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Drosophilin A methyl ether (DAME) and other chlorinated dimethoxybenzenes in fungi and forest litter from Sweden

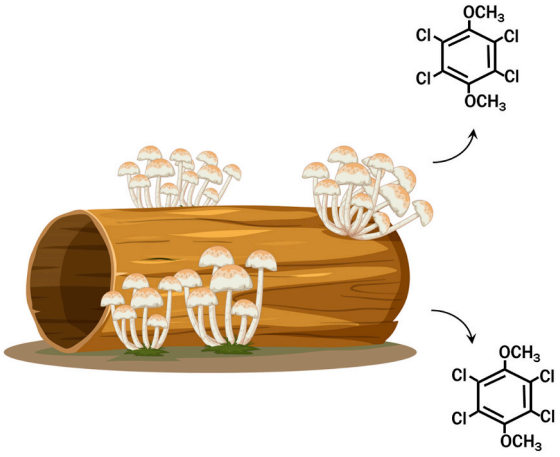
Terry F. Bidleman [a,*], Lars Ericson [b], Per Liljelind [a], Mats Tysklind [a]

[a] Department of Chemistry, Umeå University, Linnaeus väg 6, SE-901 87, Umeå, Sweden
[b] Department of Ecology and Environmental Science, Umeå University, Linnaeus väg 6, SE-901 87, Umeå, Sweden

HIGHLIGHTS

- A screening method was developed for chlorinated fungal metabolites.
- Drosophilin A methyl ether (DAME) was identified in in fungi and forest litter.
- Five fungal species were suggested to produce DAME *de novo*.
- Other chlorinated dimethoxybenzenes were found in some specimens.

GRAPHICAL ABSTRACT



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ABSTRACT

Fungi and substrates undergoing fungal decomposition were collected from forests in northern and southern Sweden and analyzed for chlorinated dimethoxybenzenes (DMBs). Specimens were fungi fruiting bodies, rotting wood, forest litter and underlying humus. Targeted compounds were DAME (1,2,4,5-tetrachloro-3,6-DMB) and related fungal secondary metabolites. A screening procedure was developed which involved soaking the specimens in ethyl acetate followed by analysis by capillary gas chromatography – mass spectrometry with mass selective detection (GC-MSD). DAME was the most frequently found (62% of 47 specimens) and often the most abundant target compound, with range and mean ± SD concentrations of <0.0017–3.81 and 0.21 ± 0.63 mg kg^{-1} ww. Based on log-log correlations of partition coefficients of hydrophobic compounds between fungal biomass/water (K_D) and octanol/water (K_{OW}), five species of fungi are suggested to produce DAME *de novo* versus bioaccumulation from forest runoff water. Full-scan mass spectra of some high-concentration specimens indicated the presence of a Cl_2DMB and a Cl_3DMB, which could not be identified further due to lack of standards, and drosophilin A (DA = 2,3,5,6-tetrachloro-4-methoxyphenol), the precursor to DAME. Tetrachloroveratrole

* Corresponding author.
E-mail addresses: terry.bidleman@umu.se (T.F. Bidleman), lars.ericson@umu.se (L. Ericson), per.liljelind@umu.se (P. Liljelind), mats.tysklind@umu.se (M. Tysklind).

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(TeCV = 1,2,3,4-tetrachloro-5,6-DMB) was found in only a few specimens. This study supports our hypothesis of fungi as a source of DAME in terrestrial runoff and indicates that other chlorinated secondary metabolites are present. DAME is widely distributed globally, and it would be good to have a better understanding of its sources and pathways as a marker of terrestrial organochlorines and their availability for bioaccumulation.

1. Introduction

Chlorine in soils is present as the anion (chloride) and covalently bound to organic matter. The organic fraction ranges from 34 to 100% in soils from diverse locations (Svensson et al., 2021). The reported range of total chlorine in forest soils of Sweden is 16–458 mg kg^{-1} dry weight, of which two-thirds or more is organically bound; the balance between organic and inorganic chlorine is maintained by rates of chlorination and dechlorination (Svensson et al., 2021, 2022). Chlorination of organic matter takes place most actively in the rhizosphere and is carried out by diverse organisms, including bacteria, fungi and vascular plants, as well as by abiotic processes (Clarke et al., 2006; Montelius et al., 2019; Öberg and Bastviken, 2012; Svensson et al., 2022).

Terrestrial fungi are prolific producers of halogenated secondary metabolites and about 80% of these contain chlorine (Cochereau et al., 2022). The chlorinated compounds in forest soils are diverse, with molecular masses ranging from simple chloromethanes (Wever and Barnett, 2017) to complex compounds such as chlorinated antibiotics and dioxins (Clarke et al., 2006; Winterton, 2000). Basidiomycetes produce chlorinated metabolites during decomposition of forest litter and are also effective at degrading some anthropogenic pollutants. In addition to chloromethanes, basidiomycetes produce chlorinated amino acids, anisyls, benzaldehydes, hydroquinones, anthroquinones, orsinols, sesquiterpenes and others (de Jong and Field, 1997). Some of the lower molecular mass compounds, as well as many nonhalogenated metabolites, have been termed "fungal volatiles" (Dickschat, 2017).

In this paper we consider the "chlorinated hydroquinone metabolite" (de Jong and Field, 1997) DAME (drosophilin A methyl ether = 1,2,4,5-tetrachloro-3,6-dimethoxybenzene) and related compounds. Drosophilin A (DA = 2,3,5,6-tetrachloro-4-methoxyphenol), the precursor to DAME, was reported in the basidiomycete *Drosophila subatrata* (now *Parasola conopilea*) over 70 years ago (Anchel, 1952; Kavanagh et al., 1952). Since then, DAME and/or DA have been identified in many genera of fungi, reviewed up to the mid-1990s by de Jong and Field (1997).

We reported DAME in air and precipitation at stations in Sweden and Finland (Bidleman et al., 2023a) and in rivers and estuaries of the northern Baltic Sea (Bidleman et al., 2023b). In these papers, we also described preliminary observations of DAME in terrestrial fungi from Sweden and suggested their likely role in supplying DAME to the atmosphere, rivers and estuaries. DAME is also widespread in air and surface water across Canada (Zhan et al., 2023) and in the North and South Atlantic Oceans (Schreitmüller and Ballschmiter, 1995). DA was found in wild boar (*Sus scrofa*) from Germany, presumably from their foraging for mushrooms (Hiebl et al., 2011).

The objectives of this study were to develop a screening method for DAME and similar halomethoxybenzenes (HMBs) in fungi and ground litter and survey these compounds in specimens from Swedish forests. Here we describe analytical methodology for HMBs, report DAME and other chlorinated HMBs in fungi and forest litter, propose a way to distinguish *de novo* production versus bioconcentration from runoff water and discuss environmental implications.

2. Materials and methods

2.1. Sample collection

Sample collection was done during October–November 2021 from forests in Västerbotten and Gävleborg counties in Sweden. The locations are shown on a map in Bidleman et al. (2023b). Specimens collected in brown paper bags were fungal fruiting bodies (sporocarps) and rotting wood, forest litter (conifer needles and deciduous leaves) and underlying humus which were infested with fungal mycelia. Two feather mosses were also included. The paper bags allowed the specimens to "breathe" and prevented moisture buildup. The specimens were refrigerated for several weeks, then transferred to plastic bags and frozen. Identification of fungi and mosses was done at the species level. Descriptions are provided in Table 1.

2.2. Extraction and analysis

Different techniques were used to achieve a uniform consistency of samples. Small soft fruiting bodies were extracted whole. Large hard ones were cut into useable pieces with a knife or saw. Portions of fruiting bodies, mosses, rotted wood and forest litter were homogenized with an electric coffee mill. Specimens with a spongy or corky texture were partially frozen, then homogenized. Dry weight (dw) was determined for most samples by oven-drying portions overnight at 70 °C. Table 1 gives the percent dw, which varied greatly from 6 to 7% for two fruiting bodies that were decomposing and in a liquifying state (18F, 24F) to over 80%.

Samples of 0.5–3 g wet weight (ww) were transferred to glass culture tubes with polytetrafluorethylene-lined caps. Ethyl acetate (10 mL) was added, the samples were vortexed for 30 s then refrigerated for 3–7 days, agitating periodically. Ethyl acetate is a common solvent for extracting secondary metabolites from fungal mycelia cultures (Riquelme et al., 2020; Silk et al., 2001; Teunissen et al., 1997).

After addition of 2,2′,6,6′-tetrachlorobiphenyl (PCB-54) internal standard to 1-mL portions of sample extracts, analytes were determined without cleanup by capillary gas chromatography−low resolution electron impact mass spectrometry (Agilent 6890 N chromatograph-5975 mass selective detector, GC-MSD, Agilent Technologies, Santa Clara, CA) and selected ion monitoring. The procedure was previously reported (Bidleman et al., 2023a, 2023b). The column was a J&W DB-5ms Ultra Inert, 30 m × 0.25 mm i.d., 0.25 μm film. The oven program was 90 °C (1 min), 1.3 °C/min to 135 °C, 5 °C/min to 180 °C, 20 °C to 250 °C (10 min). Inlet and transfer line temperatures were 250 °C; source and quadrupole temperatures were 230 and 150 °C.

Targeted compounds were dichloro-, trichloro- and tetrachloro-DMBs (Cl_2DMBs, Cl_3DMBs and Cl_4DMBs). Standards of Cl_4DMBs (drosophilin A methyl ether, DAME = 1,2,4,5-tetrachloro-3,6-DMB and tetrachloroveratrole, TeCV = 1,2,3,4-tetrachloro-5,6-DMB), Cl_3DMB (3,4,5-trichloroveratrole, TriCV = 1,2,3-trichloro-4,5-DMB) and Cl_2DMB (chloroneb = 1,4-dichloro-2,5-DMB) were obtained from AccuStandard (New Haven, CT, U.S.A.). Ions monitored (**quantifying**/qualifying) were 274/**276**, 259/261 (Cl_4DMBs), **240**/242/244 (Cl_3DMBs) and **191**/193, 206/208, (Cl_2DMBs). The internal standard PCB-54 was quantified with ion **290** and $^{13}C_6$- pentachloroanisole spike surrogate with ion **284**. Analytical injection standards for quantification varied from 250 to 5000 pg μL^{-1}, and response factors (area/pg injected) were linear over these ranges.

2.3. Quality control

Quantification was judged successful when the ratio of quantifying/qualifying ions was within 20% of values for standards. Several samples showed no peaks for target compounds which were above baseline noise. In these cases, the baseline noise was integrated over the limits of

peak elution times and results were expressed as instrumental detection limits (IDL). Averaged over several samples and replicate determinations, this procedure gave limits of detection LOD = mean IDL + 3xSD. LODs were 0.0017 (n = 22), 0.0013 (n = 74), 0.0027 (n = 9) and 0.0022 (n = 42) mg kg^{-1} for DAME, TeCV, Cl_2DMB and Cl_3DMB, respectively. For statistical evaluations, 1/2 of the LOD was substituted in cases of nondetection (Hites, 2019). In some cases, specimens plus ethyl acetate were spiked with 260 ng $^{13}C_6$-pentachloroanisole to check recoveries during the extraction process. The mean recovery was 109 ± 4% (n = 16). We did not adjust for recovery. Replicates (n = 2–4) of 29 specimens were analyzed, of which 23 had detectable DAME levels. For these 23, the difference among replicates was 3–41%, with mean ± standard deviation of 14 ± 11%. No difference in DAME concentrations was found for 3- or 7-day extraction times, the 7/3-day ratio averaged 0.99 ± 0.23 (n = 16).

3. Results and discussion

3.1. Compound identification

DAME was identified by a retention time which matched that of a standard within 0.02 min and by agreement within 20% between sample and standard ratios of monitored ions within chlorine clusters (259/261, 274/276). Extracts of some higher-concentration specimens were transferred to isooctane, cleaned by shaking for 30 s with 95% sulfuric acid, and full-scan spectra were acquired by GC-MSD (Fig. 1). Spectra of the suspected DAME peak in specimens 9L,16F, 17F and 32F matched the DAME spectrum in the National Institute of Standards and Technology (NIST) library with >90% probability, while the match for specimen 44B was 80%. Drosophilin A (DA), the precursor to DAME (de Jong and Field, 1997), was tentatively identified in 16F and 17F, with a spectrum that matched DA in the NIST library with >90%; however, we

Table 1
Specimens from Swedish forests.[a]

Specimen[b]	Location[c]	Fungi fruiting bodies	% dry weight	major group	biology	substrate
16F	GB	*Thelephora terrestris*	20.0	Basidiomycota	ectomycorrhizal	pine
17F	GB	*Hydnellum mirabile*	17.8	Basidiomycota	ectomycorrhizal	pine (and spruce)
9F	VB	*Microphale perforans*		Basidiomycota	saprotroph	carpet of spruce needles
12F	VB	*Stereum hirsutum*		Basidiomycota	white rot	dead gray alder
38F	VB	*Stereum subtomentosum*	80.4	Basidiomycota	white rot	*Alnus incana*, old windthrow
32F	GB	*Kuehneromyces mutabilis*	20.8	Basidiomycota	saprotroph	birch stump
11F	VB	*Jackrogersella multiformis*	85.6	Ascomycota	soft rot	dead downy birch wood
47F	VB	*Cladonia stellaris*	83.8	Ascomycota	lichen	very dry pine heath
13F	GB	*Phellinopsis conchata*	81.2	Basidiomycota	white rot	*Salix cinerea*
14F	GB	*Fomitiporia hippophaeicola*	78.0	Basidiomycota	white rot	*Hippophae rhamnoides*
21F	GB	*Hymenochaete tabacina*	33.5	Basidiomycota	white rot	cut aspen trunk
42FM	VB	*Stereum sanguinolentum*	60.3	Basidiomycota	white rot	young pine windthrow
18Fd	GB	*Hydnum repandum*	6.0	Basidiomycota	ectomycorrhizal	spruce
8F	VB	*Gleophyllum separium*	60.5	Basidiomycota	brown rot	spruce windthrow
15F	GB	*Lactarius rufus*		Basidiomycota	ectomycorrhizal	pine
28F	GB	*Phellinus igniarius*	36.7	Basidiomycota	white rot	birch
5F	VB	*Phellinus pini*	48.8	Basidiomycota	white rot	pine
29F	GB	*Clitocybe fragrans*	36.4	Basidiomycota	saprotroph	dry pine heath
1F	VB	*Fomitopsis pinicola*	40.8	Basidiomycota	brown rot	spruce stump
2F	VB	*Fomitopsis pinicola*		Basidiomycota	brown rot	spruce stump
3F	VB	*Fomes fomentarius*	69.2	Basidiomycota	white rot	downy birch
4F	VB	*Sarcodon squamosus*		Basidiomycota	ectomycorrhizal	dry pine heath
6F	VB	*Galerina sideroides*		Basidiomycota	saprotroph	dead pine
7F	VB	*Gyromitra infula*		Ascomycota	ectomycorrhizal	wood litter, rowan
33FM	VB	*Nectria cinnabarina*	81.8	Ascomycota	hemibiotrophic	*Ribes spicatum*, dead shoots
35F	VB	*Phellinus igniarius*	86.3	Basidiomycota	white rot	*Alnus incana*, old windthrow
35M	VB	*Phellinus igniarius*	84.5	Basidiomycota	white rot	rotten wood
37F	VB	*Plicatura nivea*	81.9	Basidiomycota	white rot	*Alnus incana*, dead stems, branches
38M	VB	*Stereum subtomentosum*	84.8	Basidiomycota	white rot	*Alnus incana*, old windthrow
39FM	VB	*Jackrogersella multiformis*		Ascomycota	soft rot	*Prunus padus*, windthrow
41M	VB	*Phellinus pini*	80.1	Basidiomycota	white rot	dying pine wood
19F	GB	*Sarcodon imbricatus*	15.0	Basidiomycota	ectomycorrhizal	spruce
22F	GB	*Climatcystis borealis*	27.4	Basidiomycota	brown rot	spruce stump
23F	GB	*Sarcomyxa serotina*		Basidiomycota	white rot	birch windthrow
24Fd	GB	*Sarcomyxa serotina*	7.1	Basidiomycota	white rot	maple windthrow
25F	GB	*Rigidoporus populina*		Basidiomycota	white rot	aspen windthrow
26F	GB	*Antrodiella pallescens*	20.9	Basidiomycota	white rot	birch windthrow
31F	GB	*Tricholoma* sp.		Basidiomycota	saprotroph	under *Prunus padus*
Mosses						
44B	VB	*Pleurozium schreberi*	86.7	Bryophyta		dry pine heath
45B	VB	*D. majus/H. splendens*	80.2	Bryophyta		mesic spruce forest
Litter, humus						
9L	VB	litter, mycelia, paired with 9F	40.0			carpet of spruce needles
46L	VB	leaf litter	70.4			mesic spruce forest
49L	VB	leaf litter	76.2			birch stand
44H	VB	humus layer, paired with 44B	38,9			dry pine heath
45H	VB	humus layer, paired with 45B	75.1			mesic spruce forest
48L	VB	leaf litter	80.4			*Alnus incana* stand
47H	VB	humus layer, paired with 47F	40.6			*Alnus incana* stand

a) Collected between 2021 and 10-07 and 2021-11-31.
b) Specimens: F = fruiting body (sporocarp), B = bryophyte, M = mycelia-infested wood, FM = mixed F and M, L = forest litter, H = humus layer.
c) VB = Västerbotten County, GB = Gävleborg County.
d) Decomposing, semi-liquid texture.

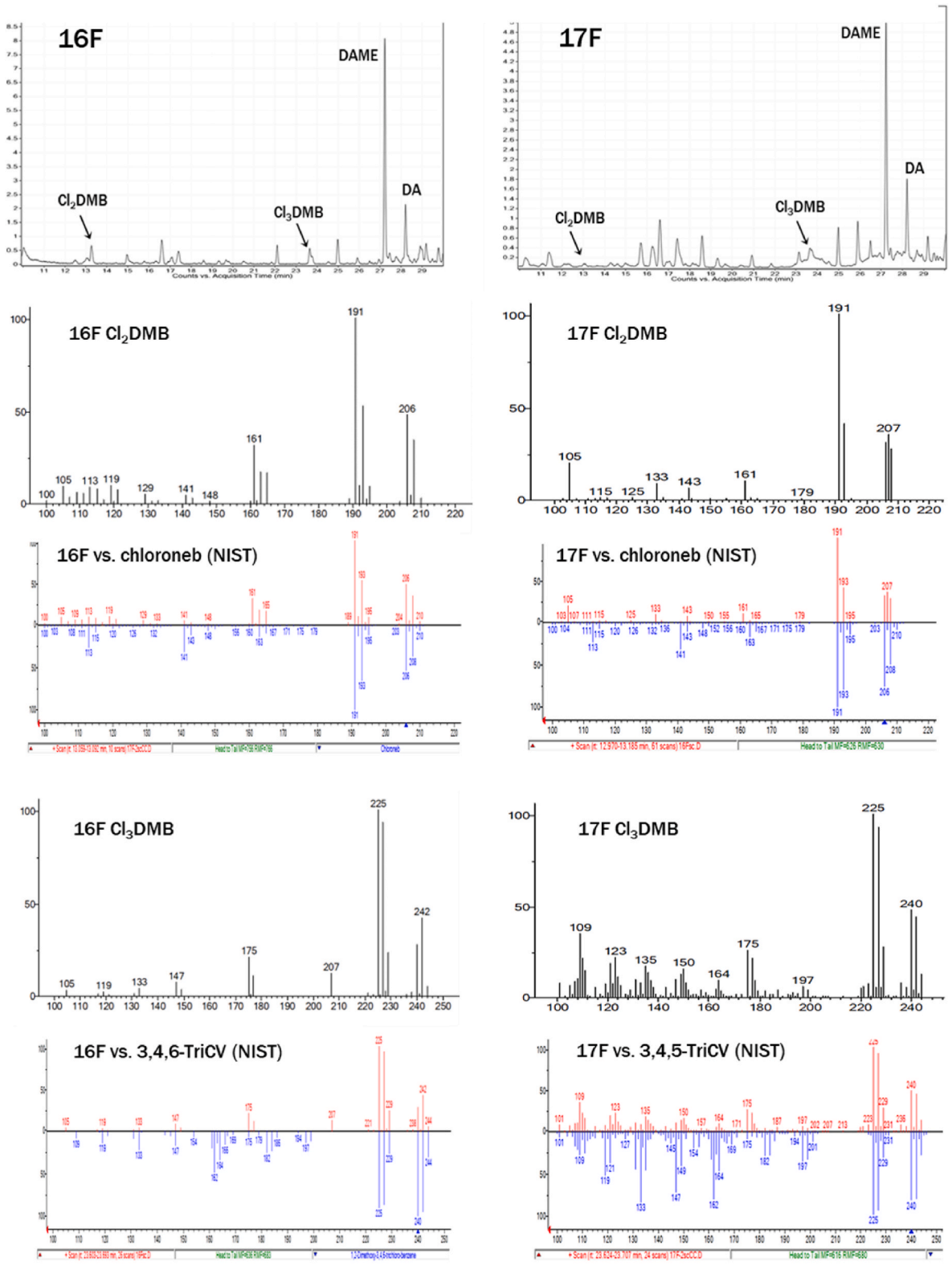

Fig. 1A. Top: Chromatograms of specimens 16F and 17F, showing DAME, drosophilin A (DA), and structurally unidentified compounds Cl_2DMB and Cl_3DMB. The middle two panels show mass spectra of the Cl_2DMB peak and the spectral match with the NIST library for chloroneb, while the bottom two panels show mass spectra of the Cl_3DMB peak and the spectral match with the NIST library for 3,4,6-TriCV or 3,4,5-TriCV. However, Cl_2DMB and Cl_3DMB are not likely to be these NIST compounds, but may be isomeric with them, for reasons explained in the text.

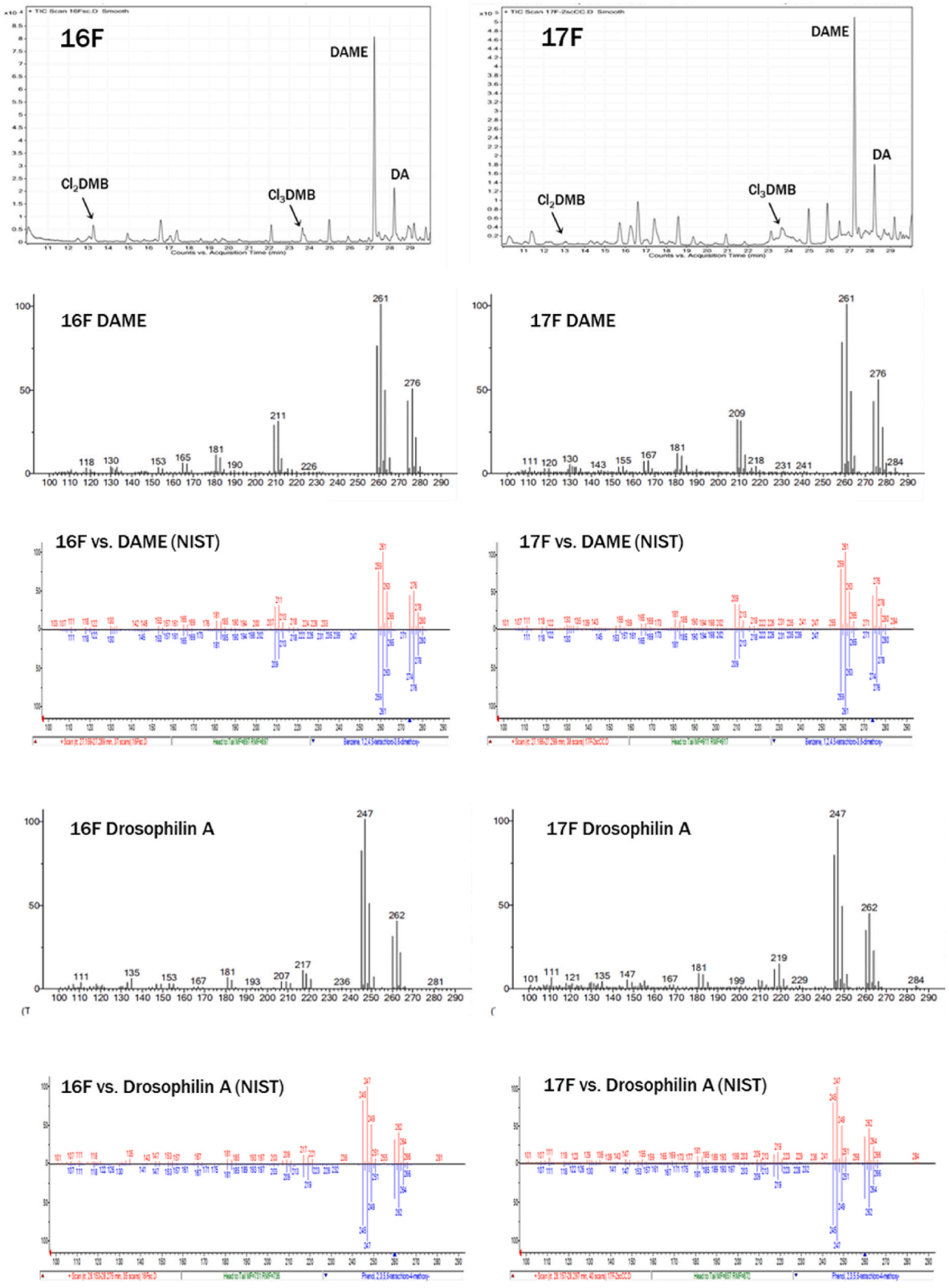

Fig. 1b. Top: Chromatograms of specimens 16F and 17F, showing DAME, drosophilin A (DA), and structurally unidentified compounds Cl_2DMB and Cl_3DMB. The middle two panels show mass spectra of the DAME peak and the spectral match with the NIST library for DAME, while the bottom two panels show mass spectra of the DA peak and the spectral match with the NIST library for DA.

did not have a DA standard for confirmation.

Chloroveratroles (CVs) have ortho-positioning of the two methoxy groups. A Cl_3DMB was tentatively identified by ratios of monitored ions in specimens 9F, 9L,12F,13F,14F,16F,17F,18F, 32F, 44B, 44H, 45B and 46L. Spectral matches with NIST library spectra for 3,4,5-TriCV or its isomer 3,4,6-TriCV in cleaned extracts were 60–90% for 16F, 17F and 32F, and comparisons to our suspected Cl_3DMB in 16F and 17F are shown in Fig. 1. However, the retention time of the Cl_3DMB was about 0.1 min later than that of a 3,4,5-TriCV standard, suggesting that our Cl_3DMB was not this isomer. We did not have a standard of 3,4,6-TriCV, but reported retention times of the two TriCVs on a nonpolar SE-30 column differ by over 2 min, with 3,4,6-TriCV eluting first (Korhonen et al., 1984). Thus, neither 3,4,5-TriCV or 3,4,6-TriCV are likely for our Cl_3DMB. We have no information about other possible Cl_3DMBs with different chlorine or methoxy positioning, and prefer the non-specific designation "Cl_3DMB", since neither TriCV has been reported in fungi. The Cl_3DMB in our specimens was quantified versus a 3,4,5-TriCV standard.

A Cl_2DMB was tentatively identified in fewer specimens (9L,12F,14F,16F, 17F, 18F, 32F and 44B) by ratios among monitored ions. Mass spectral matches with chloroneb (1,4-dichloro-2,5-DMB) in the NIST library were 56–72% for 16F, 17F and 32F, and are shown in Fig. 1 for 16F and 17F. However, the purported Cl_2DMB is not chloroneb because of its much shorter retention time in our specimens (13.0 min versus 16.7 min for a chloroneb standard). A large retention time range, from 12 to 18 min on a nonpolar SE-30 column, has been reported for dichloroveratroles (DiCVs) by Korhonen et al. (1984), but we have no information about DiCVs with other positioning of methoxy groups. The Cl_2DMB in our specimens was quantified versus chloroneb.

Several Cl_2DMBs have been reported in fungi (Fig. 2), which are suggested candidates for our compound: 1,3-dichloro-2,4-DMB (Wang et al., 2018), 1,3-dichloro-2,5-DMB (de Jong and Field, 1997; Silk et al., 2001; Spinnler et al., 1994), 2,5-dichloro-1,3-DMB (Rinkel et al., 2018), 1,4-dichloro-2,5-DMB (chloroneb) (Riquelme et al., 2020) and 1, 5-dichloro-2,3-DMB (Wang et al., 2013; Schalchli et al., 2015). Many chlorinated compounds were found in forest soil from southeast Sweden, including 2-chloro-1,4-DMB and 1,3-dichloro-2,5-DMB (Hjelm et al., 1996).

Specimens 16F and 17F were examined by GC-High Resolution Accurate Mass (HRAM) using an Agilent 7250 GC/Q-TOF system (Agilent Technologies, Santa Clara, CA) with the quadrupole in Total Transmission Ion (TTI) mode, and the above J&W DB-5ms Ultra Inert column, operating at a mass resolution of 25,000 FWHM with a TOF accuracy of 2 ppm or better. Exact masses derived from these runs (16F, 17F, respectively) were DAME [M^+] 273.9127, 273.9128 (NIST 273.9122, 1.8–2.2 ppm deviation), Cl_3DMB [M^+] 239.9517, 239.9515 (NIST 239.9512 for 3,4,5-TriCV, 1.3–2.1 ppm deviation, Cl_2DMB [M^+] 205.9905, 205.9905 (NIST 205.9901 for chloroneb, 1.9 ppm deviation).

3.2. Concentrations and distribution of DAME and other compounds

DAME was the most frequently found and often the most abundant chlorinated DMB, above the LOD of 0.0017 mg kg^{-1} ww in 29 of 47 specimens of various types (Table 2).

Concentrations above 1 mg kg^{-1} ww were found in fruiting bodies of fungi *Thelephora terrestris* (16F), *Hydnellum mirabile* (17F) and in litter sample 9L, which contained fruiting bodies of *Microphale perforans* (9F). Specimens having concentrations between 0.1 and 1.0 mg kg^{-1} ww were fruiting bodies of *Microphale perforans* (9F) and *Stereum hirsutum* (12F), wood infested with *Stereum subtomentosum* (38 M), mosses *Pleurozium schreberi* (44B) and *Dicranum majus/Hylocomium splendens* (45B), and litter sample 46L. It is interesting that rotting wood containing *Stereum subtomentosum* mycelia showed 10x higher DAME concentration than the fruiting body itself, whether compared on a ww or dw basis (cf 38 M and 38F, Tables 1 and 2). Much higher DAME concentrations, up to 30,000 mg kg^{-1} dw, and DAME crystals, were reported in the decaying heartwood of the mesquite (*Prosopis juliflora*) while the fruiting bodies of *Phellinus badius* living on the tree contained 24,000 mg kg^{-1} dw of DAME (Garvie et al., 2015).

DAME has not previously been reported in mosses, so this finding may appear surprising. However, boreal forest mosses such as *Hylocomium splendens* and *Pleurozium schreberi*, are associated with a highly diverse fungal community with taxa of a broad range of life strategies (pathogenic, endophytic, saprotrophic and ectomycorrhizal) (Davey et al., 2017; Kauserud et al., 2008). This may suggest that the occurrence of DAME in the moss samples was from associated fungal mycelia (unidentified) rather than the mosses themselves.

The mean ± SD, median and geometric mean for the set of 47 samples of all types were 0.21 ± 0.63, 0.0038 and 0.0082 mg kg^{-1} ww, when nondetectables were replaced by the LOD/2 = 0.00085 mg kg^{-1} ww (Section 2.3). The distribution of DAME concentrations is shown in Fig. 3.

Other quantified compounds were Cl_3DMB and Cl_2DMB with unspecified positioning of substituents (Section 3.1) (Table 2) and

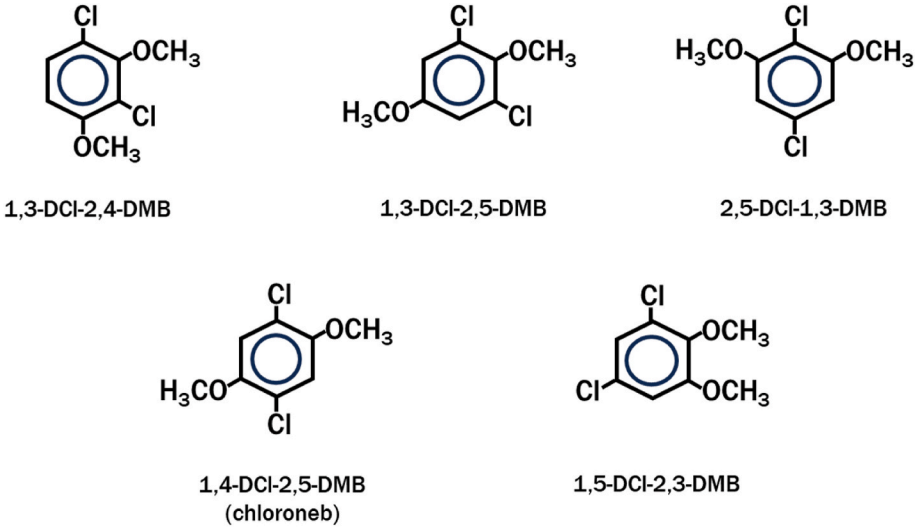

Fig. 2. Cl_2DMBs (DCl-DMBs) reported in fungi. 1,3-DCl-2,4-DMB (Wang et al., 2018), 1,3-DCl-2,5-DMB (de Jong and Field, 1997; Silk et al., 2001; Spinnler et al., 1994), 2,5-DCl-1,3-DMB (Rinkel et al., 2018), 1,4-DCl-2,5-DMB (chloroneb) (Riquelme et al., 2020) and 1,5-DCl-2,3-DMB (Wang et al., 2013; Schalchli et al., 2015). Many chlorinated compounds were found in forest soil from southeast Sweden, including 2-chloro-1,4-DMB and 1,3-dichloro-2,5-DMB (Hjelm et al., 1996).

Table 2
Chlorodimethoxybenzenes in specimens from Swedish forests.[a]

			mg kg^{-1} ww[d,e]			
Specimenb	**Locationc**	**Fungi fruiting bodies**	DAME	**TeCV**	**Cl_3DMB**	Cl_2DMB
16F	GB	*Thelephora terrestris*	3.81	0.039	0.39	0.59
17F	GB	*Hydnellum mirabile*	1.29		0.10	0.16
9F	VB	*Microphale perforans*	0.87		0.029	
38M	VB	*Stereum subtomentosum*	0.54	0.033	0.012	0.019
12F	VB	*Stereum hirsutum*	0.28		0.029	0.037
38F	VB	*Stereum subtomentosum*	0.053			
32F	GB	*Kuehneromyces mutabilis*	0.030		0.088	0.15
11F	VB	*Jackrogersella multiformis*	0.015			
47F	VB	*Cladonia stellaris*	0.011			
13F	GB	*Phellinopsis conchata*	0.0095		0.0028	
14F	GB	*Fomitiporia hippophaeicola*	0.0057		0.0072	0.054
21F	GB	*Hymenochaete tabacina*	0.0047			
42FM	VB	*Stereum sanguinolentum*	0.0044			
35M	VB	*Phellinus igniarius*	0.0044			
8F	VB	*Gleophyllum separium*	0.0038			
15F	GB	*Lactarius rufus*	0.0028			
18F	GB	*Hydnum repandum*	0.0027		0.0026	0.019
28F	GB	*Phellinus igniarius*	0.0025			
5F	VB	*Phellinus pini*	0.0023			
41M	VB	*Phellinus pini*	0.0018			
29F	GB	*Clitocybe fragrans*				
1F	VB	*Fomitopsis pinicola*				
2F	VB	*Fomitopsis pinicola*				
3F	VB	*Fomes fomentarius*				
4F	VB	*Sarcodon squamosus*				
6F	VB	*Galerina sideroides*				
7F	VB	*Gyromitra infula*				
33FM	VB	*Nectria cinnabarina*				
35F	VB	*Phellinus igniarius*				
37F	VB	*Plicatura nivea*				
39FM	VB	*Jackrogersella multiformis*				
19F	GB	*Sarcodon imbricatus*				
22F	GB	*Climatcystis borealis*				
23F	GB	*Sarcomyxa serotina*				
24F	GB	*Sarcomyxa serotina*				
25F	GB	*Rigidoporus populina*				
29F	GB	*Clitocybe fragrans*				
31F	GB	*Tricholoma* sp.				
Mosses						
44B	VB	*Pleurozium schreberi*	0.43	0.091	0.059	0.034
45B	VB	*D. majus/H. splendens*	0.29		0.0065	
Litter, humus						
9L	VB	litter, mycelia, paired with 9F	1.57	0.0033	0.028	0.010
46L	VB	leaf litter, spruce	0.23	0.0043	0.015	
45H	VB	humus layer, paired with 45B	0.087	0.0033	0.0040	
44H	VB	humus layer, paired with 44B	0.047	0.016	0.041	
49L	VB	leaf litter, birch	0.040			
48L	VB	leaf litter, gray alder	0.016			
47H	VB	humus layer, paired with 47F	0.0059			

a) DAME = 1,2,4,5-tetrachloro-3,6-dimethoxybenzene, TeCV = tetrachloroveratrole = 1,2,3,4-tetrachloro-5,6-dimethoxybenzene, Cl_2DMB and Cl_3DMB = structurally unidentified dichloro- and trichlorodimethoxybenzenes.
b) Specimens: F = fruiting body (sporocarp), B = bryophyte, M = mycelia-infested wood, FM = mixed F and M, L = forest litter, H = humus layer.
c) VB = Västerbotten County, GB = Gävleborg County.
d) See Table 1 for conversion to dry weights.
e) Blank space means below detection or not measureable because of chromatographic interference. LODs, mg kg^{-1} ww: DAME 0.0017, TeCV 0.0013, Cl_2DMB 0.0027 and Cl_3DMB 0.0022.

tetrachloroveratrole (TeCV = 1,2,3,4-tetrachloro-5,6-dimethoxybenzene). The means ± SD for Cl_3DMB and Cl_2DMB in positive samples were 0.058 ± 0.10 mg kg^{-1} ww (n = 14) and 0.12 ± 0.19 mg kg^{-1} ww (n = 9). Correlations were significant for log Cl_3DMB with log DAME (r^2 = 0.44, p = 0.0071) and log Cl_2DMB with log Cl_3DMB (r^2 = 0.58, p = 0.017) (Fig. 4A and B), while correlation of log Cl_2DMB with DAME was not significant (r^2 = 0.06, $p > 0.05$, Fig. 4C). TeCV was infrequently found, in 16F (*Thelephora terrestris*) at 0.042 mg kg^{-1} ww, in moss 44B (*Pleurozium schreberi*) at 0.11 mg kg^{-1} ww, in 38 M (wood infested with *Stereum subtomentosum*) at 0.033 mg kg^{-1} ww and in litter/humus samples at 0.0033–0.016 mg kg^{-1} ww (n = 3).

DAME and TeCV were routinely found in air and precipitation sampled on the Swedish west coast and in Subarctic Finland (Bidleman et al., 2023a, 2023b). Mean concentrations of DAME and TeCV in air from 2018 to 2019 were 34 ± 23 and 12 ± 7.2 pg m^{-3} on the west coast; 41 ± 43 and 2.1 ± 1.5 pg m^{-3} in Finland. Concentrations of DAME and TeCV in precipitation in 2018–2019 were 37 ± 30 and 22 ± 18 pg L^{-1} on the west coast; 72 ± 57 and 6.4 ± 3.5 pg L^{-1} in Finland. Garvie et al. (2015) hypothesized that biomass burning might release DAME to the atmosphere, but no significant correlations were found for airborne DAME or TeCV with combustion markers such as polycyclic aromatic hydrocarbons (PAHs) and benzo[a]pyrene (BaP) in Sweden or Finland (Bidleman et al., 2023a). Also, DAME and TeCV were not correlated with retene (a marker of biomass burning) in Canada (Zhan et al., 2023). However, the Garvie et al. (2015) hypothesis is not necessarily refuted because biomass burning may not be the dominant source of PAHs in the

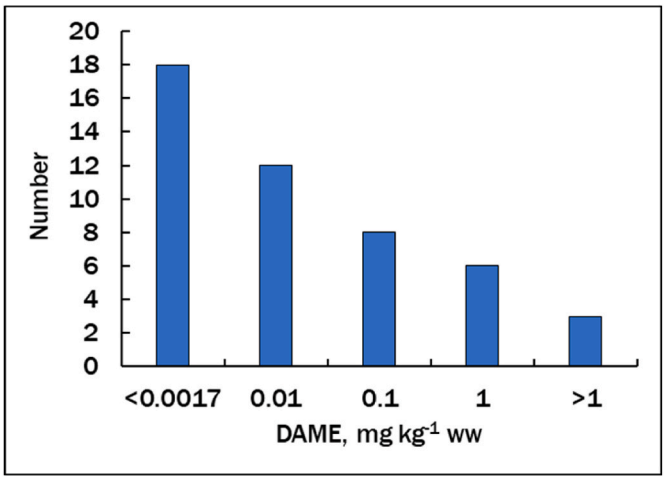

Fig. 3. Distribution of DAME concentrations in 47 specimens of various types (Table 2).

regions sampled.

The source of DAME in air is suspected to be from the terrestrial environment (Bidleman et al., 2023a, 2023b). TeCV in air might also be of terrestrial origin, but there is only weak evidence in this study of production by fungi fruiting bodies. TeCV was found in forest litter and humus (Table 1), suggesting production in the rhizosphere. Another possible source of TeCV is bacterial O-methylation of the chloroguaiacols produced in bleached Kraft mill effluent (Brownlee et al., 1993; Neilson et al., 1984). TeCV in the atmosphere might be deposited by precipitation (Bidleman et al., 2023a) or scavenged in the forest canopy by sorption to foliage (the "forest filter effect", McLachlan and Horstmann, 1998; Su et al., 2007) and subsequently deposited. TeCV and lower chlorinated veratroles were widespread in air and surface water across Canada (Zhan et al., 2023), and strong correlations between DAME and TeCV in air were found in both Canada (Zhan et al., 2023) and Finland (Bidleman et al., 2023a). However, no evidence of elevated TeCV in the vicinity of pulp and paper production facilities was found in the Zhan et al. (2023) study. These observations suggest natural rather than anthropogenic sources of TeCV.

3.3. Production versus bioconcentration

The DAME in forest specimens may be derived from *de novo* production and by bioconcentration of DAME from waters of various sources: precipitation, canopy throughfall and water flowing through forest ground cover. DAME and DA are also metabolites of the wood preservative pentachlorophenol (Varela et al., 2015; Xiao and Kondo, 2020), although this is not expected to be a contributor in the areas where our specimens were collected. Concentrations of DAME in precipitation collected at Råö on the Swedish west coast (57.39 N, 11.91E) and at Pallas, Finland (68.00 N, 24.23 E) in 2018–2019 averaged 37 and 72 pg L^{-1} respectively (Bidleman et al., 2023a), while higher levels were found in rivers of Västerbotten County, averaging 307 pg L^{-1} in 2017–2022, presumably due to the contribution from terrestrial runoff (Bidleman et al., 2023b).

To assess whether levels of DAME in the various specimens were likely from *de novo* production or bioconcentration, we sought a relationship between the fungi/water distribution coefficient (K_D, L kg^{-1} = fungi (mg kg^{-1})/water (mg L^{-1}) and the octanol-water (K_{OW}) distribution coefficient of hydrophobic organic compounds. Eleven such studies are summarized in Tables 3 and 4, and Fig. 5, in which fungal biomass was equilibrated with aqueous solutions of polycyclic aromatic hydrocarbons (PAHs), pesticides, chlorobenzene or a polychlorinated biphenyl. We did not include reports for ionizable phenolic compounds (e.g., phenol, chlorophenols) because even though DAME is derived from a chlorinated methoxyphenol (DA), it is a neutral compound. The K_D in each case was taken from reported literature values or calculated from the reported Freundlich sorption parameters, and for all compounds in Table 4 followed the relationship with $r^2 = 0.664$ (Fig. 5):

$$Log\ K_D = 1.0974 \times log\ K_{OW} - 0.9405 \quad (1)$$

Table 3
Studies of sorption to fungal biomass.

Organism	Pretreatment	Compounds[a]	Reference
"consortium of white rot fungi"	oven-dried	NAPH, ACE, FLE, PHEN, PYR	Chen et al. (2010)
Rhizopus oryzae	cell walls	NAPH, FLE, PHEN, PYR	Ma et al. (2011)
Phanerochaete chrysosporium	autoclaved pellets	PHEN, PYR	Ding et al., 2913
Phanerochaete chrysosporium	autoclaved pellets	PHEN	Zhang et al. (2022)
Phanerochaete chrysosporium	freeze-dried pellets	PHEN	Gu et al. (2015)
Gomphidius viscidu	mix of autoclaved and active mycelia	ANTH	Huang et al. (2010)
Ophiostoma stenoceras	live biomass	chlorobenzene	Cheng et al. (2019)
Mucor racemosus, Sporothrix cyanescens, Rhizopus arrhizus	autoclaved	PCNB	Lièvremont et al. (1998)
Rhizopus oryzae	autoclaved	lindane	Ghosh et al. (2009)
Rhizopus oryzae	autoclaved pellets	lindane	Young and Banks (1998)
Rhizopus oryzae	autoclaved	lindane, diazinon, 2-CB	Bell and Tsezos (1987)

a) Abbreviations: NAPH = naphthalene, ANTH = anthracene, ACE = acenaphthene, FLE = fluorene, PHEN = phenanthrene.
PYR = pyrene, PCNB = pentachloronitrobenzene, 2-CB = 2-chlorobiphenyl.

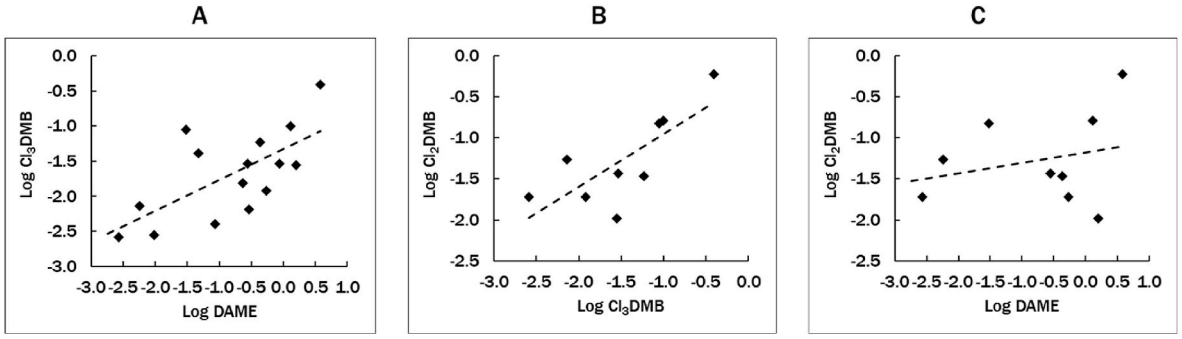

Fig. 4. A. Log-log correlation of structurally unidentified Cl_3DMB with DAME ($r^2 = 0.44$, $p = 0.0071$), B. structurally unidentified Cl_2DMB with Cl_3DMB ($r^2 = 0.58$, $p = 0.017$) and C. Cl_2DMB with DAME ($r^2 = 0.06$, $p > 0.05$).

Table 4
Distribution coefficients between fungi[a] and water, K_D, L kg^{-1} and octanol-water, K_{OW}.

Compound[b]	Log K_{OW}	Log K_D	Reference
NAPH	3.29	2.696	Chen et al. (2010)
ACEN	3.92	3.263	
FLE	4.18	3.333	
PHEN	4.45	3.994	
PYR	4.90	4.759	
NAPH	3.3	3.3	Ma et al., (2011)[c]
FLE	4.2	3.8	
PHEN	4.4	4.2	
PYR	4.9	4.6	
PHEN	4.45	3.606	Ding et al. (2013)
PYR	4.90	4.243	
PHEN	4.45	3.294	Zhang et al. (2022)
PHEN	4.45	4.058	Gu et al. (2015)
ANTH	4.45	4.642	Huang et al. (2010)
chlorobenzene	2.84	1.899	Cheng et al. (2019)
PCNB	4.22	3.197	Lièvremont et al. (1998)
PCNB	4.22	3.077	
PCNB	4.22	2.936	
lindane	3.83	3.240	Ghosh et al. (2009)
lindane	3.83	3.521	Young and Banks (1998)
lindane	3.83	3.362	Bell and Tsezos (1987)
diazinon	3.81	3.529	
2-chlorobiphenyl	4.53	4.719	

a) See Table 3 for list of fungi used.
b) Abbreviations: NAPH = naphthalene, ANTH = anthracene, ACE = acenaphthene.
FLE = fluorene, PHEN = phenanthrene, PYR = pyrene.
PCNB = pentachloronitrobenzene.
c) Values estimated from Fig. S2 in their paper.

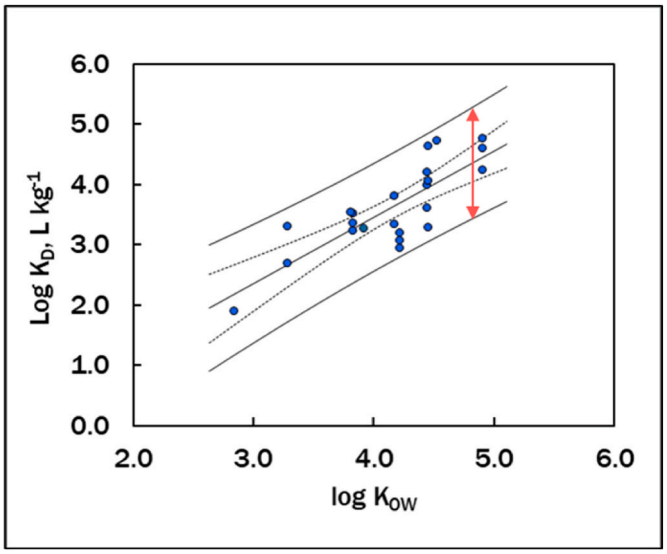

Fig. 5. Relationship between the fungi/water (K_D, L kg^{-1}) and octanol-water (K_{OW}) distribution coefficients for the compounds in Table 2, with mean and predicted 95% confidence intervals shown by the curved dotted and solid lines, respectively. Log K_D = 1.0974*log K_{OW} -0.9405, r^2 = 0.664. The mean log K_D = 4.39 at the assumed DAME K_{OW} = 4.86 with a predicted 95% confidence interval of 3.47–5.32 (orange arrow).

K_{OW} is not known for DAME, but log K_{OW} for TeCV (an isomer of DAME) is 4.86 at 25 °C (Lun et al., 1995). Assuming this log K_{OW} also applies to DAME, the predicted value of log K_D/L kg^{-1} is 4.39 with a 95% confidence interval of 3.47–5.32 (Fig. 5). The central value and (95% prediction range) for K_D = 2.45 x10^4 (2.95 x 10^3 - 2.09 x 10^5) L kg^{-1}. The average concentration (C_W) of DAME in Västerbotten rivers in 2017–2022 was 307 pg L^{-1} (3.07 x 10^{-7} mg L^{-1}), which we assume came from terrestrial runoff (Bidleman et al., 2023b). This assumption is open to question, because water in rivers comes not only from surface runoff but also groundwater flow (Laudon and Sponseller, 2017). However, we have no information on DAME in groundwater. The predicted lower and upper concentrations of DAME in fungi in equilibrium with the above water concentration are K_D*C_W = 2.95 x 10^3 L kg^{-1} x 3.07 x 10^{-7} mg L^{-1} = 0.0009 mg kg^{-1}, and 2.09 x 10^5 L kg^{-1} x 3.07 x 10^{-7} mg L^{-1} = 0.064 mg kg^{-1}.

The upper end of this 95% prediction range, *viz.* 0.064 mg kg^{-1}, appears to be a reasonable dividing point in this study to judge *de novo* production versus bioconcentration. According to this criterion, a few basidiomycetes in Table 2 may be DAME producers, *viz. Micromphale perforans* (9F), *Stereum hirsutum* (12F), *Thelephora terrestris* (16F), *Stereum subtomentosum* (38 M) *and Hydnellum mirabile* (17F). However, the only reliable way to confirm this would be to culture these species in the laboratory and test them for DAME synthesis. Teunissen et al. (1997) screened 92 basidiomycetes strains in laboratory cultures and found only five that produced DAME.

3.4. The role of chlorine availability

Species reported to produce DAME are *Agaricus bisporus*, *Agaricus arvensis*, *Bjerkandera adusta, Hypholoma fasciculare, Mycena megaspore, Peniophora pseudeopini, Phellinus fastuosus, Phellinus robineae, Phellinus yucatensis* (de Jong and Field, 1997; Teunissen et al., 1997), *Phellinus badius* (Garvie et al., 2015) and *Phylloporia boldo* (Riquelme et al., 2020). It is curious that the above *Phellinus* species produce DAME in high concentrations, ranging from 70 to 24000 mg kg^{-1} (de Jong and Field, 1997; Garvie et al., 2015; Teunissen et al., 1997), whereas the species examined by us (5F, 28F, 35F) contained DAME levels that were barely above detection or not detectable. In the case of *Phellinus badius*, the DAME was coincident with very high concentrations of chlorine, 22, 000–28,000 mg kg^{-1} in fresh basidiocarps and 4800 mg kg^{-1} in decaying mesquite heartwood (Garvie et al., 2015). Chlorine in wood and bark of four summer-harvested tree species in Finland was much lower, ranging from 30 to 330 mg kg^{-1}, while the chlorine content of different parts of the same tree (wood, bark, shoots, foliage) ranged up to 1090 mg kg^{-1} (Werkelin et al., 2005, 2011). Of the five basidiomycetes found to produce DAME by Teunissen et al. (1997), production in culture media by four species ranged from 138 to 1380 mg L^{-1} while the range for *Phellinus fastusosus* was 1100–11,000 mg L^{-1}. The culture media used by Teunissen et al. (1997) contained 58 mg L^{-1} NaCl, within the chloride range of Swedish soils (Svensson et al., 2011, 2021). Riquelme et al. (2020) observed an increase in production of DAME, DA and chloroneb by *Phylloporia boldo* upon addition of KCl to culture medium. It may be that production of DAME and other chlorinated fungal metabolites is chlorine-limited in our study areas. Such availability could be influenced by the atmospheric deposition of chlorine and its biogeochemical cycle in specific microenvironments (Svensson et al., 2021), factors that should be considered in future investigations.

3.5. Limitations of the study

The ability to identify HMBs is hampered by lack of analytical standards. Standards for DAME and some of the chloroveratroles are available commercially, but HMBs with other positioning of methoxy groups are not, and workers in previous studies often synthesized their own. Assessment of production versus bioconcentration (Section 3.2) is uncertain due to limited knowledge concerning sorption of HMBs by fungi. Studies reported in Tables 3 and 4 were done with other compounds and were conducted with fungi that received harsh pretreatment before use. Experiments of biosorption of HMBs by fungi under realistic field exposure conditions are needed. Although our survey included more fungi for HMBs than in most other studies, those in Tables 1 and 2 represent only about 1% of the fungi species that have been identified in Sweden (approximately 2700, https://svamparisverige.se/start/a

bout/). The possible influence of chlorine availability should be considered in future investigations (Section 3.3).

3.6. Environmental significance

DAME is globally distributed in the atmosphere (Bidleman et al., 2023a, 2023b; Schreitmüller and Ballschmiter, 1995; Zhan et al., 2023) and enters northern Baltic estuaries by terrestrial runoff (Bidleman et al., 2023b). Atmospheric deposition of airborne DAME may also contribute to coastal and offshore waters (Bidleman et al., 2023b; Zhan et al., 2023). This study provides evidence of forest fungi and litter as sources of DAME and possibly other chlorinated DMBs in streams, and it is likely that input comes from fungi in non-forested regimes as well. Bioaccumulation by fish has been shown for DAME (Fernando et al., 2018; Renaguli et al., 2020), TriCV and TeCV (Neilson et al., 1984). Predictions from structure-activity relationships suggest some toxic properties for DAME (Zhan et al., 2023). Threshold toxic concentrations to zebra fish (*Brachydanio rerio*) embryos and larvae have been determined for TriCV, TeCV, pentachloroanisole and a trichlorotrimethoxybenzene (Neilson et al., 1984).

4. Conclusions

This study supports our hypothesis of fungi as a source of DAME in terrestrial runoff and indicates that other chlorinated secondary metabolites are present as well. Five fungal species may be heretofor unidentified producers of DAME and its precursor DA was found in two of them. The simple procedure presented here of soak-extraction followed by GC-MSD is suitable for screening DAME and related compounds in fungi and forest litter. We suggest fruitful extensions of this work on several fronts: laboratory experiments to verify production by candidate species, field studies to extend the survey and examine spatial and temporal variability, biosorption experiments by fungi and forest litter to determine partitioning with runoff water. Considering the global spread of DAME, it would be good to have a better understanding of its sources and pathways, as a terrestrial marker and availability for bioaccumulation.

Credit author statement

Bidleman: Conceptualization, investigation, methodology, writing. Ericson: Conceptualization, investigation, sample collection and identification. Liljelind: Investigation, methodology, Tysklind: Conceptualization, resources, funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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