Characterization of Microbial Volatile Organic Compounds (MVOC) Emitted by *Stachybotrys chartarum.*

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Abstract

Stachybotrys chartarum is a filamentous fungi usually found in water-damaged buildings. Severe illnesses including pulmonary, immunologic, neurologic, and oncogenic disorders have been reported after indoor exposure to this mold. Toxicity has been associated to the production of secondary metabolites (i.e., mycotoxins), and the emission of by-products, specifically microbial volatile organic compounds (MVOC). This study used three toxigenic strains of *S. chartarum* found in water-damaged buildings. The test strains were cultivated on sabouraud dextrose agar (SDA) plates and gypsum wallboard. The inoculated substrates, with their subsequent fungal growth, were incubated in a closed glass environmental growth chamber maintained at room temperature and constant relative humidity. Atmospheric gas samples were collected for four consecutive weeks using Tenax TA tubes. The tubes were thermally desorbed and the MVOC's were identified by using gas chromatography/mass spectrometry. The data showed that anisole was emitted from both substrates used and its concentration remained constant throughout the 28 days incubation period. Other MVOC's identified at different times during the incubation period, were 3-octanone, styrene, 3-methyl-anisole and 4-methyl-anisole. Future studies will include the utilization of other common building materials (ceiling tile, wood, carpet) as substrates for the cultivation of *S. chartarum* and the determination of MVOC emissions. Although MVOC have been studied extensively for other filamentous fungi such as *Aspergillus* and *Penicillium*, not much research have been done for MVOC's emitted by *S. chartarum*. These studies are expected to provide useful knowledge for the identification of the unique MVOC produced by *S.chartarum* in order to effectively detect and control the toxigenic mold.

Introduction

Stachybotrys chartarum is a filamentous fungi usually identified in water-damaged dwellings and in indoor environments with improper moisture management [1]. It has been speculated that the presence of this mold may trigger sick building syndrome (SBS) symptoms such as allergic reactions (e.g., irritated eyes, nose and throat) [2-4]. Severe illnesses including pulmonary, immunologic, neurologic, and oncogenic disorders have been reported after indoor exposure with this mold. Toxicity has been associated with the production of spores, mycotoxins and emissions of volatile by-products.

Microbial volatile organic compounds (MVOC) are secondary metabolites that may linked to some of the adverse respiratory conditions generated by *S. chartarum* [2-4].

Recent MVOC studies suggest that their emissions patterns could be used as a tracer of mold contamination because they vary from fungi to fungi, and they easily diffuse through weak barriers like wallpaper and small crevices. In buildings were occupants complaint of poor indoor air quality and SBS, MVOCs are detected prior to visual mold growth. [5]

The purpose of this research was to determine, under laboratory conditions, the identity of MVOCs emitted by three toxigenic strains of *Stachybotrys chartarum* in an effort to generate an MVOC fingerprinting system to identify this mold. These studies are expected to advance our basic understanding of the physiology of *S.chartarum* and provide useful knowledge for the detection and control of this toxigenic mold.

Materials and Methods

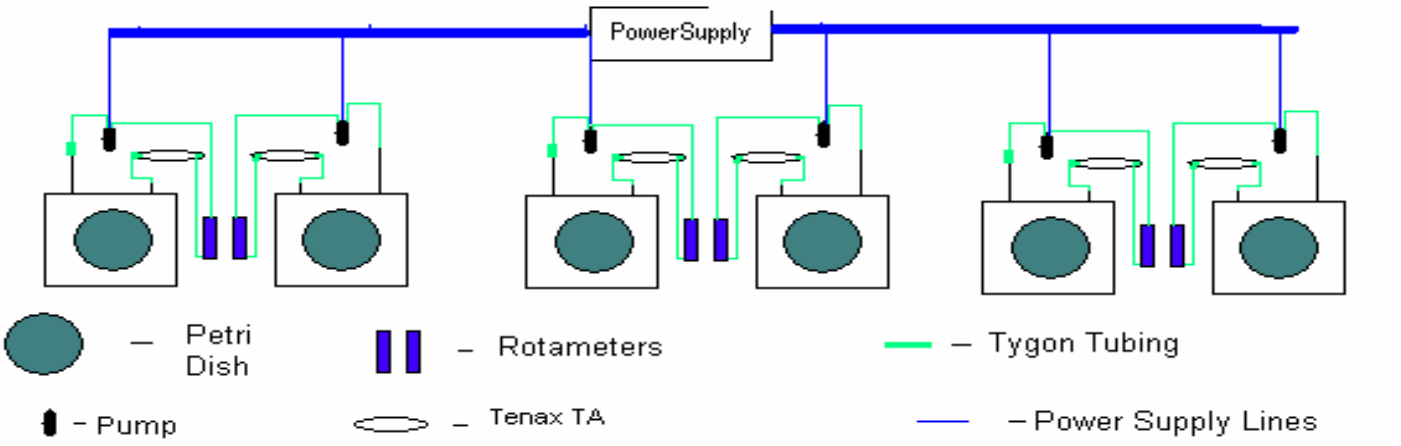
Spores from three toxigenic strains of *Stachybotrys chartarum* were used in this study. Strains RTI 3454 and RTI 3514 were isolated from water-damaged homes and were obtained from the Research Triangle Institute Collection (Research Triangle Park, N.C.). Spores from ATCC 201210 were obtained from the American Type Culture Collection (Manassas,VA). Spore suspensions were prepared as described in Crow et al.[6] with modifications for harvesting mold spores [7].

Sabouraud Dextrose Agar (SDA) and gypsum board were chosen as cultivation media. The dimensions of the gypsum wallboard coupons were 3 x 1.5″ (7.62 x 3.81 cm). All substrates were steam – sterilized by autoclaving prior to inoculation.

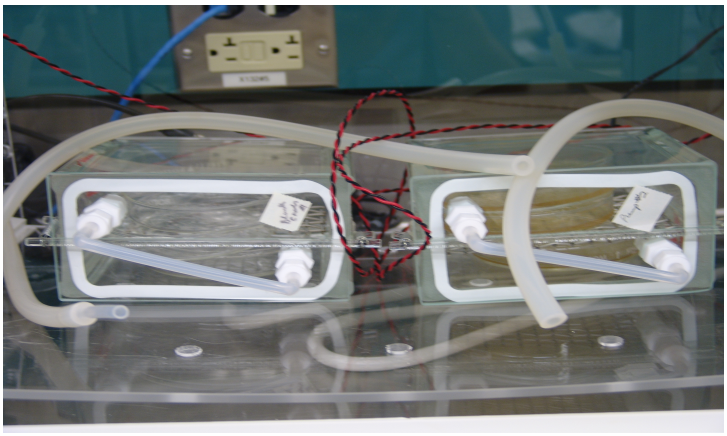
The inoculated substrates with its subsequent fungal growth were contained in a glass environmental growth chamber to quantitatively determine MVOC emissions (Fig 1). These containers consisted of all-glass thin-layer chromatography (TLC) chambers, 4 ¾ W x 2 ½ D x 4 ½″ H (12 cm x 6.4 cm x 11.5 cm) (General Glassblowing Co., Inc., Richmond, CA) which were modified to include a face plate with two ¼″ Teflon bulkhead unions (with fritted glass disks), three culture plates with a test substrate, a wire mesh separator, 0 to 1 lpm rotometer and an individual small sample pump. The size of each chamber was approximately 820 ml. Each strain was tested in duplicates – two TLC chambers per strain. Each test included a control consisting of a TLC chamber with no substrate and a second control consisting of a TLC chamber with substrate without mold spores. The MVOC sampling media were Tenax TA tubes (Supelco). Tenax TA is preferred because of is its efficiency to trap VOC and semi-VOC from air, however it is not as efficient in collecting lower molecular weight VOC's.

Samples were taken at the initial setup of the chambers and then once a week for about four weeks. SDA agar plates were inoculated initially to evaluate MVOC emissions from nutrient-rich substrates, and to ascertain the feasibility of the experimental design. Three inoculated agar plates were introduced into each of the chambers, and the air from the headspace was collected onto Tenax TA tubes for 90 minutes at a nominal airflow of 0.05 L per minute one week after inoculation. Weekly headspace samples were collected for the following 4-5 weeks, for a total of 4-5

Figure 1 Experimental Set-up



The experimental setup allows for easily introducing the sorbent tubes into the sample loop, without the need to open the growth chambers. A miniature pump draws the headspace from the chambers into the sorbent tube. The sample loop continues to a rotameter, where airflow is measured, and is then transferred back into the growth chambers, thus providing a completely enclosed sample system



samples per chamber. Once the culture media studies were completed, gypsum board was tested and evaluated according to the same procedure.

MVOC samples from the Tenax TA were thermally desorbed according to published procedures described in EPA Method TO −17, and analyzed using a HP 6890/5973 GC/MS with PE ATD 400 system. For the MVOC emissions using SDA as a substrate, the instrument was calibrated with ethylbenzene, decane, and dodecane. For the MVOC emissions using gypsum board as substrate , the instrument was calibrated with 3-methyl-1-butanol (isoamylalcohol), styrene, 3-octanone, anisole (methoxy-benzene), cyclohexanol, 4-methylanisole (1-methoxy-4-methyl-benzene), 3-methylanisole (1-methoxy-3-methyl-benzene), napthalene, and 3,5-dimethoxytoluene. For the instrument calibration, the relative response factor (RRF) method based on peak areas of extracted ion profiles for target analytes relative to the internal standard was used. Gas phase d8-toluene was used as the internal standard. The calibration standards were prepared at five levels ranging from approximately 4 to 400 ng/μL in CH_3OH. The calibration curve was constructed by spiking 2 μL of the prepared standards to Tenax TA tubes in triplicate at each concentration level.

Results and Discussion

The experimental design included two substrates, SDA and gypsum wallboard. SDA is a rich nutrient media consisting of glucose and peptone used for the cultivation of fungi. SDA was used to qualitatively characterize MVOC emissions from metabolically active cultures of *S. chartarum* and to ascertain the feasibility of the experimental design. Gypsum wallboard is a cellulose-rich, building material frequently utilized by *S. chartarum* as a nutrient source when indoor conditions are favorable [1]. Results from the SDA tests were used to characterize and quantify MVOCs emitted from gypsum wallboard. Table 1 summarizes the MVOCs emissions on SDA. Those reported as Group 1 are MVOC emissions detected for the three strains tested. Those designated as Group 2 were those emitted by at least two of the strains. All of them have been previously reported as fungal metabolites [5, 8]. Anisole, 4-methyl anisole, 3-methyl anisole and 3,5 dimethoxy-toluene were the most frequently detected within the 28d incubation period followed by 3-octanone, D-limonene, styrene, and napthalene .

Table 2 summarizes the MVOCs emitted by two of the three strains of *S. chartarum* on gypsum wallboard. Strain 3514 had not been tested at the time this report was prepared. Anisole emissions were detected for both strains after 1 week of incubation and maintained constant during the 28 days incubation period. Wilkins et al. (2000) previously reported this aromatic ether as a predominant MVOC emitted by *S. chartarum* growing on gypsum wallboard [5].

Table 3 summarizes the MVOC emissions of *S. chartarum* growing on SDA and gypsum wallboard. Results showed that only anisole and styrene were commonly produced between the two substrates utilized. These results were expected since production of volatiles is variable and dependent on both fungal species and the substrates used [8]. Fig. 2 shows the MVOC concentrations of *S. chartarum* growing on gypsum wallboard (graphs are depicted in duplicates for each of the strains tested). Of particular interest is the production of anisole. The emissions of this ether were detected

Table 1 MVOC emissions of *Stachybotrys chartarum* growing on sabouraud dextrose agar

	RTI 3454					RTI 3514					ATCC 201210				
	24h	7d	14d	21d	28d	24h	7d	14d	21d	28d	24h	7d	14d	21d	28d
MVOC- Group 1[a]															
anisole		++	++				++	++	++	++		++	++	++	+
4-methyl-anisole		++	++				++	++	+			++	++		
3-methyl-anisole		++	++				++	++				++	++		
3,5-dimethoxy-toluene		++	++				++	++	+	++		++	++	++	
3-octanone		++							++	+				++	
styrene	+		+				++			+			++	+	
napthalene	+						+	+			++				
D- limonene		++						+			++		+		
MVOC- Group 2[b]															
2-methylfuran methyl ester propanoic acid							++				++		++		
								+		++		++	++	+	
1,3 octadiene			+	+	++		+	++	++						
dimethyl-disulfide						++	++	++	+	++	++	++	++	++	++
2-butanol							++	++	++	++		++	++	++	++
2-methyl-3-buten-2-ol								+	+			+	++	++	++
3- octanol									+	++				++	
1-octen-3-ol methyl-ester benzoic acid									+	++				++	++
							++	++				++	+		

++ = emisions detected in both chambers

+ = emissions detected in one chamber

[a] MVOC Group 1: detected in all the strains tested

[b] MVOC Group 2; detected in at least two of the strains tested

Table 2 MVOC emissions of *Stachybotrys chartarum* growing on gypsum wallboard

	RTI 3454						ATCC 201210					
	24h	7d	14d	21d	28d		24h	7d	14d	21d	28d	
MVOC- Group 1[a]												
anisole		++	++	++	+			++	++	++	++	
styrene					++				+			
methyl ester propanoic acid				+	+						++	
3-hydroxy,2-butanone		+	+	+	+		+		+	++	++	
dodecamethylcyclohexasiloxane			++						+			
2-1-cyclopent-1-enyl-1-methylethylcyclopentanone		++	++	++	+					+		
3- furanmethanol	++	++	+				+					
MVOC- Group 2[b]												
trimethylsilanol		++		+								
3-methyl, 3-buten-1-ol		++	++									
3-octanone				++								
1-hydroxy,2-propanone				+								
3-methyl, 2-buten-1-ol				+								
6-methyl, 5-hepten-2-one					+							
++ = emisions detected in both chambers												
+ = emissions detected in one chamber												
[a] MVOC Group 1: detected in all the strains tested												
[b] MVOC Group 2; detected in at least one of the strains tested												

Table 3 Classification of MVOC emissions from *Stachybotrys chartarum*

Substrate	Compounds	Strains ATCC 201210	RTI 3454	RTI 3514
Sabouraud Dextrose Agar	**Alcohols**			
	2-butanol	+		+
	2-methyl-3-buten-2-ol	+		+
	3-Octanol	+		+
	1-Octen-3-ol	+		+
	Ethers			
	Anisole	+	+	+
	4-methyl anisole	+	+	+
	3-methyl anisole	+	+	+
	2-methylfuran	+		+
	Esters			
	methyl ester propanoic acid	+		+
	methyl ester benzoic acid	+		+
	Hydrocarbons			
	1,3 octadiene		+	+
	Ketones			
	3-octanone	+	+	+
	Sulfur compounds			
	Dimethyl disulfide	+		+
	Terpenes and terpene derivatives			
	Limonene	+	+	+
	Styrene	+	+	+
	Napthalene	+	+	+
	3,5 dimethoxytoluene	+	+	+
Wallboard	**Alcohols**			
	Trimethylsilanol		+	
	3-furanmethanol	+	+	
	3-methyl-3-buten-1-ol		+	
	3-methyl-2-buten-1-ol		+	
	Ethers			
	Anisole	+	+	
	Esters			
	methyl ester propanoic acid	+	+	
	Hydrocarbons			
	Dodecamethylcyclohexasiloxane	+	+	
	Ketones			
	3-hydroxy,2-butanone	+	+	
	cyclopentanone	+	+	
	3- octanone		+	
	1-hydroxy-2-propanone		+	
	6-methyl-5-hepten-2-one		+	
	Sulfur compounds			
	Terpenes and terpene derivatives			
	Styrene	+	+	

after 7 days and maintained throughout the 28 days incubation period. On gypsum wallboard its concentration ranged between 2.8 – 9.2 μg/m^3 for ATCC 201210 and 3.0 –10.0 μg/m^3 for RTI 3454 (Fig. 2). The ketone 3-octanone emitted only by strain RTI 3454 when grown on gypsum wallboard was the other volatile that was quantified; its concentrations ranging between 2.1 – 2.5 μg/m^3. Fig. 2 shows cyclohexanol among the volatiles detected early during the incubation period for both strains tested, however this VOC was also detected on the control chambers in amounts comparable to those detected for the tested strains (data not shown). Cyclohexanol emissions may originate from the environmental conditions or some of the components of the experimental set-up. We were not able to quantify any of the other MVOC previously detected on SDA – 4-methylanisole, 3-methylanisole, styrene, napthalene, and 3,5-dimethoxytoluene .

Conclusion

In summary, our study showed that the MVOC anisole (methoxy-benzene) was produced by the three strains of *S. chartarum* when grown on SDA. MVOC anisole was also emitted by two of the strains tested when grown on gypsum wallboard. Our MVOC emission pattern for both substrates is similar to those reported previously for this toxigenic mold [5]. We were able to demonstrate that our experimental set-up is capable of measuring MVOCs using different substrates and these could be maintained at a constant level throughout the incubation period.

Future research will include studying additional toxigenic strains of *S. chartarum* and developing MVOC emission patterns using other building materials such as ceiling tiles, carpet and wood.

Acknowledgements

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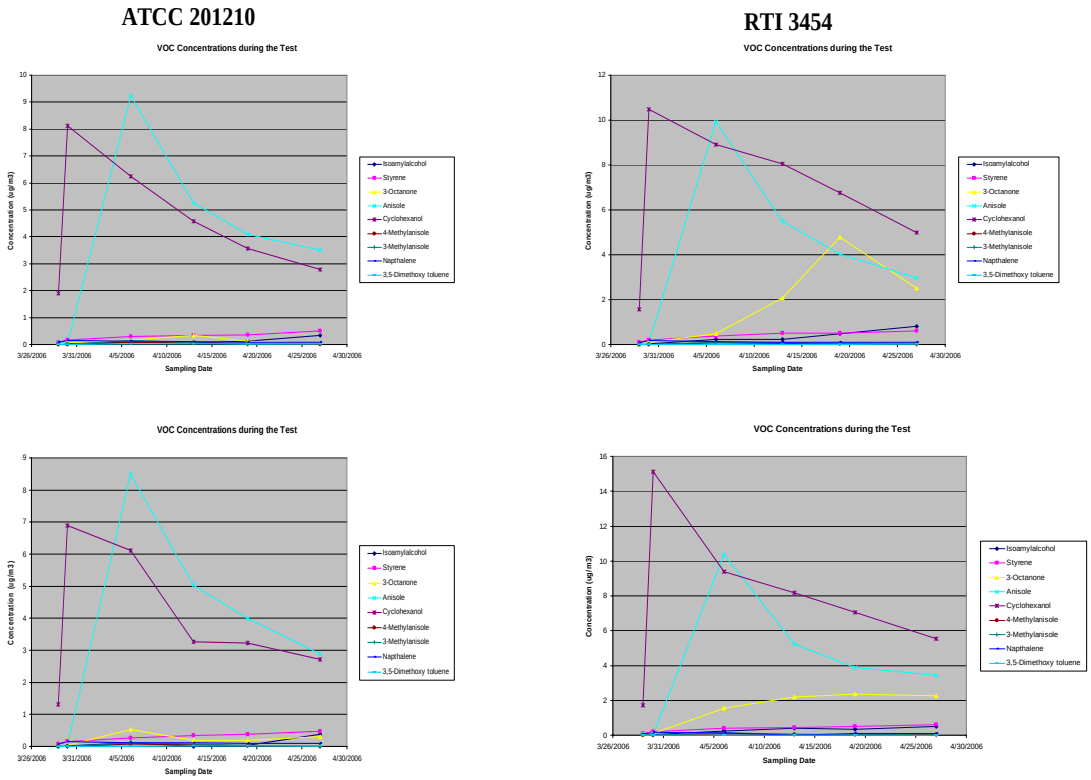
Figure 2 MVOC Concentrations of *Stachybotrys chartarum* growing on gypsum wallboard



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