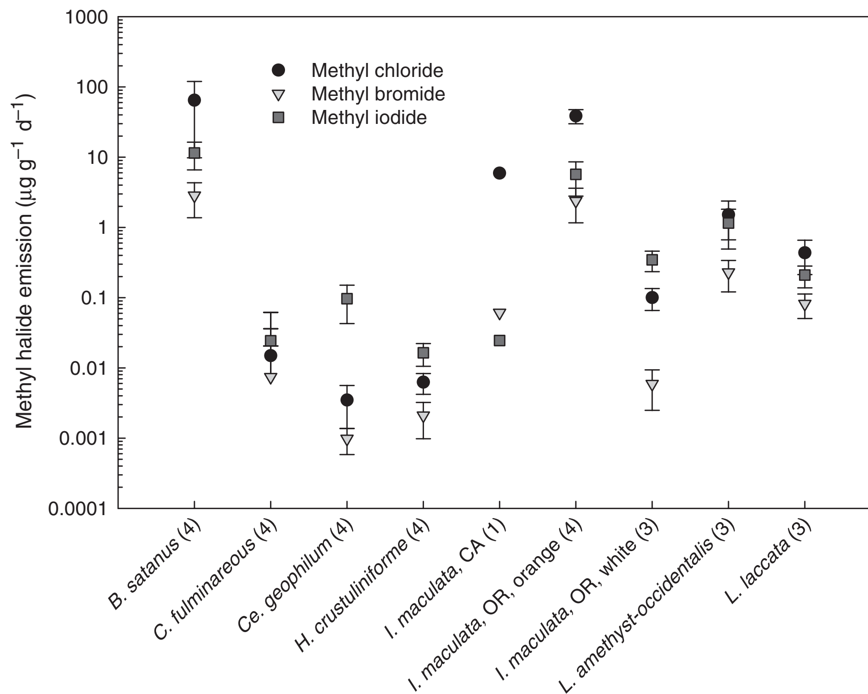

Fig. 1 Emission of methyl chloride (•) methyl bromide (▼) and methyl iodide (■) by various ectomycorrhizal isolates. Fungi were grown in media containing 700 ppm Cl^-, 16 ppm Br^-, and 3.5 ppm I^-. Values in parentheses after names indicate number of sample replicates. Note log scale of emissions. Symbols represent means ± 1SD.

averages of two separate sets of *Ce. geophilum* analyzed on different dates, with different colony sizes, and at potentially different growth stages was better than 40% (Table 2).

Statistics

In Test 1, methyl halide production was compared among isolates by using a repeated measures analysis of variance (ANOVA) on ranked data, with isolates as the independent variable and halide type (e.g. chloride, bromide, or iodide) as the source of repetition. In Test 2, linear regressions were used to determine relationships between halide concentration in the media and emission rates of methyl halides by *Ce. geophilum*.

Results

Test 1: Comparisons of ectomycorrhizal isolates

Emissions of methyl halides by the nine ectomycorrhizal isolates varied significantly and over several orders of magnitude (Fig. 1; $F_{8,21} = 59.83$, $P < 0.0001$). Most fungal isolates could be assigned to one of three groups based on overall emissions. Highest rates were observed in the orange morphotype of *I. maculata* from Oregon and in *Boletus satanus*. The moderate group included *L. laccata* and *Laccaria amethyst-occidentalis*. The lowest producers were *Cortinarius fulmineous* and *H. crustuliniforme*. The remainder displayed broad variation among types of methyl halides. For example, in *Ce. geophilum*, emissions of methyl iodide were much greater than those of methyl chloride or methyl bromide, making classification of this species difficult.

In addition, the 'preferred halide' (i.e. the methyl halide with highest rates of production) differed among isolates – in five isolates, methyl chloride was preferred. In contrast, methyl iodide was the leading product of the remaining four isolates, despite media concentrations 1000-fold less than those of chloride. In no case was methyl bromide preferred (media concentrations of bromide were ten times those of iodide and 100 times less than those of chloride). These differences in preference among isolates contributed to a significant isolate-by-halide interaction ($F_{16,42} = 4.02$, $P = 0.0001$). Across isolates, under natural substrate concentrations, methyl chloride was produced in significantly greater amounts than were the others ($F_{2,16} = 31.898$, $P = 0.0001$).

Test 2: Exponential relationships between halide availability and methyl halide emission in Ce. geophilum

Methyl bromide and methyl iodide emissions from *Ce. geophilum* were positively and exponentially related to media halide concentration (Fig. 2; methyl bromide:

Table 2 Emissions of methyl halides from several ectomycorrhizal isolates at 0.2 and 20.0 mm media halide concentration

	Emission rate ($\mu g\, g^{-1}$ fungus d^{-1})[a]	
	0.20 mM halide in media	20 mM halide in media
Methyl chloride		
Ce. geophilum (test 2)	bdl[b]	0.0019 ± 0.0002
Ce. geophilum (test 3)	bdl	0.0056 ± 0.0004
H. crustuliniforme	bdl	0.0027 ± 0.0053
I. maculata, OR, orange	230 ± 70	nd[c]
L. laccata	60 ± 49	130 ± 220
Methyl bromide		
Ce. geophilum (test 2)	0.0059 ± 0.0006	0.15 ± 0.02
Ce. geophilum (test 3)	0.0031 ± 0.0007	0.021 ± 0.004
H. crustuliniforme	0.00006 ± 0.00013	nd
I. maculata, OR, orange	3.3 ± 1.3	nd
I. maculata, OR, white	0.0018 ± 0.0009	nd
L. laccata	5.4 ± 3.9	320 ± 360
Methyl iodide		
Ce. geophilum (test 2)	1.9 ± 0.1	18 ± 2
Ce. geophilum (test 3)	2.1 ± 1.9	nd
H. crustuliniforme	0.28 ± 0.17	2.9 ± 1.6
I. maculata, OR, orange	30 ± 37	nd
I. maculata, OR, white	23 ± 36	nd
L. laccata	0.15 ± 0.10	0.09 ± 0.11

[a]Values are means ± 1SD.
[b]bdl: below detection limit.
[c]nd: not determined.

exponential $= e^{1.731[Br]}$, $r^2 = 0.997$, $P < 0.001$; methyl iodide: exponential $= e^{1.345[I]}$, $r^2 = 0.972$, $P < 0.001$). Methyl iodide at 20 mM may be within saturation concentrations, as the data from the lower three concentrations are much more self-consistent (exponential $= e^{1.621[I]}$, $r^2 = 0.998$, $P < 0.001$). If iodide is within saturation concentrations at 20 mM then the effects of increased halide concentration on methyl halide production within the unsaturated range is nearly identical between bromide and iodide. It was not possible to determine the effect of chloride concentrations on methyl chloride production, as concentrations lower than 20.0 mM produced emissions that were below the detection limit of the instrument.

Test 3: Effects of high and low availability of halides for several ectomycorrhizal isolates

The fungal isolates in Test 3 demonstrated broad variations in emissions of methyl chloride, methyl bromide and methyl iodide, consistent with observations from Test 1 (Table 2). Emissions from the isolates of *Ce. geophilum* that were tested for methyl halide emission dependence on media halide concentration (Test 2) are also included in Table 2. The ratios of emissions between 0.2 and 20.0 mM media halide concentrations differed among fungal isolates and halide type (Table 2). For example, methyl bromide emissions from *Ce. geophilum* increased by a factor of 16 (range equals 5–32), while production from *L. laccata* rose 60-fold (range equals 0–650). In terms of methyl iodide, *Ce. geophilum* and *H. crustuliniforme* displayed similar increases in emissions (factors of 9.5 and 10.5 with ranges of 8–12 and 3–41, respectively). In contrast, methyl iodide production by *L. laccata* actually dropped in response to an augmentation of iodide in the growth media. High concentrations of iodide have previously been shown to diminish fungal growth (Harper & Kennedy, 1986), although negative effects on ectomycorrhizal biomass were not observed in our experiment (data not shown).

Discussion

Our broad taxonomic sampling of common ectomycorrhizal fungi indicated that this functional group produces methyl halides at rates equivalent to or greater than those documented in other non-anthropogenic

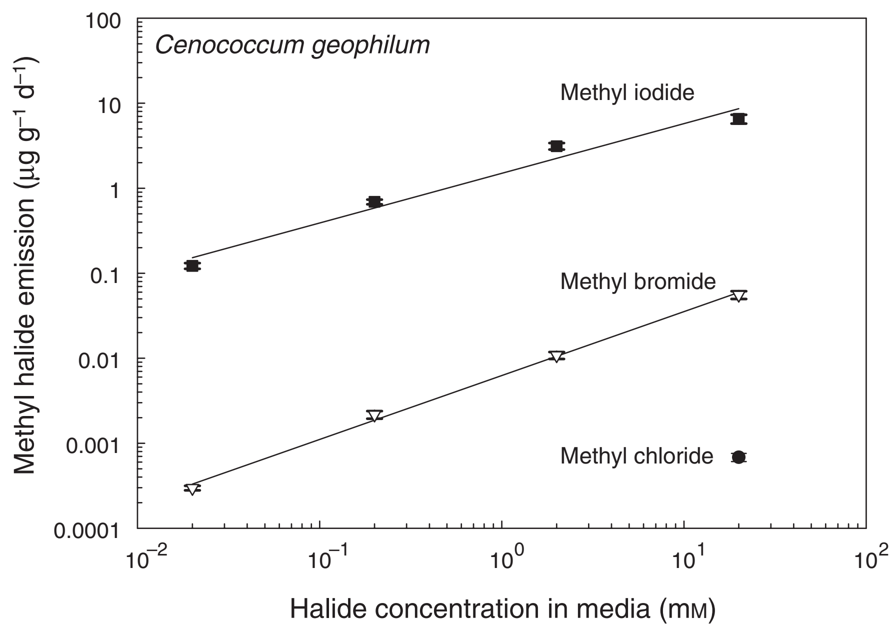

Fig. 2 Relationships between methyl halide emissions by *Ce. geophilum* and halide concentrations in growth media. Regression lines indicate least-squares best fit (±95% confidence intervals). Note log–log axes. Symbols represent means ± 1SD.

sources. Specifically, emission rates of methyl bromide from some ectomycorrhizal isolates (Fig. 1) were orders of magnitude higher than those of other natural producers (4–36 ng g^{-1} day^{-1}) (Manley & Dastoor, 1988; Saini *et al*., 1995; Gan *et al*., 1998; Redeker *et al*., 2000; Rhew *et al*., 2000), and methyl iodide emissions were within the same range of other non-fungal sources (14 ng g^{-1} day^{-1} to 650 μg g^{-1} day^{-1}) (Manley & Dastoor, 1988; Saini *et al*., 1995; Gan *et al*., 1998; Redeker *et al*., 2000) (Fig. 1). Due to methodological differences, we cannot directly compare our results to data from Watling & Harper (1998) to assess similarity between emissions of methyl chloride from white rot fungi and ectomycorrhizal fungi. Our laboratory results were consistent with recent field measurements of methyl halide emissions from surface soils (Dimmer *et al*., 2001; Varner *et al*., 2003); ectomycorrhizal fungi in the upper soil layers could potentially contribute to this flux.

The mechanisms of methyl halide production by ectomycorrhizal fungi are unclear at this stage. White rot fungi generate methyl chloride as a tool to produce veratryl alcohols during woody tissue (lignin) decomposition (Watling & Harper, 1998), but most ectomycorrhizal fungi are not strong lignin degraders. In addition, no lignin was present in the growth media in our experiment.

We observed that different ectomycorrhizal isolates had varying affinities or processing capacities for chlorine, bromine, and iodine (Fig. 1). Iodide appeared to be the preferred methylation substrate for most isolates, in accordance with observations of white rot fungi (Harper & Kennedy, 1986). However, there were significant differences in how much methyl chloride was produced by fungi relative to methyl iodide (Fig. 1, Table 2). Methyl bromide did not seem to play a key role in these reactions as, within the subset of fungi that we observed, it was never the primary methyl halide released. The capacity to generate particular methyl halides may be related to the ecology of the ectomycorrhizal fungi in question, but additional work will be required to address this issue. These gases may serve as methyl donors, defensive insecticides, or may merely be inadvertent metabolic byproducts (Manley, 2002).

Rates of methyl bromide emission may be genetically controlled to some extent, since two of the greatest emitters, *L. amethyst-occidentalis* and *L. laccata*, were congeners (Fig. 1). However, production rates did not fall strictly along taxonomic lines. For instance, the *I. maculata* isolate from southern California and the white morphotype from Oregon had similar rates (0.06 and 0.006 μg MeBr g^{-1} fungi day^{-1}, respectively), whereas the orange morphotype from Oregon produced more than 30 times as much (2.39 μg g^{-1} fungi day^{-1}). Likewise, there was no clear correlation between taxonomic relationship and production of methyl chloride and methyl iodide (Fig. 1).

Our results suggest that chloride is generally not a competitive inhibitor for methyl bromide or methyl iodide production. Emissions from fungi on media with single halides (Test 3) were similar to emissions from

Test 1, in which all three halides were combined in one medium (Fig. 1, Table 2). For example, in *Ce. geophilum*, *H. crustuliniforme*, and *L. laccata*, emissions from fungi on single-halide media with 20.0 mM chloride were identical, within reproducibility limits, to those on three-halide media, in which chloride concentrations were also 20.0 mM. This pattern also generally held for methyl bromide and methyl iodide.

These findings have implications for previous estimates of methyl halide production by white rot fungi. Harper & Kennedy (1986) assumed that chloride would competitively inhibit methylation of bromide and iodide in white rot fungi, since chloride concentrations in the dead plant material typically decomposed by this group are much higher than concentrations of bromide or iodide. Therefore, methyl iodide production from white rot fungi was expected to be minimal even though emission rates of methyl iodide are higher than emission rates of methyl chloride when each halide is provided singly. However, if this assumption is not true, emissions of methyl bromide and methyl iodide from white-rot fungi may be globally significant. A direct test for competitive inhibitions among halides in white rot fungi would be germane, since at this point it is unclear whether results from ectomycorrhizal fungi can be applied to other types of fungi.

Lee-Taylor & Holland (2000) estimated that methyl bromide emissions from white-rot fungi are globally significant, but our findings indicate that caution should be applied when considering their extrapolation efforts. When calculating global fluxes, they assumed equal efficiency in conversion of chloride and bromide to methyl chloride and methyl bromide, respectively. For ectomycorrhizal fungi, the relative efficiency of methyl chloride, methyl bromide and methyl iodide conversion appeared to vary among species and even within morphotypes of the same species (Table 2).

Previous research has clearly shown that soil bacteria are capable of utilizing methyl halides as substrates (Miller *et al*., 1997; Hines *et al*., 1998) and abiotic mechanisms of methyl halide production in soils have been identified (Keppler *et al*., 2000). These processes are likely to significantly influence the escape of fungal-generated methyl halides from soil systems. To fully understand the fungal/soil system we will need to pursue studies that determine microbiological biomass and species diversity in specific ecosystems, the depth of microbiological biomass in the soil, the actual substrates that provide halogens to fungi and bacteria, and the reaction of individual species to varying substrate halide concentrations and temperature. Studies that measure soil depth profiles, soil diffusion rates, and soil pore water concentrations of methyl halides will also be necessary for accurate assessment of the role of soil-atmosphere interactions in methyl halide budgets. Additional investigations of this new fungal source are especially critical considering the well-established responses of ectomycorrhizal fungi to different aspects of global change.

Acknowledgements

We thank Prof. Steve Manley and Prof. David Harper for sage advice and manuscript editing, Prof. Ralph Cicerone for lab space and equipment, K. Lamb and A. Lindahl for technical assistance, and L. M. Egerton-Warburton, D. McKey, and R. Molina for fungal cultures.

This work was supported by NSF Ecosystems grant DEB-0122445 to KKT, NSF grant ATM-0121153 to Ralph J. Cicerone, and by a grant from the University Research Foundation, University of Pennsylvania, to KKT and Ralph J. Cicerone.

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Appendix A. Natural halide concentrations in plants and soil

Chlorines

Chlorine concentrations in soil vary depending on distance from the ocean. Coastal soils in Norway contain an average chloride concentration of 920 ppm. Other regions, including inland sites in Norway and forests and upland fields in Japan, have lower average chloride concentrations of 265, 228, and 114 ppm (Kabata-Pendias & Pendias, 1992).

Bromine

Bromine concentrations in soils range from 0.5 to 515 ppm, averaging 5 ppm (Kabata-Pendias & Pendias, 1992; Yuita, 1994). Higher concentrations are found in coastal regions, while lower concentrations are found in well-leached soils. Bromide values range from 0.5 to 120 ppm in plants and 2 to 36 ppm in fungi (Kabata-Pendias & Pendias, 1992; Yuita, 1994).

Iodine

Iodine concentrations are more difficult to ascertain, since iodide, iodate and other ionic forms are formed in the soil as a function of soil moisture (Kabata-Pendias & Pendias, 1992). Estimates for total iodine content in soils range from 0.05 to 149 ppm, with average values of 5 ppm (Kabata-Pendias & Pendias, 1992; Yuita, 1994). Iodine concentrations are higher near seacoasts. Leaching lowers iodine content, but less effectively than it lowers bromine content, due to adsorption of iodine by organic molecules. Iodide concentrations in plants vary from 0.01 to 11 ppm, while those in fungi span 5.2 to 9.5 ppm (Kabata-Pendias & Pendias, 1992).