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Spore trap analysis and MSQPCR in evaluating mold burden: a flooded gymnasium case study

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TABLE OF CONTENTS

1. ABSTRACT

A school gymnasium was accidentally flooded by the fire-suppression sprinkler system. The surface water was removed immediately and, after 10 days, a professional firm engaged to dry the environment. Twenty five days after the flooding, the school decided to evaluate whether there was any mold growth in the gymnasium. The inspector used two approaches, traditional air samples or a DNA-based analysis of dust samples. Thirty five-minute air samples (for total of 75 L of air each sample) were collected with Air-O-Cell™ (AOC) cassettes and the mold structures (MS) were quantified by microscopy. These samples were compared to two identical outdoor air samples. As an alternative, two dust samples were collected and quantified by mold specific quantitative PCR (MSQPCR). Comparisons of indoor to outdoor mold concentrations in air samples were inconclusive, but applying MSQPCR to the investigation of this water-damaged environment provided a more reliable and useful answer to the extent of mold contamination than did the spore trap analysis.

2. INTRODUCTION

The World Health Organization (WHO) in its "Guidelines for Indoor Air Quality: Dampness and Mould" stated "Persistent dampness and microbial growth on interior surfaces and in building structures should be avoided or minimized, as they may lead to adverse health effects" (1). So how does the indoor air community determine if the mold exposure in a building has been "minimized"? The American Society for Testing and Materials (ASTM) International has standardized the visual inspection process for mold growth (2). However, mold is not always obvious (3). Often a building's mold contamination is evaluated by comparing indoor and outdoor mold concentrations in air samples (4). Spore trap analysis of molds, collected using short duration air sampling, represents the most common type of air sample collected to evaluate a possible mold problem indoors. These air samples are enumerated by microscopic observation.

This approach has several limitations. First, air sampling times are usually short, often less than ten

minutes, because longer times result in spore traps that are too densely covered. Second, most molds cannot be identified to the species level simply by microscopic observation of the spores alone. Third, there is no agreed upon or scientifically validated method to compare or interpret the results (5,6).

In order to solve these problems, researchers at the US Environmental Protection Agency (EPA) developed a DNA-based method of mold analysis called mold specific quantitative PCR (MSQPCR) (7,8). This technology removes the human element from the analysis, since it uses an instrument called a sequence detector to identify and quantify the molds in a sample. The interpretation of the results from this analysis is then related to a national scale of home mold burdens called the Environmental Relative Moldiness Index or ERMI^{sm} (8).

The ERMI value is computed by quantifying 36 indicator mold species in a dust sample. There are 26 Group 1 molds that indicate water-damage and 10 Group 2 species that are often found in homes, even without water-damage (8). Although the ERMI scale was created for homes, it can be used to estimate the mold burdens of other indoor environments on a comparative basis.

The goal of this study was to compare two methods of mold problem evaluation in a flooded school gymnasium. The first method utilized the traditional comparison of indoor and outdoor air samples. In the second method, dust samples were analyzed by MSQPCR and the evaluation made by using the ERMI scale.

3. METHODS

3.1. Study design

A sprinkler system accidentally poured water onto a wooden gymnasium floor for one to three hours. Standing water was removed immediately but no attempt was made for 10 days to dry the area beneath the wooden flooring. At that time, a professional firm was engaged to remove the remaining water, which took 3.5 days. The airborne and surface mold samples were collected 25 days after the initial water intrusion occurred. All sampling occurred in November 2008.

3.2. Sampling strategy

The 689 m^2 gym floor was divided into 30 sections with dimensions of 4.15 by 5.5 meters, approximately (Figure 1) based on the Environmental Monitoring & Measurement Advisor (EMMA) program designed to determine the number of AOC samples to be collected ("HotSpot-Calc 2.0; National Science Foundation, Washington, D.C). This method helps calculate the grid size and number of samples necessary to detect a single localized area of pollution with a specified probability of missing the "hot spot". This program uses selected grid shapes, hot spot shapes and identifies samples to be taken to achieve a 95% confidence level (+ 5%).

3.3. Air Sampling

Five-minute AOC samples were collected sequentially in the center of each section of floor using Buck BioAire™ Bioaerosol sampling pumps (AP Buck Inc., Orlando, FL) at a flow rate of 15 liters per minute (LPM) for a total of 75 liters (a total of 0.075 m^3 each sample). The flow rate was verified with dryCal® DC-Lite Calibrator (Bios International Corporation, Butler, NJ, USA) before and after each sample. Outdoor samples were collected simultaneously with indoor sampling at locations 1 and 34 (Figure 1). A transition hallway sample between indoor and outdoor conditions was collected at position 37 (Figure 1). Air samples at positions 2 and 3 were set up to parallel the dust samples taken at positions 35 and 36. Air samples were collected at a vertical height of 1 m.

3.4. Dust sampling

Since this was a fairly recent water-problem, the selection of the location for the dust samples was based-on a need to cover both sides and near the middle of the gymnasium. Surface dust samples were collected at positions 35 and 36 by vacuuming 2 m^2 for 5 minutes with a Mitest™ (Indoor Biotechnologies Inc, Charlottesville, VA, USA) dust collector-fitted vacuum. The dust collector was labeled and placed into a new Ziplock™ bag before shipment to the laboratory for analysis.

3.5. Spore trap analysis

Spore trap analysis was performed by Mycometrics (Monmouth Junction, NJ) an American Industrial Hygiene Association certified laboratory. The impaction area of the AOC slide was stained with lacto-fuchsin and analyzed by microscopy using a Nikon Eclipse E200 Microscope (Nikon Instrument inc., Melville, NY). The entire impaction area was analyzed (100% reading) at 400 X or higher magnification powers, as needed. The minimum detection for microscopic analysis of air samples is one cell. The cells are identified using standard reference material (9,10,11).

3.6. MSQPCR analysis of dust samples

MSQPCR analysis was performed by Mycometrics (Monmouth Junction, NJ). Dust retrieved from the dust collector was sieved through a 300 micro meter pore size nylon mesh screen (Gilson, Lewis Center, OH). Five mg of sieved dust was placed into a sterile 2 mL screwed-capped extraction tube pre-loaded with 0.7 mm zirconia/silica beads (GeneRite, North Brunswick, NJ). Each dust sample was spiked with 1 x 10^6 conidia of *Geotrichum candidum* as an external reference, and was then extracted by a rapid mechanical bead-milling method at 5,000 rpm for 1 minute (12) and DNA purified with the use of DNA-EZ extraction kit (GeneRite, North Brunswick, NJ).

The extraction tube was shaken in the bead beater (Biospec Products, Bartlesville, OK) for one minute at maximum speed, followed by one minute of top speed centrifugation (16,000 x g) (Model 5415D, Eppendorf, Westbury, NY) to pellet the beads and debris. The crude extract was filtered through the pre-filter provided in the DNA-EZ kit to remove potential PCR interferences. The filtrate was mixed with 600 micro liter of binding buffer and was further purified according to the manufacturer's protocol provided in the DNA-EZ extraction kit. Parallel

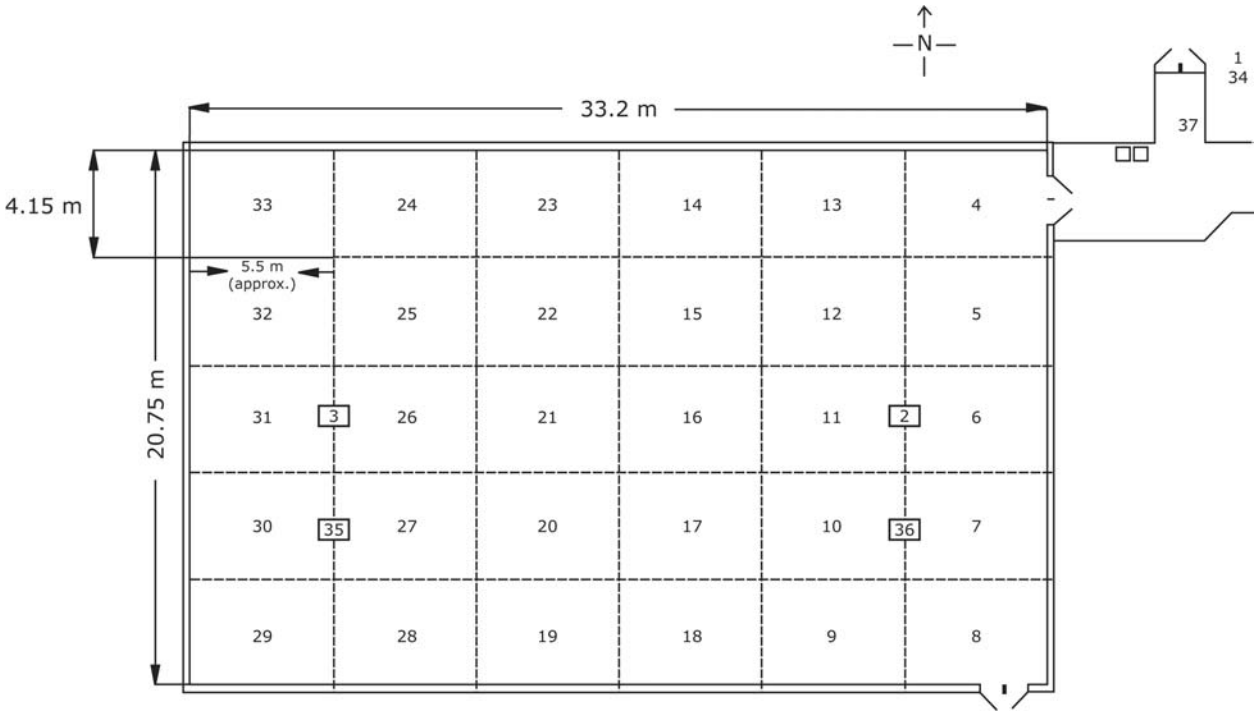

Figure 1. Location of samples associated with the gymnasium. Outdoor air samples were collected at positions 1 and 34; a hallway air sample was collected at position 37 (two small squares on map represent storage containers for gymnasium gear); indoor air samples at positions 2 to 34 and vacuum dust samples from positions 35 and 36.

analysis of a method blank (which is simply an empty extraction tube containing no dust) was performed to ensure that there was no contamination of the samples during the extraction process.

3.7. DNA-based analysis

Methods and assays have been reported previously for performing MSQPCR analyses (12,13,14) and preparing standard calibration curves for target conidia or spore equivalents. Methods for estimating the amplification factors and extrapolating spore or conidia sensitivities of the assays from the standard curves have also been described (13,14).

Briefly, the standard reaction assays contained 12.5 micro liter of "Universal Master Mix" (Applied Biosystems Inc., Foster City, CA), one micro liter of a mixture of forward and reverse primers at 25 micro molar each, 2.5 micro liter of a 400 nano molar TaqMan probe (Applied Biosystems Inc.), 2.5 micro liter of 2 mg/ml fraction V bovine serum albumin (Sigma Chemical, St. Louis, MO) and 2.5 micro liter of DNA free water (Cepheid, Sunnyvale, CA). To this mix was added 5 micro liter of the DNA extract from the sample.

Reactions were performed on the Applied Biosystems Sequence Detector Model 7900 following the manufacturer's instructions. Standard thermal cycling conditions consisted of 2 minutes at 50^{o}C, 10 minutes at 95^{o}C, followed by 40 cycles of 15 seconds at 95^{o}C for template denaturation and 1 minute at 60^{o}C for probe and primer annealing and primer extension . The Cycle threshold (C_t) determinations were automatically performed by the instrument using default parameters. Assays for each target species and the internal reference (*Geotrichum candidum*) were performed in separate tubes of the 96-well plate format.

3.8. Statistical analysis

The ERMI was calculated by taking the sum of the logs of the concentrations of the 26 Group 1 species and subtracting the sum of the logs of the concentrations of 10 Group 2 species (8). For computation of the ERMI, the concentrations in cell equivalents per mg of dust (CE mg^{-1} dust) of fungal species not detected in the dust sample were set to the minimum detection limit (MDL) of one (1) CE mg^{-1} dust before log transformation.

4. RESULTS

4.1. Spore trap data

The average MS concentration for the 30 short indoor AOC samples was 271 MS/m^3 air but varied from 52 to 707 MS/m^3 air (Table 1). The MS concentrations in the two outdoor AOC samples were 187 and 253 MS/m^3 air and 65 MS/m^3 air for the sample collected in the hallway exit (Table 2). Outdoor sample #1 contained more MS than 14 of the inside samples. Outside sample # 34 had more MS than 18 of the inside air samples. The hallway air sample, # 37, had fewer MS than any sample except for inside air sample # 6, which had only 52.

Table 1. Number of mold structures in indoor spore trap samples

Sample No.	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Total mold structures	107	93	52	840	266	279	227	347	520	159	292	307	253	187	479
Occurrence %															
Alternaria					5										
Arthrinium			25						3	8	4				
Ascospores	25	43					18	4	3	8		17	11		
Bipolaris/Dreschlera						5	18	8	3				5		
Chaetomium															
Cladosporium	37				5	5		4	8	8	14		11	14	3
Hyphal fragments	12	14		2	10	5	18	8	5	17	4	9	21	7	3
Myxomycetes											4	9			
Asp-Pen-like	25	43	75	98	80	86	47	77	67	50	73	65	53	79	95
Peronospora-like									13						
Curvularia										8					
Nigrospora															
Stachybotrys-like															
Sample No.	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**
Total mold structures	186	120	146	173	173	120	134	172	120	226	374	707	227	253	585
Occurrence %															
Alternaria		11											6		
Arthrinium				8						6	7	4			2
Ascospores	7	23	9			23	20	23	33	6	14	11	12	16	7
Bipolaris/Dreschlera				8					11	6	7				
Chaetomium								8							
Cladosporium	15		36		8			31			21	2	18	16	2
Hyphal fragments	7		18	23	23	11	30	8	23	12	3	6	12	21	5
Myxomycetes		44			16						7				2
Asp-Pen-like	72	23	36	62	54	67	50	16	33	71	39	77	53	47	68
Peronospora-like															2
Curvularia															7
Nigrospora								8							
Stachybotrys-like								8							2

Total number of mold structures per m^3 of air in the 30 indoor air samples (#4 to #33) collected with Air-O-Cell cassettes and quantified by microscopy. The percentage of total structures for each genus/category identified is also given. (All empty cells are non-detects, based on a limit of detection of one spore or cell).

Table 2. Number of mold structures in outdoor or hallway spore trap samples

Sample No.	**1**	**34**	**37**
Total mold Structures	187	253	65
Occurrence %			
Alternaria			
Arthrinium	14		20
Ascospores	14	11	20
Bipolaris/Dreschlera	7	16	
Chaetomium	7	5	
Cladosporium	21	11	
hyphal fragments	14	16	20
Myxomycetes	21	5	20
Asp-Pen-like	0	32	20
Peronospora-like			
Curvularia			
Nigrospora			
Stachybotrys-like			
Torula		5	

Total number of mold structures per m^3 of air in two outdoor air samples (# 1 and 34) and one exterior hallway sample (# 37) collected with Air-O-Cell cassettes and quantified by microscopy. The percentage of total structures for each genus identified is also given. (All empty cells are non-detects, based on a limit of detection of one spore or cell).

Asp-Pen-like spores were the most common cell type in the AOC samples, with an average occurrence of 59% and a range of 16% to 95% (Table 1). Ascopsores, *Cladosporium* and hyphal fragments were the only other commonly found structures in the AOC samples, averaging 11%, 9%, and 11%, respectively, of the total MS observed.

4.2. MSQPCR data

The surface dust samples had ERMI values of 8 and 11 (Table 3), which would place these samples in the upper 25% of mold burdens for U.S. homes (8). *Aureobasidium pullulans* was found in relatively high numbers in the dust. Also, high concentrations of *Aspergillus* cells, especially *A. niger*, as well as *Paecilomyces variotii*, were very common in both dust samples (Table 3). *Penicillium* cells were only found in low concentrations compared to *Aspergillus* cells.

5. DISCUSSION

The mold burden is based on the ERMI scale (8). The higher the ERMI value, the more likely there is water-damage and mold growth. An ERMI value above 5, places a home in the top quartile for mold burden on the ERMI

Table 3. Total number of mold cells per mg of dust

Sample No.	**35**	**36**
Mold		
Aspergillus flavus	43	23
Aspergillus fumigatus	7	4
Aspergillus niger	38000	8700
Aspergillus ochraceus	9	20
Aspergillus penicillioides	21	20
Aspergillus restrictus	ND	ND
Aspergillus sclerotiorum	3	ND
Aspergillus sydowii	16	7
Aspergillus unquis	3	1
Aspergillus versicolor	ND	ND
Aureobasidium pullulans	26000	21000
Chaetomium globosum	6	6
Cladosporium sphaerospermum	1	2
Eurotium group	34	34
Paecilomyces variotii	3700	1300
Penicillium brevicompactum	ND	ND
Penicillium corylophilum	ND	<1
Penicillium group 2	15	10
Penicillium purpurogenum	ND	6
Penicillium spinulosum	ND	ND
Penicillium variabile	2	9
Scopulariopsis brevicaulis	ND	ND
Scopulariopsis chartarum	ND	4
Stachybotrys chartarum	3	3
Trichoderma viride	ND	ND
Wallemia sebi	6	28
Sum of the Logs (Group 1)	**24.52**	**24.65**
Acremonium strictum	ND	ND
Alternaria alternate	35	29
Aspergillus ustus	310	150
Cladosporium cladosporioides 1	160	200
Cladosporium cladosporioides 2	1	1
Cladosporium herbarum	68	9
Epicoccum nigrum	1	1
Mucor group	5700	1000
Penicillium chrysogenum 2	900	260
Rhizopus stolonifer	29	16
Sum of the Logs (Group 2)	**16.23**	**13.50**
ERMI (Group 1 - Group 2)	**8.29**	**11.15**

Results in cell equivalents per mg of dust for the mold analysis in the two indoor dust samples (#35 and #36) quantified by mold specific quantitative PCR and the resulting ERMI values.

scale (8). The analysis of the two surface dust samples by MSQPCR measured ERMI values of 8.3 and 11.1, respectively. (The maximum standard deviation of any ERMI value is +/- 3.) These ERMI values placed the environment in the top 25% of the ERMI scale.

On the other hand, the short-term air samples could not be interpreted. Normally only one or two air samples inside would be compared to one or two air samples from outside. Depending on which sample location was chosen indoors, it could be concluded that there was more mold inside than outside or more mold outside than inside. Outdoor samples #1 and #34 had more mold structures observed than 47% and 64% of the indoor samples, respectively. So even the high density sampling used here could not lead to a conclusion about whether there was a mold problem in this gymnasium. Even more importantly, since most mold spores cannot be identified to the species level by microscopy alone, the populations can not be scientifically compared.

For example, Asp/Pen-like structures dominated the AOC results. Asp/Pen is a categorization of convenience that only indicates that the observed "structure" was a small round object that could not be accurately identified. If *Aspergillus* concentrations inside were comparable to *Penicillium* concentrations outside, the Asp/Pen concentrations would appear to be about the same inside and outside. Without more accurate identification, comparisons of air samples cannot be scientifically valid. Other molecular based technologies, for example Molecular ID (Abbott Laboratories, Chicago IL) can be used to identify isolated colonies but cannot be used in complex mixtures of mold cells. Mold Specific Quantitative PCR provides for both identification and quantification of complex mixtures of cells.

Without species specific identification and quantification, it is not possible to usefully interpret a mold contamination issue. The Department of Housing and Urban Development (HUD) in its Report to Congress described the situation succinctly (5): "Another problem is the difficulty in interpreting test results, since mold spores are ubiquitous and there is no consensus among experts regarding what constitutes acceptable indoor spore concentrations in indoor air or house dust, or which species are most problematic." In addition, others (6) recently summarized the results of indoor/outdoor comparison by noting that: "professional judgment in the evaluation of airborne mold sampling data leads to inconsistent conclusions regarding the presence of an indoor mold source."

Short-term air samples have been used historically for mold contamination analysis because they are seemingly simple to obtain, they can be taken in a matter of a few minutes and they are relatively inexpensive to analyze. Obtaining dust samples for MSQPCR analysis can also be rather simple, inexpensive and fast. However, MSQPCR analysis is about 5 times more expensive per sample than an air sample. This is because the analytical instrument used for MSQPCR analysis is expensive. However, this study suggests that fewer samples are needed for MSQPCR than air samples to describe the mold problem. Also, short duration air samples, analyzed by microscopy and evaluated by comparisons of the indoor and outdoor air, have been shown to be unreliable for describing mold contamination (15,16,17,18).

On the other hand, dust samples have been used to represent the longer term mold burden. Others (19) observed that dust samples provide a better indication of the cumulative microbial populations compared with short-duration air samples. Molds were found to gradually increase in concentration in floor dust (20). As long as there is moisture and available growth substrate for the molds, then the molds will continue to grow and reproduce. Even if the moisture becomes limiting, the mold spores already produced will have the opportunity to disperse unless the substrate is removed. The on-site inspector recommended a full remediation of the gymnasium, including removable of contaminated material, and the school system proceeded to complete the remediation.

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Abbreviations: L: liter; m: meter; mL: milliliter; mm: millimeter; mg: milligram

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