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ORIGINAL PAPER

Substantial production of drosophilin A methyl ether (tetrachloro-1,4-dimethoxybenzene) by the lignicolous basidiomycete *Phellinus badius* in the heartwood of mesquite (*Prosopis juliflora*) trees

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Abstract Toxic organohalogen pollutants produced as by-products of industrial processes, such as chloroform and polychlorinated dibenzo-*p*-dioxins, also have significant natural sources. A substantial terrestrial source of halogenated organics originates from fungal decay of wood and leaf litter. Here we show that the lignicolous basidiomycete *Phellinus badius* deposits up to 30,000 mg of the halogenated metabolite drosophilin A methyl ether (DAME, tetrachloro-1,4-dimethoxybenzene) per kilogram of decayed heartwood in the mesquite *Prosopis juliflora*. DAME occurs as clusters of glassy crystals up to 1 mm long within the decayed heartwood. In addition, the *Phellinus badius* basidiocarps contain an average of 24,000 mg DAME/kg dried fruiting body, testifying to the significant translocation and accumulation of Cl accompanied by DAME biosynthesis. The high DAME concentrations attest to the substantial Cl content of the heartwood, which averages near 5,000 ppm, with Cl/K near 1:1, consistent with an inorganic chloride precursor. *Phellinus badius* has a circumglobal distribution in the tropics and subtropics, where it is widely distributed on hardwoods and commonly associated with decay of mesquite. There is the potential for extensive DAME formation within decayed heartwood worldwide given the extensive range of *Phellinus badius* and its propensity to form DAME within mesquites. Further, DAME production is not limited to *Phellinus badius* but occurs in a range of lignicolous basidiomycetes, suggesting a significant natural reservoir for this chloroaromatic with potential environmental implications.

Keywords DAME · *Phellinus badius* · *Prosopis juliflora* · Chlorinated hydroquinone metabolites · Drosophilins · Organohalogen pollutants

Introduction

More than 5,000 organohalogen compounds are produced via geological and biochemical processes (Gribble 2012; Schwandner et al. 2013; Winterton 2000). Many that show persistent bioaccumulative and toxic effects usually associated with human activities, such as chloroform, methyl chloride, and polychlorinated dibenzodioxins, have significant natural sources (Harper 1994; Khalil et al. 1999; Lavric et al. 2004; Yokouchi et al. 2007). Certain life forms emit organochlorine compounds, including fish, sponges, insects, fungi, and humans (Gribble 1998; Gribble 2012). In particular, biosynthesis of organohalogens is widespread among many families and genera of basidiomycetous fungi, which produce a large range of low-molecular-weight compounds (de Jong et al. 1994; deJong and Field 1997; Field et al. 1995; Harper et al. 1988; Ortiz-Bermúdez et al. 2007; Teunissen et al. 1997; Verhagen et al. 1998). These organohalogens can accumulate in higher organisms. For example, some free-ranging wild boar meat from southern Germany contains high

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concentrations of tetrachloro-*p*-methoxyphenol; the chloroaromatic likely derives from boar eating basidiomycetous fungi (Hiebl et al. 2011). Chlorinated organics have garnered significant public attention as many show significant toxicity, bioaccumulate, and can persist in the environment. As a result, it is imperative to better understand the sources, fluxes, and mechanisms behind the biogeochemical chlorine cycling.

The Cl inventory in soils is divided between inorganic and organically bound forms, and the interplay between the two is poorly constrained. Forests in temperate ecosystems are important mediators in Cl cycling (Redon et al. 2011), because of the significance of the organochlorine turnover in forest ecosystems (Leri et al. 2007; Leri and Myneni 2010). In some forested soils, organic chlorine constitutes 100 % of the total Cl inventory, with up to 690 mg Cl/kg of soil (Redon et al. 2011). Concentrations of organically bound Cl in soils are mediated primarily by microbiota, in particular the basidiomycetes, which produce a wide range of halogenated metabolites (deJong and Field 1997; Field et al. 1995). Organochlorine formation is a dynamic process occurring during biodegradation of lignin (Öberg and Bastviken 2012; Öberg et al. 1997; Ortiz-Bermúdez et al. 2007).

Lignin is, after cellulose, the most abundant terrestrial biopolymer on Earth, occurring importantly as a structural component in plants (Boerjan et al. 2003). The chlorination of lignin by fungi likely occurs through the secretion of haloperoxidases, enzymes that catalyze the H_2O_2-dependent oxidation of Cl- to a reactive Cl electrophile, such as hypochlorous acid (HOCl), which then chlorinates the lignin (Ortiz-Bermudez et al. 2003; Reina et al. 2004). Recent work has demonstrated the global role of fungal chloroperoxidase-mediated chlorination of lignin (Ortiz-Bermúdez et al. 2007).

Most organohalogens produced by lignicolous basidiomycetes are aromatic (deJong and Field 1997; Field et al. 1995; Teunissen et al. 1997), the most common being the chlorinated anisyl and chlorinated hydroquinone metabolites (deJong and Field 1997; Field et al. 1995; Teunissen et al. 1997). The methyl ethers of the tetrachlorinated hydroquinone metabolites are called drosophilins (Fig. 1). The first such compound was isolated from the basidiomycete *Psathyrella conopilus* (= *Drosophila subatrata*) and was called drosophilin A (abbreviated DA), named after the earlier genus name of the fungus (Kavanagh et al. 1952). DA is tetrachloro-4-methoxyphenol (Anchel 1952). The first reference to drosophilin A methoxy ether is from *Phellinus fastuosus* (= *Fomes fastuosus*) from India (Singh and Rangaswa 1966), with reported concentration of 2,400 mg drosophilin A methyl ether (DAME)/kg of dry fungus. Concentrations to ~1,000 mg DAME/kg of dried fruiting body were also found in *Phellinus robiniae* (Dominguez et al. 1972). Another basidiomycete, *Mycena megaspora*, formed long white crystalline needles of DAME while growing on malt agar (van Eijk 1975). DA and DAME are produced by a range of lignicolous basidiomycetes, in particular several species of *Phellinus* (deJong and Field 1997; Teunissen et al. 1997). The purpose of DA and DAME biosynthesis is poorly understood (Teunissen et al. 1997). Ligninolytic activity occurs together with DA and DAME production, suggesting a physiological role for the tetrachlorinated hydroquinone metabolites in lignin degradation. In addition, these compounds show antimicrobial activity that may act as a chemical defense against other lignicolous or antagonizing organisms that are competing for the substrate (Anchel 1952; Teunissen et al. 1997).

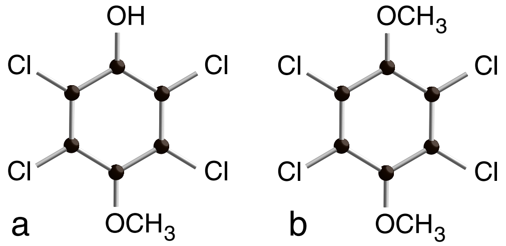

Fig. 1 **a** Drosophilin A (DA) also called tetrachloro-4-methoxyphenol. **b** Drosophilin A methyl ether (DAME) also called tetrachloro-1,4-dimethoxybenzene

In addition to their ability to produce a range of organochlorine compounds (deJong and Field 1997; Field et al. 1995; Teunissen et al. 1997), taxa of *Phellinus* are of interest because of their potential dietary, medicinal, and pharmacological benefits (e.g., Dai et al. 2010; Lee et al. 2011; Meera and Janardhanan 2012). Fungi produce a range of bioactive metabolites, some of which are used for their medicinal properties (Dai et al. 2010). Several hundred species of *Phellinus* have been described (www.mycobank.org), most of which are lignicolous, growing on and in living trees or dead wood. One species used for medicinal purposes, *Phellinus linteus* [= *Phellinus yucatensis*], contains DAME (Hsu et al. 1971). Extracts of this mushroom are consumed as a tea in eastern Asia, suggesting the possible consumption of and accumulation of DAME within humans.

Most studies of the fungal production of organochlorine compounds are conducted under laboratory or in vitro conditions. In contrast, we describe the formation of large quantities of the chloroaromatic drosophilin A methyl ether by the basidiomycete *Phellinus badius* in the mesquite tree *Prosopis juliflora*.

Materials and methods

Study site and samples collected

The serendipitous discovery of large quantities of drosophilin A methyl ether in mesquite trees and subsequent association with *Phellinus badius* occurred as a result of the May 8th, 2011, Pickett fire east of Phoenix, Arizona (centered around 33° 16.427′N, 111° 10.454′W in Alamo Canyon). Thick stands of mature mesquite (*Prosopis juliflora*) grow along the canyon and creek bottoms. Many of these trees were consumed by the fire, which characteristically burned near the base of the tree with the fire spreading within the decayed heartwood.

On May 15th, 2011, the senior author was collecting ash for a mineralogical study and encountered a pungent, at times overpowering, chemical odor in and around the burnt and smoldering mesquite forest. Smoldering followed the easy-to-burn decayed heartwood, leaving hollowed out areas, the top interior surfaces commonly contained fur-like masses composed of lustrous golden to clear needle-like crystals (Fig. 2). The largest mass was ~10×30 cm, with crystals up to 2 cm long. These crystals were the source of the pungent chemical odor in the smoldering mesquite forest.

The origin of the pungent crystals was determined by sectioning a fallen ~25-cm diameter log, hollowed out at one end by fire and containing the pungent crystals, until unburnt wood was encountered. This unburnt wood showed a core of pale yellow and porous decayed heartwood surrounded by dense brownish red, fresh heartwood. The decayed heartwood also possessed the pungent odor of the fur-like masses. Optical microscopic examination of the decayed heartwood shows masses of golden-brown mycelial felts studded with white to glassy clear crystals, with individual elongated, euhedral crystals to 1 mm long (Fig. 3a).

A search for fungi on the mesquite trees with heartwood rot in the study area revealed a perennial bracket fungus growing on trunks near ground level of several trees. Twenty-four basidiocarps were found along a 2-km stretch of Alamo Canyon. Fresh specimens are bright yellowish brown and produce an abundance of dark reddish-brown spores (Fig. 3b). The old specimens are hard and woody with a thick cracked crust. The characteristics of this fungus match that of the basidiomycete *Phellinus badius* (Gilbertson et al. 1976). Several basidiocarps were collected, all of which are consistent with *Phellinus badius*. The fungus was common on mature mesquite trees growing along the riparian areas of Alamo Canyon.

X-ray diffraction

Single-crystal data collection was performed on a Bruker SMART APEX single-crystal diffractometer. Data were acquired at 123 K from a 0.262×0.064×0.062-mm crystal. Powder X-ray diffraction patterns were acquired with a Rigaku D\Max-IIB powder XRD employing Cu $K\alpha$ radiation. Representative pieces were crushed and deposited onto a single-crystal low-background quartz slide covered with a thin coating of Vaseline. Samples were scanned from 5° to 65° 2θ.

Decayed heartwood extractable compounds

Soluble and crystallizable materials were extracted from gram-sized pieces of *Phellinus badius* basidiocarps and decayed heartwood using chloroform at 50 °C for 24 h. The solvent was decanted and allowed to evaporate. The resultant crystalline material was weighed and identified by powder X-ray diffraction.

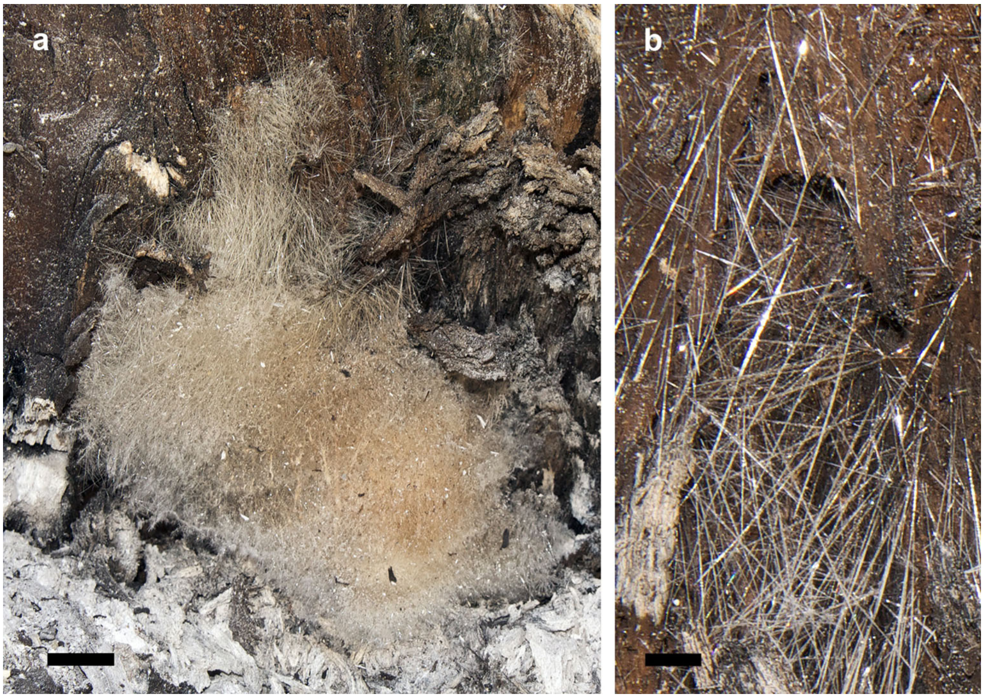

Fig. 2 Photographs of DAME on a smoldering mesquite tree in Alamo Canyon, Arizona. **a** Large fur-like mass of DAME crystals stained light brown by smoke. In the foreground is white mesquite ash. The crystals adhere to a shell of unburnt mesquite wood. *Scale bar*=1 cm. **b** Close-up photo showing individual DAME needles. *Scale bar*=1 mm

Fig. 3 **a** Photograph of elongated glassy DAME crystals in decayed heartwood. The crystals are nestled on a bed of golden-brown mycelia. Also visible are smaller white crystals of Ca oxalate. *Scale bar*=200 µm. **b** The basidiocarp of *Phellinus badius* (sample B4 in the text and Table 1). This year's spore-producing basidiocarp is yellowish brown, with rusty brown spores staining the tree bark below. The older basidiocarps are blackened, woody, and rimose. *Scale bar*=1 cm

Proton-induced X-ray emission

Proton beams were accelerated at low energy (1.90 MeV) with a 1.4-MeV Tandetron tandem accelerator (Cockroft-Walton type manufactured by General Electric). The proton beam of 1×1 mm crosses a 7.8-µm-thick kapton foil window before entering the sample chamber and striking the sample. The sample chamber is evacuated to low vacuum to avoid air signal and X-ray absorption. A Canberra Si(Li) detector (detector resolution 5.9 keV line is 168.0 eV) is placed at 47° from the normal of the sample surface, which is oriented at 45° with respect to the incoming proton beam. No filters were used in front of the detector for the low-energy, light-element analyses. Proton current incident on the sample was ~0.5 nA.

Proton-induced X-ray emission (PIXE) was used to measure minor elements of Na and above in sections of heartwood and *Phellinus badius* basidiocarps. Sections of heartwood were cut and filed flat and smooth. Sanding was not used as grains from the sandpaper became embedded in the wood and contributed to the PIXE spectra. Multiple points were analyzed on each section of heartwood. The fresh cut surface of the basidiocarps were filed giving ~2 g of powder that was compressed with a pellet press into 1-cm diameter disks. Spectra were acquired from areas ~1×1 mm. Each spectrum was acquired for a total of 20,000 counts (approximately 5 to 7 min).

The PIXE data were processed with the GUPIX software (www.physics.uoguelph.ca/PIXE, updated 2005). The PIXE spectra showed peaks for Na, Mg, Al, Si, P, S, Cl, K, Ca, and minor Fe. The NIST biological standard reference material Bovine Liver (SRM-1577) was used for calibration of the PIXE data.

Results

Fur-like tufts composed of pungent, transparent, needle-like crystals were collected within hollows of nine smoldering trees. In all cases, the tufts were accompanied by an overpowering, pungent chemical odor. Single-crystal structure analysis of a crystal from the large tuft (Fig. 2a) is monoclinic, space group $P2_1/c$, with a=4.0456(4), b=14.3251(14), c=9.1775(10)Å, β=104.2577(13)°, Z=2, and V=515.49(9) Å^3. The structure solved by direct methods is consistent with tetrachloro-1,4-dimethoxybenzene (Wieczorek 1980), and so the details of the data collection and structure refinement are not shown. For historical reasons (see explanation in the "Introduction"), this compound is called drosophilin A methyl ether and abbreviated as DAME—an acronym that will hereafter be used. Powder XRD patterns from all the tufts match the powder pattern calculated from the single-crystal DAME data (Fig. 4): no other reflections are present. Thus, the abundant crystalline fur-like masses in the smoldering mesquite trees is DAME, and is hence the source of the pungent chemical odor in the smoldering mesquite forest.

In order to determine the origin of the DAME tufts, sections of 12 unburnt logs were collected from separate trees with heartwood rot; in all cases, the decayed wood smelled strongly of DAME. Chloroform extracts were taken from five of the rotten heartwood samples: powder XRD patterns from

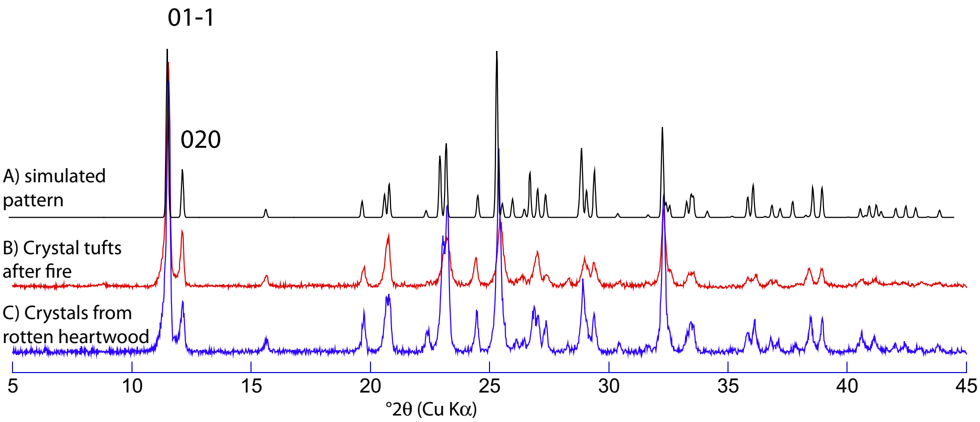

Fig. 4 (*A*) Simulated powder XRD pattern of DAME calculated from the single-crystals structure data. (*B*) Representative powder pattern for one of the crystal tufts in the smoldering mesquite wood. (*C*) Representative powder pattern of the crystalline material after chloroform extraction from the rotten heartwood

the white crystalline powder after evaporation of the chloroform match the calculated powder XRD pattern of DAME (Fig. 4). No other reflections were present. Optical microscopic examination of the rotten heartwood showed clusters of glassy crystals of DAME up to 1 mm long, within voids and associated with the fungal hyphae (Fig. 3a). Quantities of DAME were determined by chloroform extraction from five trees with heart-rot; the decayed heartwood contained high concentrations of DAME, viz., 31.4±7 g DAME/kg of decayed heartwood. The association with the hyphae is consistent with a fungal source for its formation. The powerful odor of DAME makes it easy to locate trees with extensive heartwood rot.

Eight *Phellinus badius* basidiocarps were collected, all smelled strongly of DAME. Four fresh, living basidiocarps (B1 to B4) were sectioned, all showed active spore production along the margin of the basidiocarp. The basidiocarps were so odorous that nitrile gloves were needed in their handling and they were cut and prepared in a fume hood. The sections show a distinctly stratified hymenophore attesting to their perennial life cycle. PIXE spectra acquired from compressed pellets made from the basidiocarps show well-resolved, intense peaks for Cl, K, and Ca, and smaller resolved peaks for Mg, Al, Si, S, and P (Fig. 5). Chlorine concentrations ranged from 22,224 to 28,588 ppm, whereas the other minor elements were more variable in their values (Table 1).

Gram-sized pieces of B1 and B2 were extracted with chloroform—a white crystalline powder remained after evaporation of the solvent. Powder XRD patterns of the crystals match the calculated powder pattern from DAME; no other reflections are present. DAME concentrations in the basidiocarps are 29.4 g/kg in B1 and 27.0 g/kg in B2, equivalent to 15.0 g Cl/kg and 13.8 g Cl/kg for B1 and B2, respectively. Chloroform extraction from a third, older woody basidiocarp resulted in 17.0 g DAME/kg.

In order to determine the source of the high levels of DAME and hence Cl in the decayed heartwood, PIXE spectra were acquired on samples of fresh heartwood. Sections of heartwood were collected from six trees within Alamo Canyon. The PIXE spectra for all samples showed well-resolved peaks for Cl, K, and Ca, which are present at the thousands of parts per million levels (Table 2). Peaks for Mg, Al, Si, P, and S were occasionally present, with maximum concentrations of a few hundreds of parts per million or less (data not shown). Trace element metals detected at the tens of parts per million include Fe, Ni, Cu, Zn, Sr, and Rb. Means for Cl ranged from 3,527 (tree 4) to 5,758 (tree 2)ppm; *K* values were similar to and paralleled those of Cl, whereas Ca are variable (Table 2). The Ca concentrations are associated with the heterogeneous distribution of Ca oxalate crystals. The Cl mean is near 5,000 ppm (4,809±1,295 ppm, *n*=30), which is well above other common trees from Alamo Canyon, i.e., *Parkinsonia microphylla* (506 ppm), *Olneya tesota* (284 ppm), *Senegalia greggii* (434 ppm), and the invasive *Tamarix ramosissima* (698 ppm). The reason for the order-of-magnitude higher Cl content of the mesquite wood relative to the others is unknown.

Discussion

The data and associations described above are consistent with *Phellinus badius* as the source of the chloroaromatic secondary

Table 1 Minor element concentrations, in parts per million, determined by PIXE from basidiocarps pellets

Basidiocarp	Mg	Al	Si	P	S	Cl	K	Ca
B1	1,237	1,283	1,604	1,788	1,654	28,588	44,921	9,586
B2	955	410	458	734	1,330	23,600	42,608	8,174
B3	1,452	304	487	1,111	1,227	22,224	21,331	17,240
B4	1,301	591	584	680	1,074	23,177	39,848	15,089

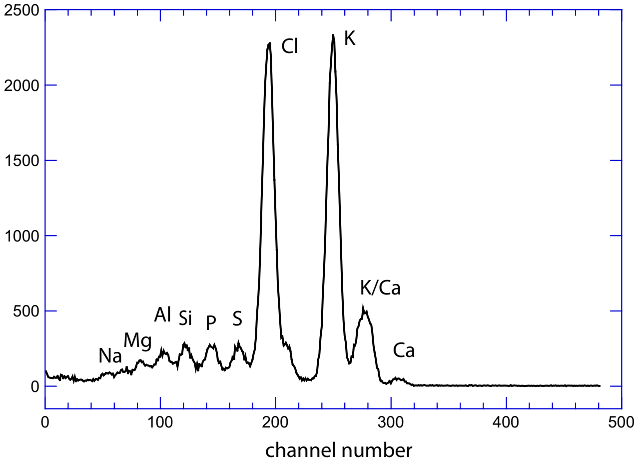

Fig. 5 Representative PIXE spectrum from basidiocarp B1 (see Table 1)

metabolite DAME in the *Prosopis juliflora* decayed heartwood. While published studies have described DAME within the fruiting body, culture, or leaf litter, this report is the first to locate significant quantities in situ at the site of the decay in a natural setting. The high concentrations of DAME in the decayed heartwood are a direct consequence of the high Cl heartwood content (Table 2). This high Cl content implies significant accumulations per tree. For example, aboveground biomass measurements (trunk and branches) from a visually average-sized mesquite tree from the study site gives a volume of 6.5×10^5 cm^3, equivalent to a biomass of 540 kg (wood density of 0.83 g/cm^3). Previous measurements of *Prosopis* show that the bulk (94 %) of the biomass is contained in the trunk and branches, with high levels of aboveground biomass and net primary production (Sharifi et al. 1982). The PIXE measurements for the heartwood yielded an average of ~5,000 ppm Cl, equivalent to ~3 kg Cl in the visually average-sized mesquite tree. These high Cl values translate to significant aboveground bioaccumulations. For example, a 1-km stretch of the study site along Alamo Canyon contains 635 mesquite trees, giving an estimated 1,900 kg of Cl in the heartwood.

The high Cl contents in the basidiocarps (Table 1) are evidence of its translocation from within the tree and accumulation in the external growing fruiting body. Fungal fruiting bodies have been shown to concentrate elements above those present in their host ecosystem (Řanda and Kučera 2004; Rudawska and Leski 2005; Vogt and Edmonds 1980), with up to 26,774 ppm Cl (Řanda and Kučera 2004). This high Cl content occurs in *Amanita rubescens*; certain species of *Amanita* also produce high concentrations of chlorinated nonprotein amino acids (deJong and Field 1997). Much of the Cl in the *Phellinus badius* basidiocarps is located within DAME, for example, fruiting body B1 contained 2.94 wt% DAME, equivalent to 15,300 ppm Cl. After chloroform extraction, B1 contained 7,100 ppm Cl, showing that the majority of Cl is extracted and contained in DAME. The high Cl concentrations in the basidiocarps are significantly above the 0.5 g Cl/kg of heartwood.

The destiny of the DAME in the heartwood with decay will in part depend on the fate of the tree, i.e., whether decay continues or the tree is consumed by fire. DAME is sparingly soluble in water and can persist in the environment for many years. For example, one of the DAME-bearing logs sampled in 2011, which was sectioned and left on the ground, was resampled in 2014 and contained abundant DAME in the rotten heartwood, even after exposure to the weather for three years. Thus, DAME can persist in decayed wood for many years. While the fate of DAME released to the environment has not been studied, DA is degraded by bacteria into recalcitrant chlorophenols (Milliken et al. 2004), suggesting that the structurally similar DAME would behave similarly.

The fire that swept through the study area partially burned many of the old mesquite trees, the majority of which had heart-rot. The smoldering fire volatilized the DAME, some of which subsequently condensed in the cooler parts of the smoldering logs. However, the overpowering smell of DAME in the smoldering mesquite forest attests to a potentially significant atmospheric pathway for this chloroaromatic.

Wood combustion is a significant source of atmospheric chloro-organics, e.g., hexachlorobenzene (Bailey 2001), chlorinated dioxins (Lavric et al. 2004), and a range of chlorinated aliphatics (Khalil et al. 1999). However, the magnitude of DAME released to the atmosphere through biomass burning is unknown. Mesquite is widely utilized for charcoal production in the borderland region of northern Sonora, Mexico, with

Table 2 Element concentrations, in parts per million, for Cl, K, and Ca in the heartwood of mesquite trees determined by PIXE. *n* is the number of separate points analyzed per sample

Tree	Cl		K		Ca	
	Average	Range	Average	Range	Average	Range
1 (*n*=5)	3,554	2,345–5,336	3,374	2,582–4,800	8,253	5,715–14,362
2 (*n*=5)	5,758	5,107–6,608	4,732	4,299–5,368	8,428	4,800–15,830
3 (*n*=5)	5,019	4,266–7,076	5,230	3,664–5,890	14,371	12,417–17,431
4 (*n*=5)	3,527	3,121–4,630	3,355	3,059–3,694	3,413	2,453–4,594
5 (*n*=6)	5,723	4,376–6,877	4,434	3,042–5,497	4,655	2,191–6,992
6 (*n*=4)	5,156	4,929–5,509	4,384	4,147–4,524	6,058	4,800–8,150

imports into the USA of around 20,000 tons per annum (Taylor 2006). Charcoal producers concentrate their efforts on large, mature trees growing along riparian areas (Taylor 2006); it is these trees, as shown in the Alamo Canyon study area, that are most likely to be infected by *Phellinus badius*. DAME was found in mesquite (*Prosopis juliflora*) infected with a fungus tentatively identified as *Phellinus* "*robiniae*" in northwest Mexico (Dominguez et al. 1972). Since *Phellinus robiniae* is restricted to trees of the genus *Robinia* in temperate regions of the USA, it is likely that this fungus was incorrectly identified and was actually *Phellinus badius*. As *Phellinus badius* is common on mesquite and responsible for decay of heartwood (Gilbertson et al. 1976), there is the potential for significant atmospheric DAME release during charcoal production throughout the Mexico/US border regions.

Phellinus badius has a circumglobal distribution in the tropics and subtropical regions (Gilbertson 1979), where it is widely distributed on hardwoods and especially trees of the Fabaceae. Given the extensive range of *Phellinus badius* and its propensity to form DAME within mesquites, there is the potential for extensive DAME formation within heartwood decay worldwide. Further, DAME production is not limited to *Phellinus badius*, but occurs in a range of basidiomycetes suggesting a substantial natural reservoir for this chloroaromatic compound with probable environmental significance.

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