GROWTH AND MYCOTOXIN PRODUCTION

BY *CHAETOMIUM GLOBOSUM*

by

MATTHEW R. FOGLE, M.S.

A DISSERTATION

IN

MEDICAL MICROBIOLOGY

Submitted to the Graduate Faculty of
Texas Tech University Health Sciences Center
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Advisory Committee

David C. Straus (Chairperson)
Robert Bright
Richard Dickerson
Joe Fralick
Abdul Hamood

Accepted

Barbara C. Pence, Ph.D.
Associate Dean of the Graduate School of Biomedical Sciences
Texas Tech University Health Sciences Center

May 2007

ACKNOWLEDGEMENTS

I was fortunate to be surrounded by many people who took an interest in my education and contributed to my project in various ways. Dr. Straus offered me a position as a technician when I completed my master's degree. Nearly two years later, Steve Wilson encouraged me to pursue a PhD. Accepting Dr. Straus' offer and following Dr. Wilson's advice are two decisions I am glad I made. Both Drs. Straus and Wilson have mentored me and helped me navigate the obstacles toward completing my doctorate. Another scientist I had the pleasure of working with was Dr. David Douglas. Dr. Douglas volunteered countless hours running samples as well as discussing my project with me. I feel that finding a better trio of scientists to work with in the future will prove to be very difficult.

I would like to thank my committee members for their time and effort: Drs. Abdul Hamood, Joe Fralick, Robert Bright and Richard Dickerson. In addition, Dr. Bright provided the human epithelial cell line used in this project. I want to thank Janet Dertien for training me in cell culture techniques and using a confocal microscope and Dr. Cynthia Jumper for providing funding and laboratory space.

While working in the Department of Microbiology and Immunology, I was fortunate to meet and spend time with many other students. Two students stand out mostly because they have tolerated me longer than any others: Drs. Kendra Rumbaugh and Nancy Carty. I appreciate their support over the last 8 years.

Funding for this project was provided in part by Grant No. T42 CCT610417-11 from the National Institute for Occupational and Environmental Health (NIOSH)/Centers

for Disease Control and Prevention (CDC) to the Southwest Center for Occupational and Environmental Health (SWCOEH), a NIOSH Education and Research Center. Isolates of *Chaetomium* species were kindly provided by Janice Jones at Aerotech Laboratories (Phoenix, AZ), Dr. Weiqun Chen at P&K Microbiology Services (Cherry Hill, NJ) and Dr. Larysa Andriychuk at the Center for Indoor Air Research (Lubbock, TX). In addition, much gratitude is given to Dr. Birgitte Andersen at the Center for Microbial Biotechnology at the Technical University of Denmark (Lyngby, Denmark) for analyzing our methanol extracts and confirming the presence of chaetoglobosins A and C.

TABLE OF CONTENTS

ABSTRACT

Sick building syndrome (SBS) is a term commonly used to describe a set of non-specific symptoms resulting from poor indoor air quality (IAQ). These symptoms include: irritation of the eyes, nose and throat, dry skin, fatigue, headache, nausea, dizziness, increased number of respiratory tract infections, hoarseness, and wheezing. Over the last several years, mounting evidence has shown that fungal contamination within buildings is associated with SBS. The focus of this project is on a filamentous fungus called *Chaetomium globosum* which produces chaetoglobosins A (Ch-A) and C (Ch-C) when cultured on building material. Both metabolites belong to a group of toxins called the cytochalasins which exert their effects on mammalian cells by binding to actin. The production of Ch-A and Ch-C may contribute to the adverse health effects described by building occupants exposed to *C. globosum*. Therefore, examination of the growth of *C. globosum* and its mycotoxin production is important with regard to determining if there is a link between adverse health effects and exposure to Ch-A and Ch-C. This study had four major objectives: (1) to determine the frequency at which *Chaetomium* species are isolated in water-damaged buildings, (2) to examine the production of Ch-A and Ch-C in isolates of *C. globosum* obtained from different buildings, (3) to examine heat stability and water solubility of Ch-A and Ch-C, and (4) to examine the effects of ambient pH on growth and mycotoxin production by *C. globosum.* We found that *Chaetomium* species were commonly isolated from water-damaged buildings. Out of 30 *C. globosum* isolates, 16 produced detectable amounts of Ch-A and every isolate produced Ch-C. *C. globosum* grows best and produces the highest amount of Ch-C at a

neutral pH. Ch-A and Ch-A were relatively stable when exposed to 50^{o}C up to 3 days; however, decreased amounts were detected at longer exposure times. Exposure to 75^{o}C and higher temperatures resulted in rapid breakdown of Ch-A and Ch-C. Both compounds were poorly soluble in water.

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Sick building syndrome (SBS) is a term commonly used to describe a set of non-specific symptoms resulting from poor indoor air quality (IAQ). These symptoms include: irritation of the eyes, nose and throat, dry skin, fatigue, headache, nausea, dizziness, increased number of respiratory tract infections, hoarseness and wheezing (17). In the past, several indoor contaminants have been associated with adverse health effects. These contaminants include tobacco smoke, radon, carbon monoxide, formaldehyde, asbestos, allergens and microorganisms. Since most urban residents are indoors over 90% of the time, IAQ has become a particularly important issue (81).

Over the last several years, mounting evidence has shown that fungal contamination within buildings is associated with SBS (27, 58). In 2002, Dr. Stephen Redd presented a report on the behalf of the Centers for Disease Control (CDC) and Prevention to Congress. Dr. Redd stated "While there remain many unresolved scientific questions, we do know that exposure to high levels of mold causes some illnesses in susceptible people. Because molds can be harmful, it is important to maintain buildings, prevent water damage and mold growth, and clean up moldy materials (75)." This point was reiterated recently when Hurricanes Katrina and Rita caused extensive flooding in New Orleans and the surrounding areas. The CDC issued guidelines emphasizing the necessity to limit exposure to mold contamination in water-damaged buildings (2, 25).

Fungi are a diverse group of organisms which share several features. These organisms are eukaryotic (i.e., they contain membrane-bound nuclei and organelles) and

commonly have a haploid genome. They possess cell walls composed mostly of chitin and glucans. They are heterotropic (i.e., they depend on preformed organic nutrients) and absorb soluble nutrients through their cell wall and plasma membrane (29). The term "mold" refers to visible growth of multi-cellular fungi made up branching filamentous structures called mycelia (2). Many building materials, such as gypsum board, ceiling tile and pressed particle board are composed of cellulose. When these materials become wetted, fungal spores that are present can germinate and grow resulting in mold contamination (84).

Human illness caused by exposure to fungi can be grouped into three major categories: infection, hypersensitivity diseases, and toxicoses. Most fungal infections are opportunistic, meaning they occur only when the immune defenses are compromised. Hypersensitivity diseases include hayfever, asthma, allergic fungal sinusitis and hypersensitivity pneumonitis (or allergic alveolitis). Toxicoses can result when mycotoxins enter the body either through ingestion, direct contact (dermal route) or inhalation (27, 50, 58). Mycotoxins are non-enzymatic metabolites produced by fungi which are injurious to another organism, especially humans or animals (55). Most mycotoxins range in size between 200 to 500 mass units and are non-volatile (80).

The focus of this project is on a filamentous fungus called *Chaetomium globosum*. *Chaetomium* species are often encountered in water-damaged buildings (62, 63). Recent work has shown that *C. globosum* produces two mycotoxins when cultured on gypsum board (63). These mycotoxins were identified as chaetoglobosins A and C (63). Both metabolites are toxic when cultured with various cell and tissue culture lines (61). In addition, chaetoglobosin A was shown to be lethal when injected into rodents (67). The

rest of this introduction will summarize the known information on *C. globosum* before outlining the major objectives of the project.

Classification

Out of the five fungal phyla, *Chaetomium globosum* is categorized under the largest group called Ascomycota and further into the order Sordariales. The ascomycetes contain almost half of all known fungi and roughly 80 % of pathogenic and opportunistic species. This order consists of approximately 120 genera and 700 species. *Chaetomium* is the largest genus with around 80 species (38). Some mycologists claim there are up to twice as many species within this genus (30). *C. globosum* is the most common and widely distributed species within this genus (85). This fungus has been isolated from plant remains, seeds, compost, paper, fruit, wood, dung and straw. This organism plays an important role in the decomposition of cellulosic substrates (30). Obsolete names for this species are *C. cinnamomeum* and *C. coprophilum* (85). Several synonyms have also been reported. These names include *C. cochliodes* (92), *C. kunzeanum, C. offine, C. setosum, C. barbatum, C. subterrraneum,* and *C. japonicum* (30). *Chaetomium* species are considered teleomorphs (i.e., existing in the sexual form). A teleomorph may have up to three anamorphs. To date, an anamorph has not been identified for *C. globosum*; however, other *Chaetomium* species do have anamorphs which are listed in Table 1 (59).

Growth and sporulation

The conditions required for fungal growth include a carbon source (and/or other nutrients), water, a tolerable pH, and the absence of any inhibitory compounds (53). *C. globosum* must have a substrate with a high amount of available water (water activity, a_w > 0.9) for growth to occur (62). The optimal pH is between 7.1 and 10.4. In addition,

temperature can also affect growth. The optimal range for *C. globosum* is 16 to 25°C. The maximum temperature that allows growth of this organism is 37°C (30).

Under the appropriate conditions, a fungal spore receives a signal to germinate and then a germ tube emerges. As the fungus grows, the hyphae elongate and eventually branch. At some point, the environmental conditions which support growth deteriorate, triggering the formation of new spores and thus, beginning of a new growth cycle (53). Buston and Basu suggested that depletion of the available carbon source may initiate sporulation of *C. globosum*, but noted that exhaustion of sugar did not always induce sporulation and concluded that other factors must be involved (19). Factors that enhance sporulation include (but are not limited to) the absence of $NaNO_3$ and K_2HPO_4 (11), and the presence of calcium (10) and carbon dioxide (21) and the accumulation of metabolic products such as organic phosphates (20, 22). As the spores mature inside a sac-like structure called the ascoma (or perithecium), the walls of the ascus are degraded leaving the spores within a mucilaginous matrix. When water comes into contact with this matrix, it expands and oozes out of the ostiole releasing the spores into the environment. The outside of the ascoma is covered with numerous hairs that aid in its dispersal by insects or other animals (53).

The spores produced by *C. globosum* are very hardy and can survive for long periods of time. Our laboratory has demonstrated that these spores can remain viable for months at room temperature (93). Other researchers have reported successful attempts at culturing this fungus after storage for a period of ten (68) or even 27 years (91). High temperatures are lethal to these spores. The thermal death point was reported to be 55 to 57°C after a 10 minute exposure (30). Temperatures below freezing appear to preserve

C. globosum spores very well. After the discovery of a frozen prehistoric man in 1991, this group isolated *C. globosum* from straw lining his boots. Thus, demonstrating that these spores can remain viable in a frozen state for over 5,000 years (43).

Infections caused by *Chaetomium* species

To date, a total of 6 cases have described superficial infections caused by *Chaetomium globosum* (Table 2). Four of these cases described nail infections (28, 44, 60, 83). Two cases described skin infections caused by *Chaetomium* species (28, 97). Prior to infection, minor trauma was stated to occur in half of these cases (28, 60).

Systemic infections caused by *Chaetomium* species are rare. Thirteen cases of invasive *Chaetomium* species infection have been reported (3, 5, 7, 9, 40, 46, 57, 86, 87, 94, 95) (Table 3). These infections generally occur only in immunocompromised patients who are susceptible to many different microbes. Patients infected with *Chaetomium* species suffered from various conditions including bone marrow transplant, leukemia, intravenous (IV) drug use, renal transplant and chronic bronchiectasis.

In 8 cases, *Chaetomium* species were isolated from brain tissue possibly indicating a tropism for neural tissue (3, 5, 7, 9, 40, 87). The findings in one report suggest that direct injection of *Chaetomium* species in the normal host may allow this fungus to establish infection (3). Results from a single animal trial support this idea. *C. strumarium* spores were injected intracranially into eight outbred mice. Four mice died after 5 days, two mice exhibited signs of neurological damage after 12 days, and the remaining two mice appeared unaffected (3).

Only 3 of the 19 cases listed in Tables 2 and 3 report successful treatment of *Chaetomium* species infection: topical oxyconazole (10%) for a cutaneous lesion (28);

intraconazole for a toenail infection (44); and amphotericin B for phaeohyphomycosis (86). *In vitro* testing of 10 anti-fungal drugs on *Chaetomium* species has been performed (39, 73, 78). None of the drugs showed fungicial activity; however, some inhibitory activity was noted with the following agents: intraconazole, ketoconazole, miconazole (39), ravuconazole, voriconazole, and albaconazole (78).

In vitro effects of chaetoglobosins A and C

The chaetoglobosins belong to a group of toxins called the cytochalasins. Cytochalasins exert their effects by binding to actin. Actin is a single globular polypeptide found in all eukaryotic cells. These proteins form filaments which comprise one of the three major components of the cytoskeleton. Actin filaments are involved in maintaining the cell's shape, locomotion, and forming cell surface projections and structures inside the cell (4). The presence of cytochalasins interfere with normal cell division of eukaryotic cells (in culture) resulting in polynucleation, and inhibition of movement and nuclear extrusion (61).

The 50% growth inhibition doses of chaetoglobosins A (3.2 - 10 μg/ml) and C (10 – 32 μg/ml) have been determined with HeLa cells. Similar effects were observed with primary cell cultures of rat liver, kidney and muscle cultured with chaetoglobosin A (89). The proposed pathway for the biosynthesis of chaetoglobosin A by *C. globosum* has been described by Probst and Tamm (72).

In vivo effects of chaetoglobosin A

The acute effects of chaetoglobosin A was examined using rodents. When ingested, chaetoglobosin A was not fatal to any rats or mice tested (up to 400 mg/kg). In contrast, all rats injected intraperitoneally (IP) with 2 to 16 mg/kg died within two hours

of exposure. Histopathological examination of the visceral organs revealed congestion (i.e., an accumulation of blood), but no other specific changes. Similar results were obtained in mice. The 50% lethal dose (LD_{50}) in mice was determined to be 6.5 mg/kg in males and 17.8 mg/kg in females when injected subcutaneously (SC). In one experiment, fourteen mice were injected SC with 5 mg/kg of chaetoglobosin A. Swelling at the site of injection occurred after 6 to 8 hours. In the nine survivors, swelling disappeared after 4 or 5 days. No histopathological changes were observed after 7 days (67).

More insight into the lethal effects of chaetoglobosin A can be provided by the work conducted with cytochalasin E. This toxin is produced by the fungi *Rosellina necatrix* and *Aspergillus clavatus,* and has the same mechanism of action (i.e., binding to actin). Cytochalasin E has a similar LD_{50} as chaetoglobosin A when injected into rodents (Table 4). Within 1 hour after IP injection of cytochalasin E, a rapid decrease in plasma proteins (i.e., albumin and globulin) occurred followed by a drop in arterial pressure. Swelling was observed at the site of injection. The permeability of the blood capillaries appeared to be affected, resulting in the death due to shock within 2 to 18 hours. Congestion was observed in the liver, kidney, lung and spleen (32).

Major objectives

Based on the evidence discussed above, we speculate that the production of chaetoglobosins A and C by *C. globosum* within buildings has the potential to lead to illness in humans. Therefore, examination of the relationship between fungal growth and mycotoxin production is necessary as a first step to determine if there is a link between adverse health effects and exposure to chaetoglobosins A and C. This study had four major objectives: (1) to determine the frequency at which *Chaetomium* species are

isolated in water-damaged buildings, (2) to examine the production of chaetoglobosins A and C in isolates of *C. globosum* obtained from different buildings, (3) to examine the heat stability and water solubility of chaetoglobosins A and C, and (4) to examine the effects of ambient pH on the growth and mycotoxin production by *C. globosum*. Completion of these four objectives will be useful in the determination of a possible role in the mycotoxins of *C. globosum* in SBS.

Table 1. Known anamorphs of *Chaetomium* species.

***Chaetomium* species (Teleomorph)**	**Genus of known anamorph(s)**
C. piluliferum	*Staphylotrichum-like* *Paecilomyces*
C. megasporum	*Staphylotrichum-like*
C. trignosporum	*Scopulariopsis*
C. amesii	*Botryotrichum*
C. longirostra	*Botryotrichum*
C. seminudum	*Botryotrichum*

Table 2. Superficial infections caused by *Chaetomium globosum*.

Genus/Species	Site of fungal isolation	Disease caused by fungus	Pre-existing conditions in patient(s)	Reference
Chaetomium globosum	Skin (Lesion on right forearm)	Cutaneous phaeohyphomycosis	Minor trauma	(28)
Chaetomium perpulchrum[1]	Fingernails	Ungula phaeohyphomycosis	Minor trauma	
Chaetomium globosum	Fingernails	Onychomycosis	Minor trauma	(60)
Chaetomium globosum	Toenails	Onychomycosis	None	(83)
Chaetomium globosum	Toenail	Onychomycosis	None	(44)
Chaetomium globosum	Skin (Zygomatic regions and auricular)	Subcutaneous phaeohyphomycosis	Dilative cardiomyopathy	(97)

[1]Guarro *et al.* later identified this isolate as *C. globosum* (39).

Table 3. Invasive infections caused by *Chaetomium* species.

Genus/Species	Site of fungal isolation	Disease caused by fungus	Pre-existing conditions in patient(s)	Reference
Chaetomium species (suspect *C. cochliodes*[1])	Extrapleural fluid	Empyema	Acute leukemia and *Pneumocystis carinii* pneumonitis	(46)
Chaetomium globosum (later identified as *C. atrobrunneum*)	Brain	Cerebral phaeohyphomycosis	Renal transplant and diabetes	(5)
Chaetomium strumarium	Brain	Cerebral mycosis (3 cases)	Intravenous drug use	(3)
Chaetomium globosum	Lung	Pneumonia	Leukemia	(95)
Chaetomium atrobrunneum	Brain	Cerebral abscess	Bone marrow transplant	(40)
Chaetomium globosum	Lung	Pleural effusion and sepsis	Bone marrow transplant	(57)
Chaetomium atrobrunneum	Brain and possibly lung	Cerebritis	Bone marrow transplant	(87)
Chaetomium perlucidum	Brain, heart, lungs and spleen	Invasive mycosis	Leukemia	(9)
	Lung		Asthma and chronic bronchiectasis	
Chaetomium globosum	Aspirates of axillary and cervical lymph nodes	Phaeohyphomycosis	Bone marrow transplant	(86)
Chaetomium globosum	Brain	Cerebritis	Intravenous drug use	(7)

[1]This species is a synonym for *C. globosum* (39, 92).

Table 4. Acute toxicity of chaetoglobosin A and cytochalasin E in rodents.

Mycotoxin	**Animal model**	**Route of injection[1]**	**Sex[2]**	**LD_{50} (mg/kg)**	**Reference**
Chaetoglobosin A	Rats	IP	M, F	<2.0	(67)
		PO	M, F	>400	
	Mice	SC	M	6.5	
			F	17.8	
		PO	M, F	>400	
Cytochalasin E	Rats	IP	M	2.60	(32)
			F	2.61 - 2.64	
		PO	M	9.10	
	Mice	IP	M	4.60	
			F	4.90	

[1]IP stands for intraperitoneal. PO stands for per os. SC stands for subcutaneous.

[2]M stands for male and F for female.

CHAPTER II

MYCOTOXIN PRODUCTION BY DIFFERENT ISOLATES OF *CHAETOMIUM GLOBOSUM*

Introduction

Sick building syndrome (SBS) is a term commonly used to describe a set of non-specific symptoms resulting from poor indoor air quality (IAQ). These symptoms include: irritation of the eyes, nose and throat, dry skin, fatigue, headache, nausea, dizziness, increased number of respiratory tract infections, hoarseness and wheezing (17). Over the last several years, mounting evidence has shown that fungal contamination within buildings is associated with SBS (27, 58). The Centers for Disease Control and Prevention (CDC) have issued guidelines on limiting exposure to mold contamination in buildings after flooding has occurred (2). Exposure to mold results in human illness through three different mechanisms: infection, allergy, and toxicity (41). Inhalation of fungal spores and their associated mycotoxins can result in adverse health effects (50, 80).

Chaetomium species are often encountered in buildings with IAQ problems (62, 63). The most common species within this genus is *Chaetomium globosum* (85) which is also the most frequently isolated species in buildings (6, 62, 63, 85). When cultured on gypsum board, *C. globosum* produces two mycotoxins called chaetoglobosins A and C (63). Both of these compounds belong to a group of toxins called the cytochalasins which exert their effects on mammalian cells by binding to actin. This protein forms

filaments which are involved in maintaining the cell's shape, locomotion, forming cell surface projections and structures inside the cell (4).

The effects of chaetoglobosins A and C can be lethal to various cell lines. The 50% growth inhibition doses of chaetoglobosins A (3.2 - 10 μg/ml) and C (10 – 32 μg/ml) have been determined with HeLa cells. Similar effects were observed with primary rat cultures of liver, kidney and muscle cultured with chaetoglobosin A (89). Furthermore, injection of chaetoglobosin A is lethal when administered at relatively low doses in animals. The 50% lethal dose of chaetoglobosin A was determined in mice to be 6.5 mg/kg in males and 17.8 mg/kg in females when injected subcutaneously. All rats injected intraperitoneally with chaetoglobosin A at doses ranging from 2 to 16 mg/kg died within two hours of exposure (67).

This study had two major objectives: (1) to determine the frequency at which *Chaetomium* species are isolated in water-damaged buildings and (2) to examine the production of chaetoglobosins A and C in isolates of *C. globosum* obtained from different buildings.

Materials and Methods

Collection of air and surface samples

Between 1999 and 2006, Assured Indoor Air Quality (AIAQ, Dallas, TX) collected air and surface samples in 794 buildings with occupant complaints. Air samples were collected with a two-stage bioaerosol sampler (Andersen Instruments Inc., Smyrna, GA) on malt extract agar plates at a calibrated flow rate of 28.4 L/min for 5 minutes. This medium is commonly used to culture fungi from air samples (18). Prior to

fungal identification, these agar plates were incubated at room temperature (RT or 25°C) for 5 to 7 days. Suspected mold contamination was collected from surfaces using one of the following methods: (1) sampling with clear adhesive tape, (2) sampling with a dry cotton swab or (3) removing bulk material (i.e. building material).

C. globosum isolates/Media

Chaetomium globosum American Type Culture Collection 16021 (ATCC, Manassas, VA) was used to determine the optimal medium for the production of chaetoglobosins A and C. The following four media (Becton, Dickinson and Company, Sparks, MD) were evaluated and prepared as per the manufacturer's instructions: Difco oatmeal agar (OA), Difco potato dextrose agar (PDA), Difco malt extract agar (MEA) and BBL corn meal agar (CMA). These media were selected since they are commonly used to culture filamentous fungi (8, 70).

Different isolates of *Chaetomium* species were obtained from the following three laboratories: Aerotech Laboratories (Phoenix, AZ), P&K Microbiology Services (Cherry Hill, NJ) and the Center for Indoor Air Research (Lubbock, TX). Each isolate was obtained from a different building. Isolates were identified as *C. globosum* based on the criteria in the Pictorial Atlas of Soil and Seed Fungi (92). Ten *C. globosum* isolates were selected from each laboratory (for a total of 30) for further analysis.

Preparation of inoculum

Each *C. globosum* isolate was cultured on PDA at RT for at least 13 days until confluent growth and sporulation were achieved. Sterile water (20 ml) was poured onto one agar plate and the fungal growth scraped off using a sterile loop. The resulting suspension was passed through a cell strainer with a 70 μm nylon membrane (BD Falcon,

Bedford, MA) to remove hyphae. Spore concentrations were determined using an improved Neubauer hemacytometer (Fisher Scientific, Pittsburg, PA). Spores were counted within an area of 1 mm^2. Each spore solution was enumerated twice and then averaged. The lower detection limit was 10,000 spores/ml. The spore solution was diluted with sterile water to obtain a concentration of 25,000 spores per ml. Twenty µl of the diluted spore suspension (containing approximately 500 spores) were transferred to the center of each agar plate. The agar plates were allowed to sit overnight to absorb the inoculum before inverting and incubating at RT.

Evaluation of growth and production of chaetoglobosins A and C

Every week colony diameters were measured at right angles on each agar plate resulting in two readings. After four weeks, 20 ml of methanol were poured onto each plate and the fungal growth scraped off using a sterile loop. The contents of five agar plates were combined into a single group. The volume of methanol was measured and recorded. The spore concentration of each group was determined using a hemacytometer as previously described. The total number of spores for each group was determined by multiplying the volume of methanol extract and the spore concentration.

For each group, the methanol extract and agar from 5 plates were placed in a 600 ml beaker and sonicated for 30 minutes in a tabletop ultrasonic cleaner (FS-60, Fisher Scientific, Pittsburg, PA). The methanol extract was then passed through a fiberglass filter (GF/D 1823, Whatman, Clifton, NJ) to remove large particulates. The methanol extract was transferred to a 1 L beaker and allowed to dry under a fume hood at RT.

The dried contents of each 1 L beaker were resuspended in 20 ml of methanol. The concentrated methanol extract was then passed through 0.45 µm glass microfiber

filters (Autovial GMF, Whatman, Clifton, NJ) into a 20 ml glass scintillation vial. The vial was placed under a fume hood to evaporate the methanol. This process was repeated twice (for a total of three times) to recover any residual material left in the beaker.

After allowing to completely dry, each scintillation vial received 2 ml of methanol. The vials were vortexed until the dried residue was dissolved. The extracts were passed through 0.2 μm syringe filters (25mm sterile nylon membrane, Fisherbrand, Pittsburg, PA) into 2 ml glass vials (C4000-1W, National Scientific Company, Rockwood, TN).

Detection of chaetoglobosins A and C was performed using an 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a UV-visible diode array detector. An Agilent Eclipse C8 analytical column (400 mm [250 plus 150 mm] by 4.6 mm; particle size, 5μm) and a 12.5 mm guard column set at 40^{o}C were used in the analyses. The flow rate was set at 1.0 ml/min. Crude toxin samples in methanol were run in a mobile phase in which the gradient changed from a 35% solution of 95% water/ 5% acetronitrile to 80% acetonitrile in 20 minutes. Samples were read at 260 nm and were analyzed using Chemstation software (Agilent Technologies, Palo Alto, CA). The detection limit for each mycotoxin was approximately 50 μg/ml.

Cell line/Medium

A human lung epithelial cell line was provided by Dr. Robert Bright at the Department of Microbiology and Immunology and the Southwest Cancer Treatment and Research Center located at Texas Tech University Health Sciences Center (Lubbock, TX). This cell line was immortalized using a retrovirus which encoded the E6 and E7 transforming proteins of human papilloma virus serotype 16 (16).

The growth medium consisted of 500 ml of RPMI 1640 medium, 50 ml of fetal bovine serum, 5 ml of 200mM L-glutamine, 5 ml of 1 M HEPES, 0.5 ml of 50mg/ml of gentamicin-sulfate, 1 ml of 250 μg/ml of fungizone and 2.5 ml of penicillium (10,000 U/ml)-streptomycin (10,000 μg/ml) mixture (Cambrex BioScience, Walkersville, MD). Cell cultures were routinely maintained in 250 ml tissue culture flasks (Cellstar, USA Scientific Inc., Ocala, FL) at 37^{o}C and 5% CO_2.

Subculturing of cells

A glass coverslip (Fisher Scientific) was placed into each well of a Costar 24 well plate (Corning Inc., Acton, MA). To improve the adhesive properties of the coverslips, each well received 500 μl of poly-D-lysine hydrobromide solution (0.1 mg/ml in 0.1 M borate buffer pH 8.4) (MP Biomedicals Inc., Aurora, OH) and was allowed to incubate at RT for 2 hours before rinsing with 500 μl of Dulbecco's PBS (DPBS) (Cambrex).

Cells grown in tissue culture flask were rinsed with 12 ml of PBS. To remove attached cells, 6 ml of trypsin-versene solution (Cambrex) were added. To stop enzyme activity, 6 ml of medium were added. Suspended cells were centrifuged at 800 rpm for 5 minutes. Supernatant was removed and pellet resuspended in 10 ml of medium. Concentration of viable cells present in medium was determined by staining with trypan blue (Sigma-Aldrich, St. Louis, MO) and counting with a hemacytometer. Cells were diluted to a final concentration of 10,000 cells/ml. Each well then received 1ml of the cell suspension.

Treatment of cells with chaetoglobosin A

Stock solution was prepared by dissolving 1 mg of chaetoglobosin A (Ch-A) in 1 ml of medium. One half ml of stock solution was passed through a 0.2 μm nylon syringe

filter (Fisher Scientific). The filter was rinsed with 0.5 ml of medium. The filtered solution was mixed with 9 ml of medium to achieve the desired concentration of 50 μg/ml. After incubating for 24 hours, cells received the medium containing Ch-A (500 μl/well).

Staining of cells with DAPI and phalloidin-FITC conjugate

After incubating the plates for 72 hours with Ch-A, cells were rinsed with 500 μl of PBS containing calcium and magnesium (Sigma-Aldrich) and then fixed in 10% formalin (Sigma-Aldrich) at RT for 5 minutes. Each well was rinsed with 500 μl of DPBS and then stored at 4^{o}C. Cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) by adding 500 μl and incubated for 5 minutes at RT. After rinsing each well with 500 μl of DPBS, 250 μl of phalloidin-FITC conjugate (P5282 Sigma-Aldrich, St. Louis, MO) was added. The plate was gently rocked at RT for 1 hour. Each well was rinsed with DPBS three times. Cells were stained with DAPI nuclei acid stain (D1306, Molecular Probes Inc., Eugene, OR). The DAPI stock solution was prepared by dissolving 10 mg in 2 ml of deionized water. For staining cells, 15 ul of this stock solution was diluted in 4.5 ml of DPBS. Next, 300 μl of the diluted staining solution was added to each well for 5 minutes at RT. Cells were then rinsed twice with DPBS.

Coverslips were attached onto glass slides with mowiol mounting medium (25 ul per coverslip). Mounting medium was prepared by mixing 4.8 g of mowiol (Sigma-Aldrich) in 9.6 ml of glycerol. After adding 12 ml of water, the solution was allowed to mix at RT for several hours. Twenty four ml of 0.2 M Tris buffer (pH 8.5) (Sigma-Aldrich) was then added and the solution heated to 50^{o}C for 1 hour with occasional mixing. Next 1.2 g of DABCO, an anti-fade reagent, (Sigma-Aldrich) was added. The

mounting medium was placed in 500 µl aliquots into 1.5 ml microcentrifuge tubes, centrifuged at 8,000 rpm for 5 minutes, and stored at -20^{o}C.

Fluorescent microscopy

For confocal laser scanning, cells were examined with an Olympus IX71 inverted microscope (Olympus, Center Valley, PA) equipped with a 488 nm argon laser and a 10X objective. Images were obtained using a Coolsnap camera (Photometrics, Tucson, AZ) and Metamorph software.

Statistical analysis

Statistical analysis was performed using the SigmaStat 2.0 software (Systat Software Inc., Richmond, CA) to determine differences in colony diameter, spore production and mycotoxin production between different media. Significance was determined using Kruskal-Wallis analysis of variance on ranks followed by Tukey's post hoc analysis ($P<0.05$). Pearson product moment correlation was used to examine the relationship between colony diameter and the production of chaetoglobosins A and C ($P<0.05$).

Results

Frequency of *Chaetomium* species in buildings with occupant complaints

Chaetomium species were isolated from air and surface samples in nearly half of these buildings (Table 5). In comparison, *Cladosporium, Pencillium, Alternaria* and *Aspergillus* species were isolated more often while *Paecilomyces* and *Stachybotrys* species were found in fewer buildings. In contrast to the other five genera, *Chaetomium*

and *Stachybotrys* species were isolated less frequently in air samples compared to surface samples.

Evaluation of various inoculum sizes on PDA

This preliminary experiment was designed to determine the optimum inoculum concentration. The center of each PDA plate was inoculated with *C. globosum* ATCC 16021 spores suspended in 20 µl of water. Colony diameters were measured after seven days of incubation at RT. The colony diameters obtained on PDA plates inoculated with 5 spores was significantly less than those obtained with 50, 500 or 5,000 spores (Figure 1). Throughout the rest of the study, the size of the *C. globosum* inoculum was set at 500 spores.

Growth and sporulation on various agar media

The objective of these experiments was to determine the optimal media for the production of chaetoglobosins A and C by *C. globosum*. At 4 weeks, the colony diameters on OA were significantly higher than those grown on PDA, MEA and CMA (Figure 2). As shown in Figure 3, the colonies on OA covered the entire surface of the agar plate (85 mm). The growth on CMA was much more diffuse compared to PDA and MEA even though the colony diameters were not significantly different (Figures 2 and 3).

Since colony diameter does not take the density of growth into consideration, spore production was measured for a group of five agar plates. The number of spores produced by *C. globosum* on OA was approximately 10-fold and 100-fold higher compared to PDA and CMA respectively (Figure 4). *C. globosum* did not sporulate on MEA after 4 weeks. Tape lifts were taken from these colonies at 4 and 6 weeks, and

perithecia and spores were not present at 4 weeks, but appeared by 6 weeks (data not shown).

Production of chaetoglobosins A and C on various agar media

Chaetoglobosins A and C were detected using HPLC. Figure 5 depicts a chromatogram along with the UV spectra of these mycotoxins which is consistent with previous work (63). Crude methanol extracts of *C. globosum* cultures were incubated with a human cell line for 3 days (Figure 6). The observed effects (i.e., misshaped cells and presence of multiple nuclei) were consistent with those cell changes caused by chaetoglobosins (89). Both metabolites were detected on OA, PDA and MEA, but not on CMA. The production of chaetoglobosin A was significantly higher on OA compared to PDA but not MEA. The production of chaetoglobosin C was significantly higher on OA compared to PDA or MEA (Figure 7). When a Pearson Product Moment Correlation was performed, a positive association ($R=0.980$, $P<0.05$) was found between colony diameter and chaetoglobosin production (Figure 8).

Production of chaetoglobosins A and C by different *C. globosum* isolates

Ten *C. globosum* isolates were selected from each supplying laboratory (for a total of 30) for further analysis. Each isolate was cultured on OA for 4 weeks at RT. Each isolate covered the surface of the OA plate 4 weeks post-inoculation (data not shown) and produced 10^6 to 10^9 spores per group (Figure 9). Out of thirty different isolates of *C. globosum* examined, sixteen produced detectable amounts of chaetoglobosin A (Figure 10) and every isolate produced chaetoglobosin C (Figure 11).

Discussion

The finding that *Chaetomium* species are isolated within the indoor air at a low frequency is consistent with previous studies. Shelton et al. examined 12,026 air samples collected from 1,717 buildings across the U.S. between 1996 and 1998. Overall, this group found *Chaetomium* species in 3% of the indoor samples (total of 9,619), and in less than 1% of the outdoor samples (total of 2,407) (79). In a survey of 68 southern California houses, *Chaetomium* species were reported in 8.8% (total of 68) of air samples (56).

Our results indicate that *Chaetomium* species are encountered in water-damaged buildings and are more likely to be isolated from surface samples than from air samples. One study conducted in 21 Denmark schools between 1981 and 1984 illustrated this point (36). Although *Chaetomium* species were not detected in air samples, this genus was isolated in 25% of dust samples taken in the same locations. Recently, Vesper et al. reported similar findings using quantitative polymerase chain reaction rather than culture-based methods. *C. globosum* was detected in 23% (total of 52) of dust samples, but not in any air samples collected in water-damaged homes (90).

Altogether, these reports indicate that *Chaetomium* species are found less often in the air than in samples collected on surfaces in buildings. This trend most likely occurs due to the tendency of the relatively large spores to settle out of the air more quickly than smaller spores (35). Another explanation for the low recovery of *C. globosum* from air samples is the use of non-specific media (6). Therefore, relying solely on air samples for detection of *Chaetomium* species likely underestimates the frequency at which this fungus is actually present in water-damaged buildings. Andersen and Nissen

recommended the use of contact plates rather than air samples methods, when the objective was to detect *Chaetomium* and *Stachybotrys* species (6).

Little information currently exists regarding the frequency that *C. globosum* isolates produce chaetoglobosins. Three early studies (15, 77, 88) examined isolates of different *Chaetomium* species for mycotoxins production using thin-layer chromatography (TLC). Several problems exist with these studies. Even though 158 *Chaetomium* species isolates were examined, only five were identified as *C. globosum*. Also, TLC is not adequate for determining the presence or absence of any mycotoxin with any degree of certainty (47). The results of this study show that all 30 isolates of *C. globosum* produce detectable levels of chaetoglobosin C and roughly half produced chaetoglobosin A when cultured on artificial media (Figures 10 and 11).

Out of the four media evaluated, *C. globosum* colonies were the largest (Figures 2 and 3) and produced the most spores (Figure 4) and chaetoglobosins A and C (Figure 7) on OA. In general, it appears that as growth increases on a given medium, so does mycotoxin production.

C. globosum mycotoxins are produced on building material as well as on agar media. Nielsen et. al detected chaetoglobosins A and C in 6 *C. globosum* isolates using TLC and HPLC. These isolates were cultured on gypsum board with wallpaper paste (63). Similarly, we detected up to 21 μg of chaetoglobosin C per square centimeter when *C. globosum* ATCC 16021 was cultured on gypsum board (without wallpaper paste) for 8 weeks at RT (data not shown).

Although spores were not produced by *C. globosum* at 4 weeks on MEA, chaetoglobosins A and C were still detected. These results indicate that the production of

these secondary metabolites is not dependent on sporulation. This is different than other reports concerning mycotoxins and spore production. Gregory et. al demonstrated that satratoxin G was localized predominately in *Stachybotrys chartarum* spores as opposed to hyphae (37). Secondary metabolites produced by other fungi can induce sporulation (e.g. *Aspergillus nidulans*, *A. terreus*, and *Fusarium graminearum*), or are produced after sporulation (e.g. *Aspergillus* species, and *Pencillium urticae*) (23).

Although *Chaetomium* species spores are not detected in air samples at a high frequency, the presence of *C. globosum* contamination within a water-damaged building should not be overlooked. Based on toxicity studies, exposure to chaetoglobosins A and C could potentially cause adverse health effects in humans. Our results show that *Chaetomium* species are commonly encountered in water-damaged buildings. In addition, all isolates of *C. globosum* we examined were capable of producing mycotoxins. Since chaetoglobosin production is independent of sporulation, these mycotoxins could be carried on fungal fragments (e.g., hyphae) as has been shown in the case of *S. chartarum* (13). Future work should focus on developing a more sensitive assay than HPLC (such as an ELISA) for detecting chaetoglobosins A and C. This technology would allow IAQ researchers to determine whether or not these mycotoxins are present in the air within *C. globosum*-contaminated buildings. Despite the fact that *Stachybotrys* species spores are also not commonly found in air samples, previous studies have detected the macrocyclic trichothecenes in the air of *S. chartarum* contaminated buildings (14, 24) as well as the sera of occupants exposed to *S. chartarum* (12, 96).

Table 5. Frequency that various fungal species were isolated indoors.

Fungal genera[1]	**Number of buildings**[2]	**Air samples**[2]	**Surface samples**[2]
Total	794	10,004	27,008
Cladosporium species	724 (91.2%)	7,160 (71.6%)	2,234 (8.3%)
Penicillium species	731 (92.1%)	3,374 (33.7%)	1,546 (5.7%)
Alternaria species	652 (82.1%)	2,822 (28.2%)	531 (2.0%)
Aspergillus species	682 (85.9%)	2,377 (23.8%)	1,483 (5.5%)
Paecilomyces species	274 (34.5%)	300 (3.0%)	119 (0.4%)
Stachybotrys species	333 (41.9%)	47 (0.5%)	655 (2.4%)
Chaetomium species	389 (49.0%)	89 (0.9%)	493 (1.8%)

[1]This table does not include every fungal genus that was isolated from these buildings.

[2]The values indicate the number of buildings or samples where various fungal genera were isolated. The percentage of the total is shown in parentheses.

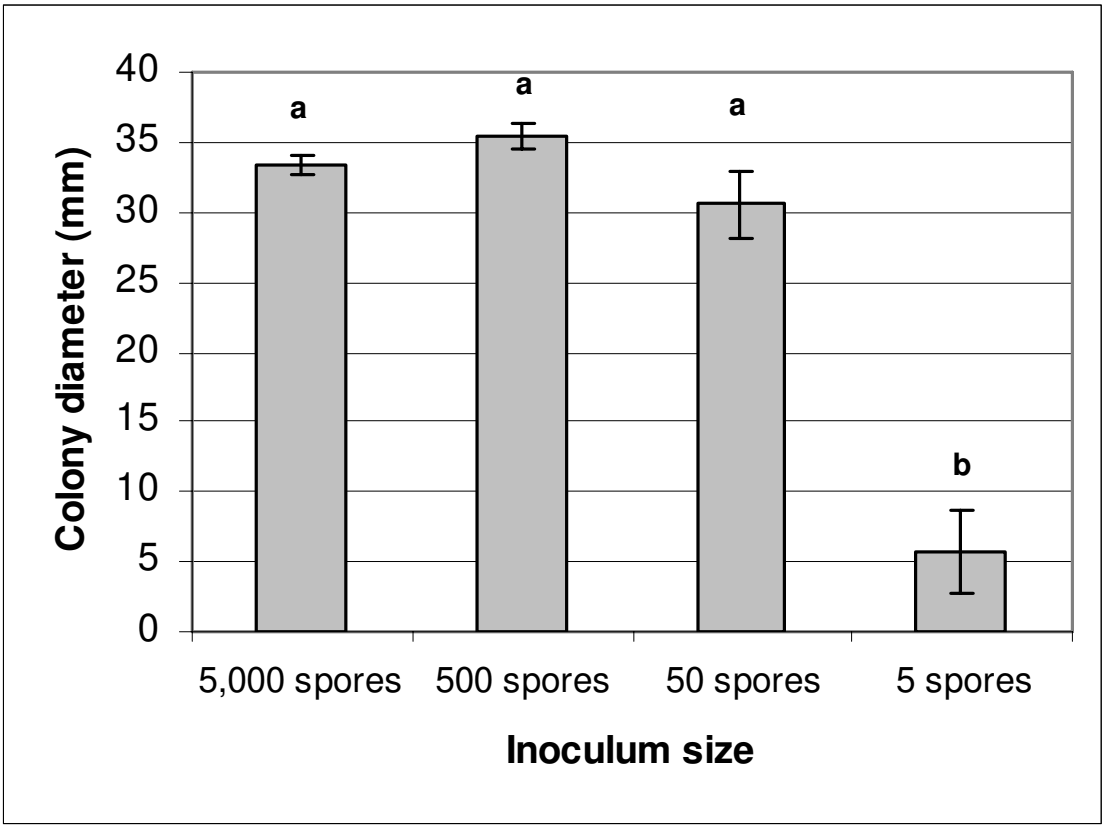


Figure 1. Effect of inoculum size on the growth of *C. globosum*. Potato dextrose agar plates were inoculated with various inoculum sizes of *C. globosum* ATCC 16021 spores suspended in 20 µl of water. Colony diameters were measured after seven days of incubation at room temperature. Mean and standard error of the mean are shown (n = 15 plates). Different subscripts denote a difference at the $P < 0.05$ significance level.

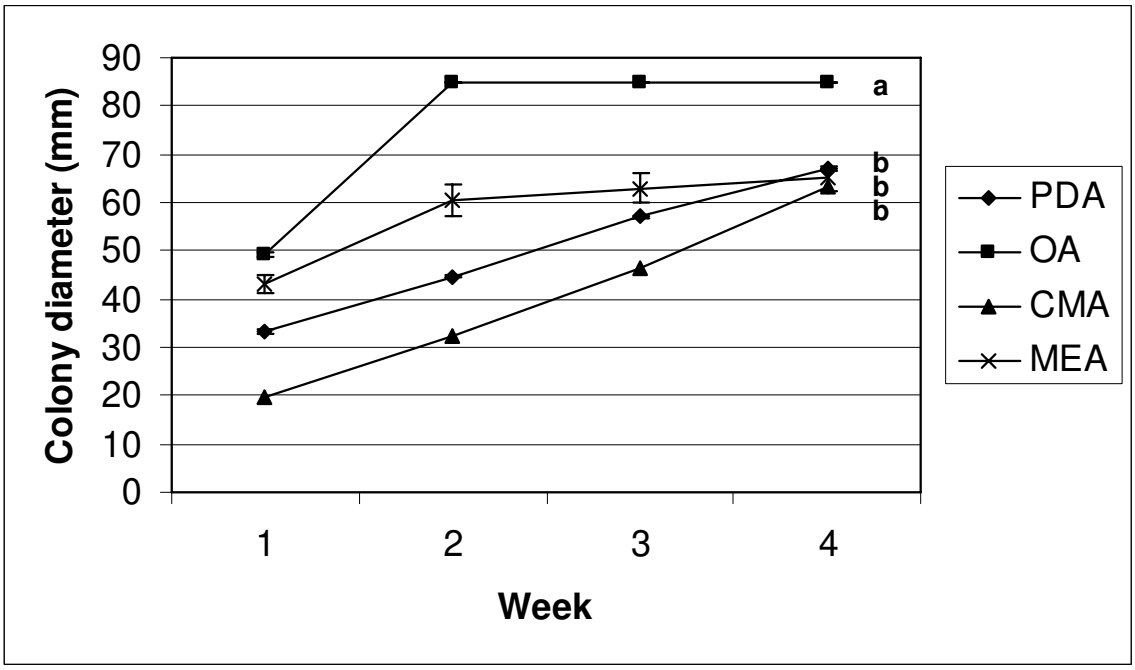


Figure 2. Colony diameters of *C. globosum* over 4 weeks on different agar media. The center of each agar plate was inoculated with 500 *C. globosum* ATCC 16021 spores suspended in 20 µl of water. The following four artificial media were evaluated: potato dextrose agar (PDA), oatmeal agar (OA), cornmeal agar (CMA) and malt extract agar (MEA). The agar plates were incubated at room temperature. Colony diameters were measured every week. Mean and standard error of the mean are shown (n = 45 plates). Different subscripts denote a difference at the $P < 0.05$ significance level.

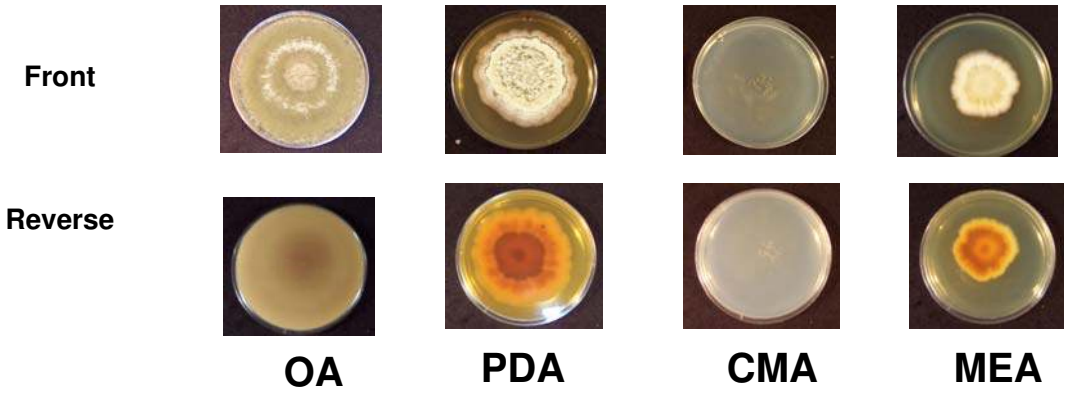


Figure 3. Photographs of *C. globosum* colonies 4 weeks post-inoculation. The center of each agar plate was inoculated with 500 *C. globosum* ATCC 16021 spores suspended in 20 µl of water. The following four artificial media were evaluated: potato dextrose agar (PDA), oatmeal agar (OA), cornmeal agar (CMA) and malt extract agar (MEA). These photographs depict the front and reverse sides of agar plates with *C. globosum* colonies after four weeks of incubation at room temperature.

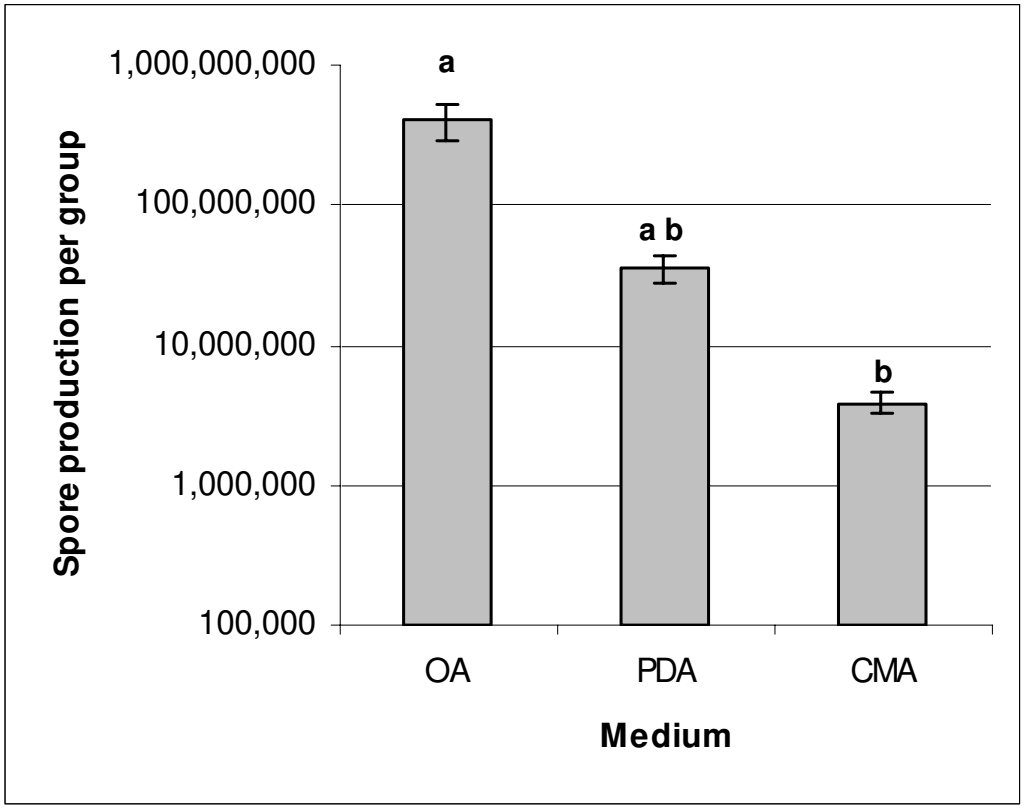


Figure 4. Spore production by *C. globosum* 4 weeks post-inoculation. The center of each agar plate was inoculated with 500 *C. globosum* ATCC 16021 spores suspended in 20 µl of water. The following four artificial media were evaluated: potato dextrose agar (PDA), oatmeal agar (OA), cornmeal agar (CMA) and malt extract agar (MEA). The number of spores (mean and standard error of the mean) produced by *C. globosum* on five agar plates is shown (n = 9 groups with 5 plates per group). Different subscripts denote a difference at the $P < 0.05$ significance level.

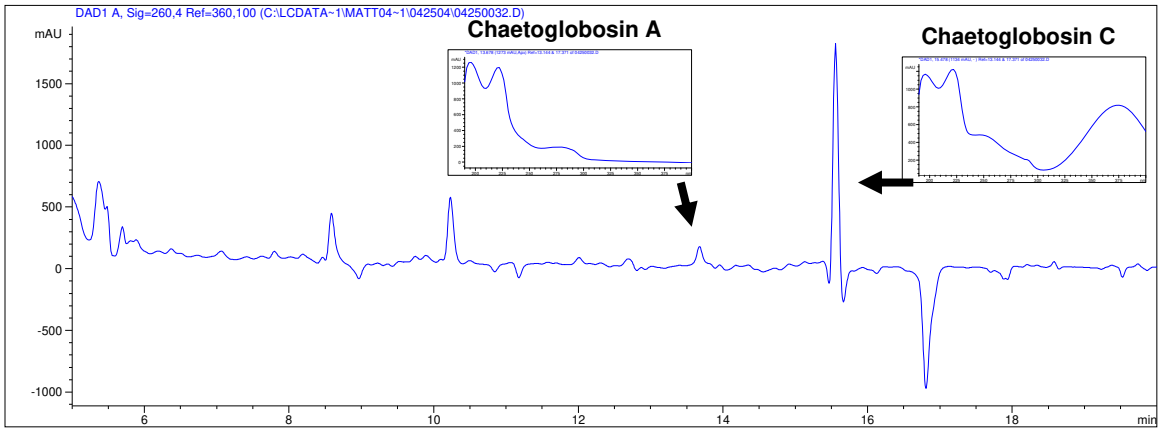
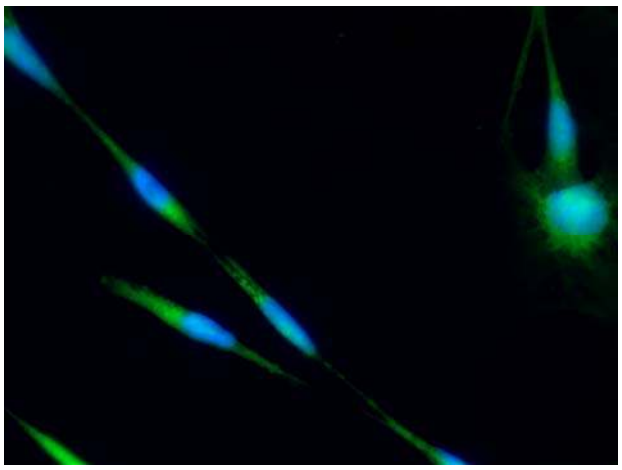


Figure 5. HPLC chromatogram with UV spectra of selected peaks inserted. The chromatogram shows the signal obtained from the methanol extract of five PDA plates with *C. globosum* ATCC 16021 growth 4 weeks post-inoculation. The retention times (min) are plotted on the x-axis and the peak sizes (in milli-absorbance units) on the y-axis. For UV spectrum analyses (insets), wavelengths (in nanometers) are plotted on the x-axis and peak sizes (in milli-absorbance units) on the y-axis.

A.



B.

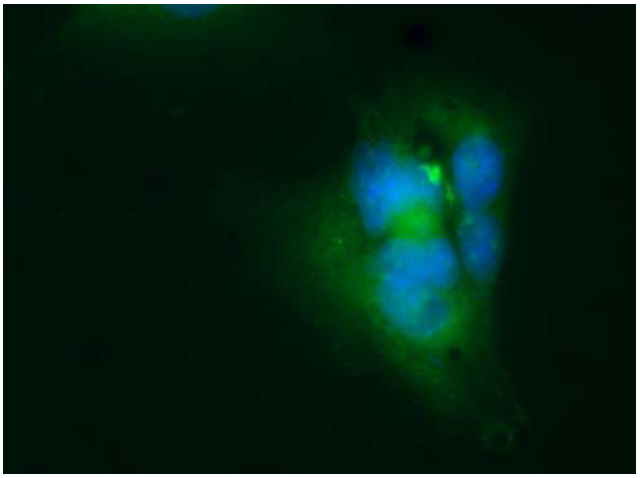


Figure 6. Effects of chaetoglobosin A on human lung epithelial cells. Micrographs (60X magnification) of human lung epithelial cells either untreated (A) or treated with 50 μg of chaetoglobosin A per ml of culture medium (B). After incubating these cells for 3 days at 37^{o}C and 5% CO_2, these cells were fixed with 10% formalin. The nuclei and actin filaments were stained with DAPI nuclei acid stain (blue color) and phalloidin-FITC conjugate (green color) respectively.

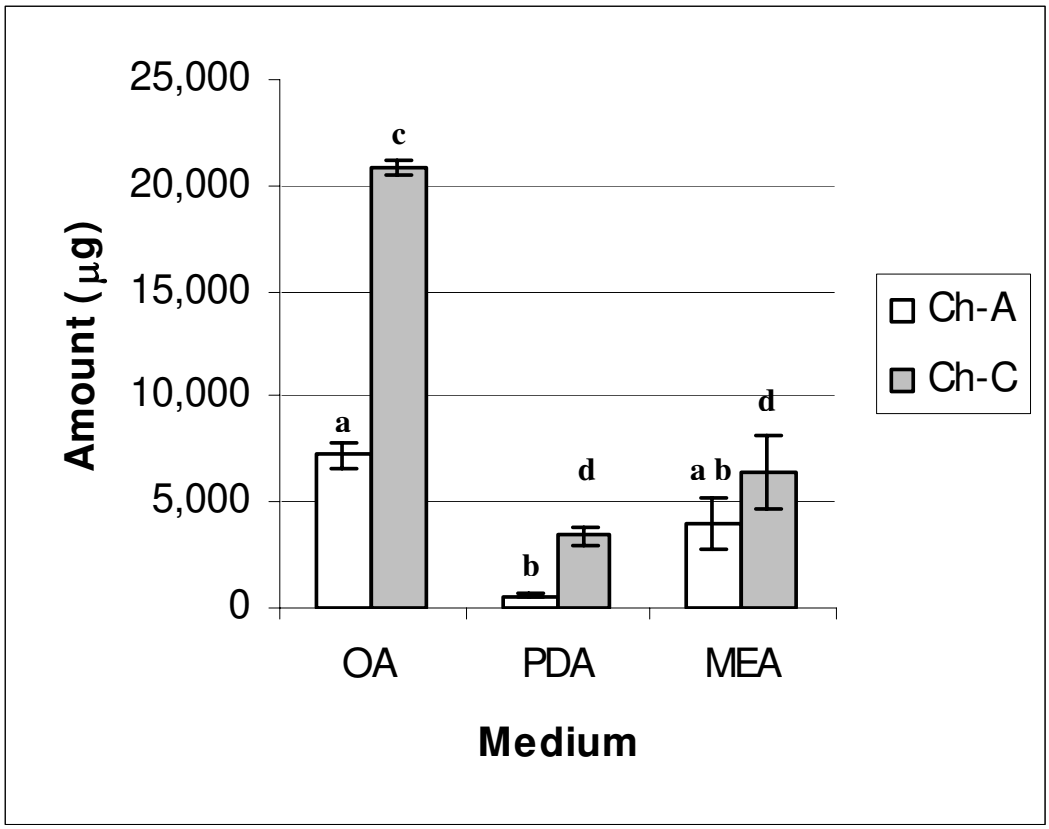


Figure 7. Production of chaetoglobosins A and C by *C. globosum* 4 weeks post-inoculation. The amount (mean and standard error of the mean) of chaetoglobosins A and C (Ch-A and Ch-C respectively) produced by *C. globosum* ATCC 16021 on five agar plates is shown (n = 9 groups with 5 plates per group). The agar plates were inoculated with 500 spores of *C. globosum* and incubated at room temperature for 4 weeks. The following four artificial media were evaluated: potato dextrose agar (PDA), oatmeal agar (OA), cornmeal agar (CMA) and malt extract agar (MEA). Different subscripts denote a difference at the $P < 0.05$ significance level.

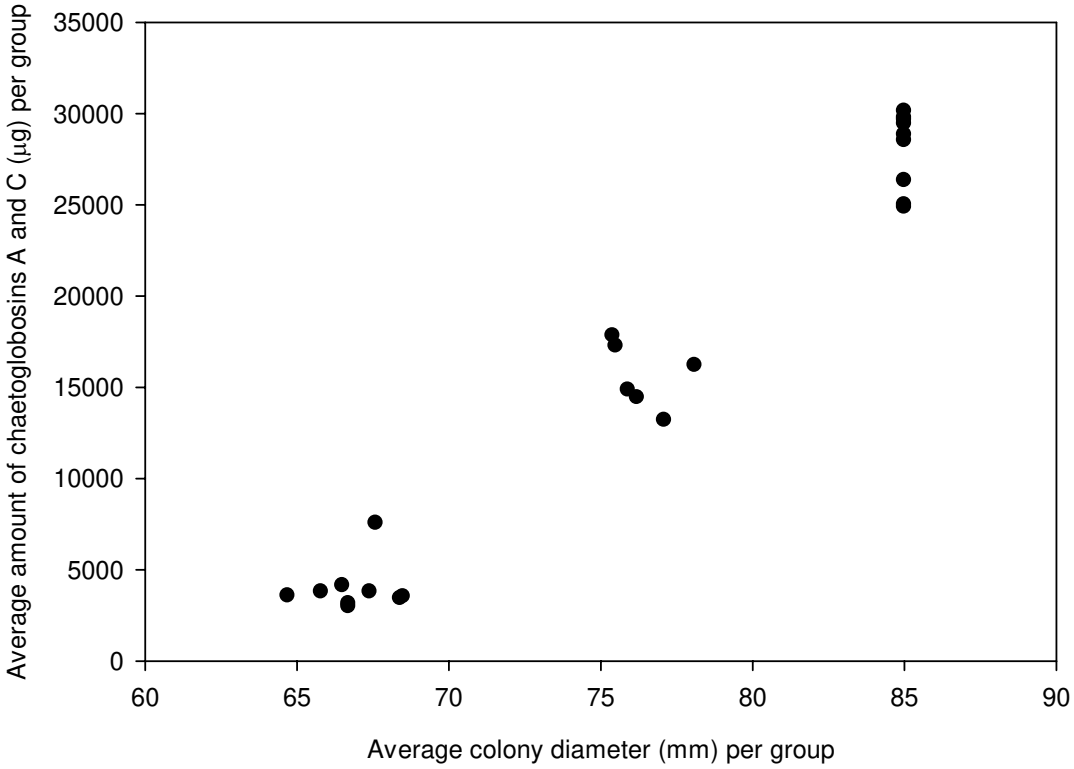


Figure 8. Relationship between growth and the production of chaetoglobosins A and C. The graph shown above depicts the average amount of both chaetoglobosins A and C produced versus the average colony diameter (4 weeks post-inoculation) per group. For this analysis (Pearson Product Moment Correlation), groups with no detectable amounts of chaetoglobosins were omitted. A positive correlation was found between colony diameter and chaetoglobosin production ($R = 0.980$, $P<0.05$, $n = 24$ groups).

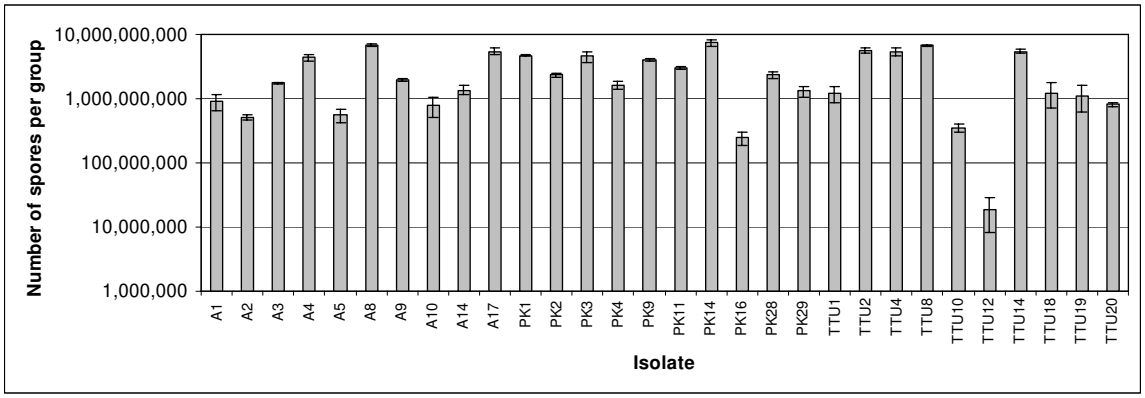


Figure 9. Spore production by different *C. globosum* isolates 4 weeks post-inoculation. The center of each oatmeal agar plate was inoculated with 500 *C. globosum* ATCC 16021 spores suspended in 20 µl of water. Plates were incubated at room temperature for 4 weeks prior to harvesting spores. The number of spores (mean and the standard error of the mean) produced by *C. globosum* on five agar plates is shown (n = 3 groups with 5 plates per group). Prefixes indicate the laboratory where each isolate was obtained: Aerotech Laboratories (A), P&K Microbiology Services (PK) and the Center for Indoor Air Research at Texas Tech University Health Sciences Center (TTU).

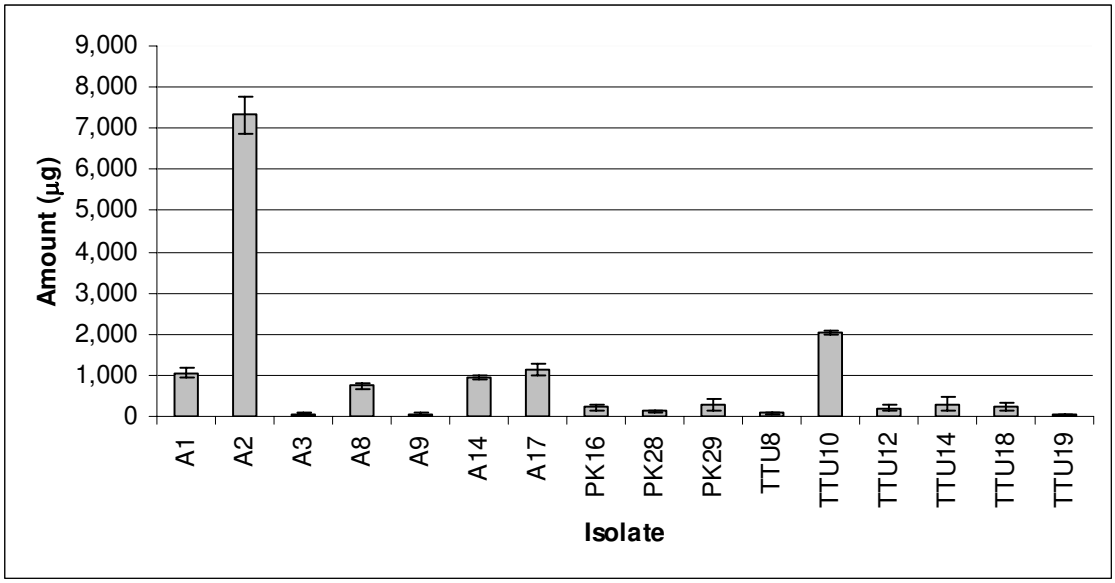


Figure 10. Production of chaetoglobosin A by different *C. globosum* isolates. The amount (mean and the standard error of the mean) of chaetoglobosin A produced on five oatmeal agar (OA) plates is shown (n = 9 groups with 5 plates per group). Prefixes indicate the laboratory where each isolate was obtained: Aerotech Laboratories (A), P&K Microbiology Services (PK) and the Center for Indoor Air Research at Texas Tech University Health Sciences Center (TTU). Each isolate was cultured on OA at room temperature for 4 weeks.

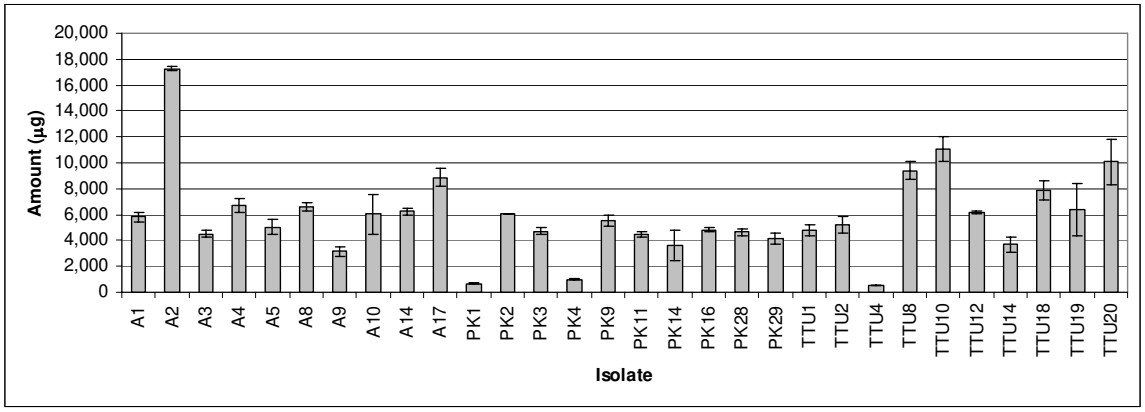


Figure 11. Production of chaetoglobosin C by different *C. globosum* isolates. The amount (mean and the standard error of the mean) of chaetoglobosin C produced on five oatmeal agar (OA) plates is shown (n = 9 groups with 5 plates per group). Prefixes indicate the laboratory where each isolate was obtained: Aerotech Laboratories (A), P&K Microbiology Services (PK) and the Center for Indoor Air Research at Texas Tech University Health Sciences Center (TTU). Each isolate was cultured on OA at room temperature for 4 weeks.

CHAPTER III

PHYSICAL PROPERTIES OF CHAETOGLOBOSINS A AND C

Introduction

Sick building syndrome (SBS) is a term commonly used to describe a set of non-specific symptoms resulting from poor indoor air quality (IAQ). These symptoms include: irritation of the eyes, nose and throat, dry skin, fatigue, headache, nausea, dizziness, increased number of respiratory tract infections, and hoarseness and wheezing (17). Over the last several years, mounting evidence has shown that fungal contamination within buildings is associated with SBS (27, 58).

Our findings as well as those from other studies demonstrate that *Chaetomium globosum* is frequently isolated from water-damaged buildings (6, 62, 63, 85). When cultured on gypsum board, *C. globosum* produces two mycotoxins called chaetoglobosins A and C (63). Both of these compounds belong to a group of toxins called the cytochalasins which exert their effects on mammalian cells by binding to actin. This protein forms filaments which are involved in various functions such as maintaining the cell's shape, locomotion, and forming cell surface projections and structures inside the cell (4). Previous work has shown that the presence of either chaetoglobosins A or C is lethal when incubated with various mammalian cell lines (89). Furthermore, injection of chaetoglobosin A is lethal when administered at relatively low doses in rodents (67).

Our previous work showed that every *C. globosum* isolate we examined could produce detectable levels of chaetoglobosin C and 16 out of 30 produced chaetoglobosin A when cultured on oatmeal agar. Further studies are required to better understand the

physical properties of these mycotoxins. This study had two major objectives: (1) to examine the stability of chaetoglobosins A and C under increasing temperatures and (2) to examine their solubility in water. Understanding heat stability and water solubility has many important implications such as safe handling in the laboratory, purification and remediation efforts.

Materials and Methods

Strains/ Preparation of medium

Two different *C. globosum* isolates were used in these experiments: American Type Culture Collection (ATCC) 16021 and Aerotech (A) 2. These isolates were grown in molasses-glucose-peptone medium (71, 72). This medium contained 20 g Grandma's molasses (Mott's Inc., Stanford, CT), 10 g D-glucose (Acros Organics, Fairlawn, NJ) and 5 g Bacto-peptone (Becton, Dickinson and Company, Sparks, MD) per liter of water. The pH was not adjusted. The medium was autoclaved at 121^{o}C for 20 minutes prior to inoculation.

Preparation of inoculum

Each *C. globosum* isolate was cultured on Difco potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, MD) at room temperature (RT) until confluent growth and sporulation were achieved. Sterile water (20 ml) was poured onto one agar plate and the fungal growth scraped off using a sterile loop. The resulting suspension was passed through a cell strainer with a 70 μm nylon membrane (BD Falcon, Bedford, MA) to remove hyphae. Spore concentrations were determined using an improved Neubauer hemacytometer (Fisher Scientific, Pittsburg, PA) as previously described

(Chapter 2). The spore solution was diluted with sterile water to obtain a concentration ranging between 25,000 to 500,000 spores per ml. One milliliter of inoculum was added aseptically to each 2 L Erlenmeyer flask containing 500 ml of medium and covered with a BugStopper 10 venting closure (Whatman, Clifton, NJ). The cultures were incubated at RT and 100 revolutions per minute (rpm) shaking for 14 days.

Collection of methanol extract

For each *C. globosum* culture, the hyphae were collected on a fiberglass filter (Whatman GF/D 150 cm diameter, Clifton, NJ) and the filtrate discarded. The filters were placed in a 1 L beaker and covered with 300 ml of methanol. After soaking in methanol overnight at RT, the filters were discarded. The methanol was evaporated under a fume hood at RT. The dried residue was resuspended in 20 ml of methanol and transferred to a 20 ml scintillation vial. The sides of the beaker were rinsed with methanol. The methanol in both the beaker and the vial was then evaporated under a fume hood. The remaining residue was resuspended in 20 ml of methanol and added to the scintillation vial.

Heat stability experiments

Various amounts of methanol extract were added to each 2 ml ampule (Wheaton cat. # 651466, Millville, NJ) to achieve approximately 1 mg of each mycotoxin. The ampules were placed under a fume hood at RT until the methanol completely evaporated and then sealed with the flame from a Bunsen burner. The ampules were exposed to 50^{o}C using an incubater (Precision, Inc., Winchester, VA) or 75^{o}C, 100^{o}C, 125^{o}C, 150^{o}C and 175^{o}C using a gravity oven (Lindberg/Blue Inc., Asheville, NC). After allowing the ampules to cool completely, each ampule was opened. The dried residue was

resuspended in 2 ml of methanol and the concentration of chaetoglobosins A and C determined using high performance liquid chromatography (HPLC) as previously described. For each set of conditions, three trials were performed with three replicates per trial for a total of nine samples.

Water solubility experiments

Each scintillation vial received 5 ml of methanol extract. After allowing methanol to evaporate under the fume hood at RT, 10 ml of water were added to each vial. Each vial was vortexed until the contents were dissolved in water. To remove any insoluble material, the suspensions were transferred to 15 ml polypropylene tubes (VWR International Inc., Aurora, CO) and centrifuged at 1,000 rpm (approximately 200 X g) for 3 minutes using a Centra-8R centrifuge (International Equipment Company, Needham, MA). Nine ml of each supernatant was transferred to a scintillation vial and the water was allowed to evaporate under a fume hood at RT. The dried residue was resuspended in 1 ml of methanol and the concentration of chaetoglobosins A and C determined using HPLC as previously described (Chapter 2). Three trials were performed with three replicates per trial for a total of nine samples.

Statistical analysis

Statistical analysis was performed using the SigmaStat 2.0 software (Systat Software Inc., Richmond, CA) to determine differences in the amounts of chaetoglobosins A and C between controls and experimental samples. Significance was determined using Kruskal-Wallis analysis of variance on ranks followed by Dunnett's post hoc analysis ($P<0.05$).

Results

Exposure to various temperatures for 1 hour or 24 hours

After 1 hour, the amounts of chaetoglobosins A and C (Ch-A and Ch-C respectively) did not significantly decrease until reaching temperatures above 125°C; however, the amount of Ch-A did drop considerably while Ch-C increased slightly at 100°C (Figure 12). The series of chromatograms (Figure 13) also illustrate this trend. The signal obtained with the control sample (Figure 13A) was virtually identical to the samples exposed to 50°C (Figure 13B) and 75°C (Figure 13C). After exposure to temperatures above 100°C, other peaks besides Ch-A and Ch-C appeared (Figures 13D-G). These peaks most likely represent breakdown products of Ch-A and Ch-C. The identity of these compounds remains unknown at this time.

Exposure to 50°C for 24 hours did not result in any loss of Ch-A and Ch-C compared to the control sample. A reduction in the amounts of both mycotoxins occurred after heating at 75°C for 24 hours; although, only Ch-A had significantly reduced levels. At 100°C, 125°C and 150°C, no Ch-A and significantly lower amounts of Ch-C were detected. Neither mycotoxin was detected after heating samples at 175°C for 24 hours (Figure 14).

Exposure to 50°C, 100°C and 150°C at various times

Heating the samples at 50°C up to 3 days did not result in any significant loss of Ch-A and Ch-C. At 4 and 5 days, the amount of Ch-A was significantly lower while the amount of Ch-C increased slightly compared to the control samples (Figure 15). The amount of Ch-A decreased after a 30 minute exposure to 100°C and continued to decrease with increasing exposure times. The amount of Ch-C appeared to increase after

30 and 60 minutes before decreasing after 150 minute exposure; however, these amounts were not significantly different from the control samples (Figure 16). After 15 minute exposure to 150°C, no Ch-A and only half the amount of Ch-C was detected compared to the control samples. Ch-C was detected between 30 and 75 minutes at significantly lower levels (Figure 17).

Solubility of Ch-A and Ch-C in water

No Ch-A was detected in the experimental samples so its solubility in water was calculated as less than 6 μg/ml. The solubility of Ch-C was determined to be approximately 10 μg/ml (data not shown).

Discussion

Chaetoglobosins A and C were relatively stable when exposed to 50°C up to 3 days (Figure 15). After 4 and 5 days, the overall amount decreased compared to the control sample suggesting that these compounds are not stable when exposed to this temperature for long periods of time. The amount of chaetoglobosin C actually increased after 3 days at 50°C (Figure 15) and after 30 and 60 minutes at 100°C (Figure 16) compared to the control sample, although not to significantly higher levels. Sekita et al. suggested that chaetoglobosin A could be converted into chaetoglobosin C "by a series of keto-enol tautomerizations (76)." Heating likely favors the keto form (chaetoglobosin C) over the enol form (chaetoglobosin A) which may explain the observed increase in chaetoglobosin C.

Exposure to 75°C and higher temperatures resulted in the rapid breakdown of chaetoglobosins A and C. Neither compound was detected after heating samples to

175°C for 1 hour. This instability of chaetoglobosins A and C when exposed to heat explains why our attempts to purify these mycotoxins were unsuccessful. In order to concentrate our samples, large volumes (2-4 liters) of methanol extract were placed on a hotplate which resulted in the loss of the vast majority of chaetoglobosins A and C.

Previous studies indicate that satratoxins G and H are somewhat soluble in aqueous solution (42, 45, 51, 52). Karunasena et al. suggested that solubility in water could allow these mycotoxins to be easily spread throughout the indoor environment away from the fungal growth site and distributed to other materials during a watering event (45, 52). It appears that the satratoxins G and H (< 1 mg/ml PBS) are more soluble in water compared to chaetoglobosins A and C (approximately 10 μg/ml water). Thus, satratoxins may pose a more significant risk to human health due to the increased risk of exposure even though the 50% lethal dose ($LD_{50)}$ is comparable. When injected intraperitoneally (IP) in mice, the LD_{50}s for satratoxin G and H are 1.23 and 5.69 mg/kg respectively (74). The LD_{50} for chaetoglobosin A was less than 2 mg/kg when injected IP into rats and between 6.5 and 17.8 mg/kg when administered to mice subcutaneously (67). Much work remains to determine whether or not a link exists between the production of these mycotoxins by *C. globosum* and the adverse health effects described by occupants in water-damaged buildings. A better understanding of the physical properties of chaetoglobosins A and C will aid IAQ researchers in establishing this link in future studies.

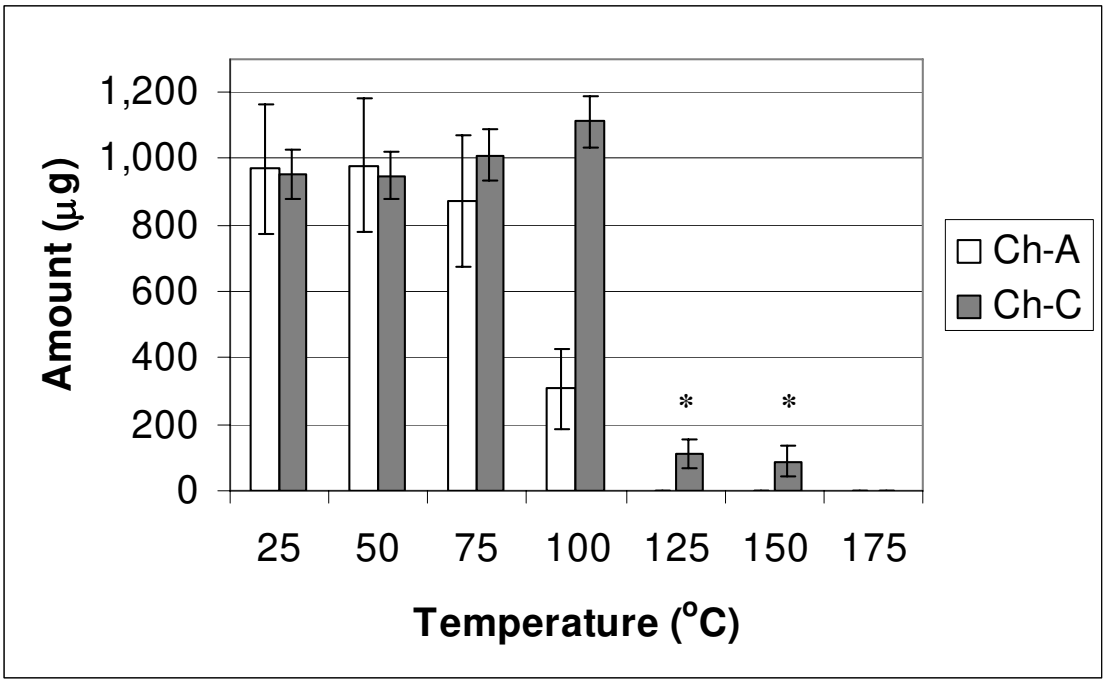


Figure 12. Exposure to various temperatures for 1 hour. The amounts (mean and standard error of the mean) of chaetoglobosin A (Ch-A) and chaetoglobosin C (Ch-C) are shown after no treatment (25°C) or a 1 hour exposure to 50°C, 75°C, 100°C, 125°C, 150°C and 175°C. Asterisks indicate a difference at the $P<0.05$ significance level.

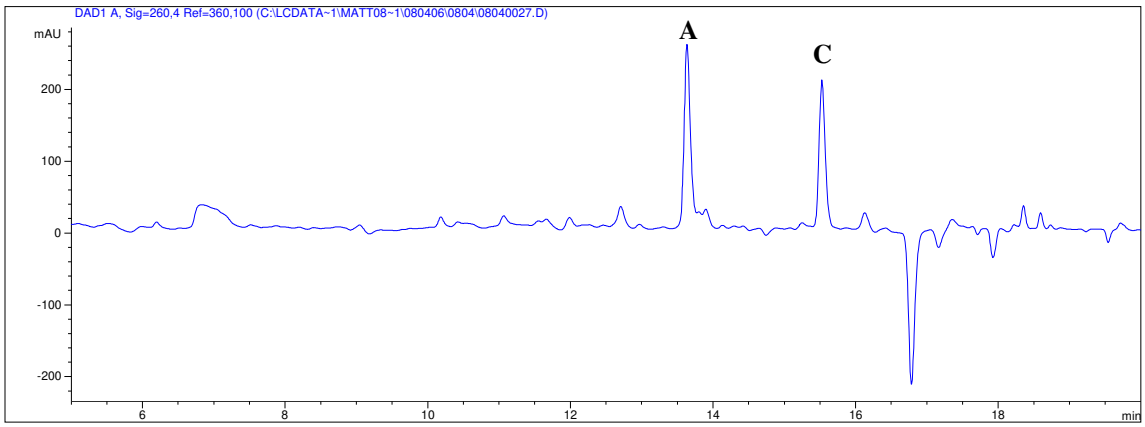


Figure 13A. HPLC chromatogram of methanol extract. The chromatograms show the signal obtained from the methanol extract of *C. globosum* after no treatment (A) or a 1 hour exposure to 50°C (B), 75°C (C), 100°C (D), 125°C (E), 150°C (F) and 175°C (G). The retention times (min) are plotted on the x-axis and the peak sizes (in milli-absorbance units) on the y-axis. Letters indicate the peak corresponding to chaetoglobosin A (A) or chaetoglobosin C (C).

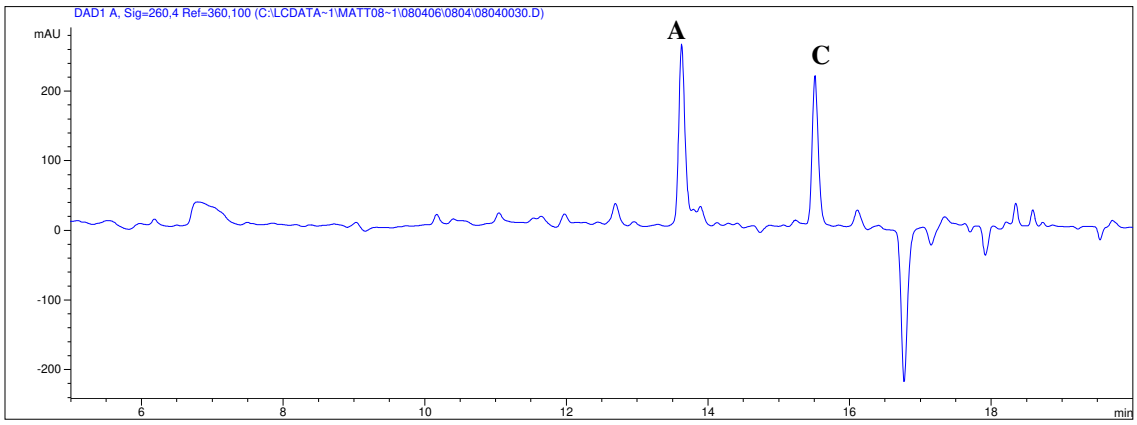


Figure 13B. HPLC chromatogram of methanol extract. The chromatograms show the signal obtained from the methanol extract of *C. globosum* after no treatment (A) or a 1 hour exposure to 50°C (B), 75°C (C), 100°C (D), 125°C (E), 150°C (F) and 175°C (G). The retention times (min) are plotted on the x-axis and the peak sizes (in milli-absorbance units) on the y-axis. Letters indicate the peak corresponding to chaetoglobosin A (A) or chaetoglobosin C (C).

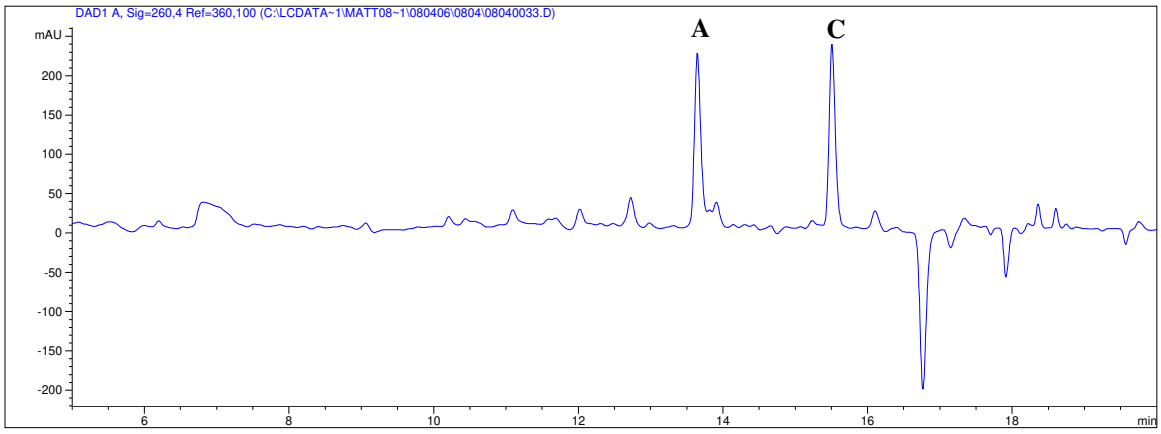


Figure 13C. HPLC chromatogram of methanol extract. The chromatograms show the signal obtained from the methanol extract of *C. globosum* after no treatment (A) or a 1 hour exposure to 50°C (B), 75°C (C), 100°C (D), 125°C (E), 150°C (F) and 175°C (G). The retention times (min) are plotted on the x-axis and the peak sizes (in milli-absorbance units) on the y-axis. Letters indicate the peak corresponding to chaetoglobosin A (A) or chaetoglobosin C (C).

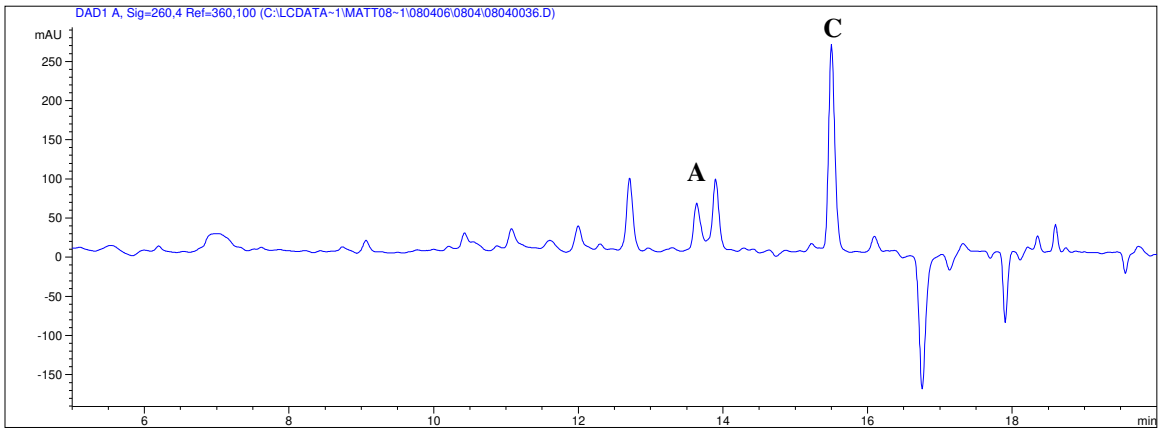


Figure 13D. HPLC chromatogram of methanol extract. The chromatograms show the signal obtained from the methanol extract of *C. globosum* after no treatment (A) or a 1 hour exposure to 50°C (B), 75°C (C), 100°C (D), 125°C (E), 150°C (F) and 175°C (G). The retention times (min) are plotted on the x-axis and the peak sizes (in milli-absorbance units) on the y-axis. Letters indicate the peak corresponding to chaetoglobosin A (A) or chaetoglobosin C (C).

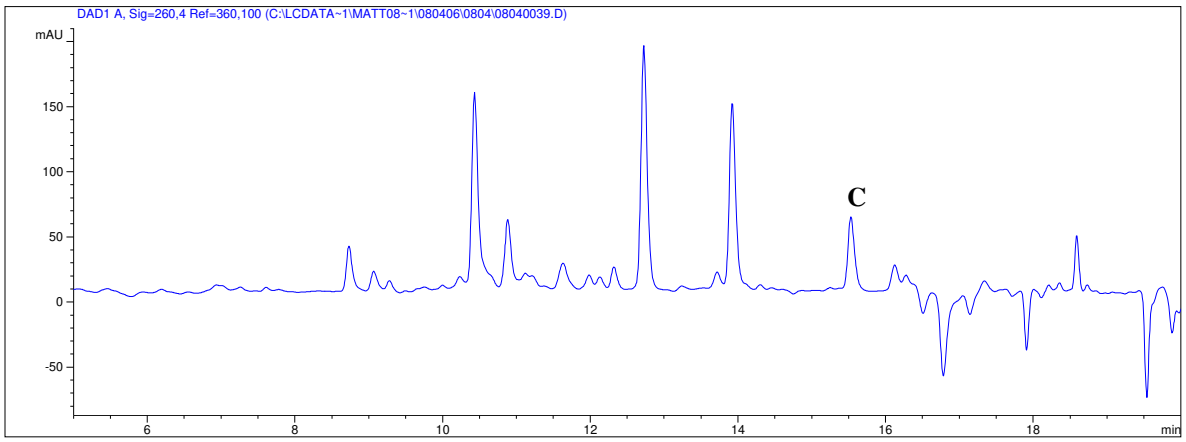


Figure 13E. HPLC chromatogram of methanol extract. The chromatograms show the signal obtained from the methanol extract of *C. globosum* after no treatment (A) or a 1 hour exposure to 50°C (B), 75°C (C), 100°C (D), 125°C (E), 150°C (F) and 175°C (G). The retention times (min) are plotted on the x-axis and the peak sizes (in milli-absorbance units) on the y-axis. Letters indicate the peak corresponding to chaetoglobosin A (A) or chaetoglobosin C (C).

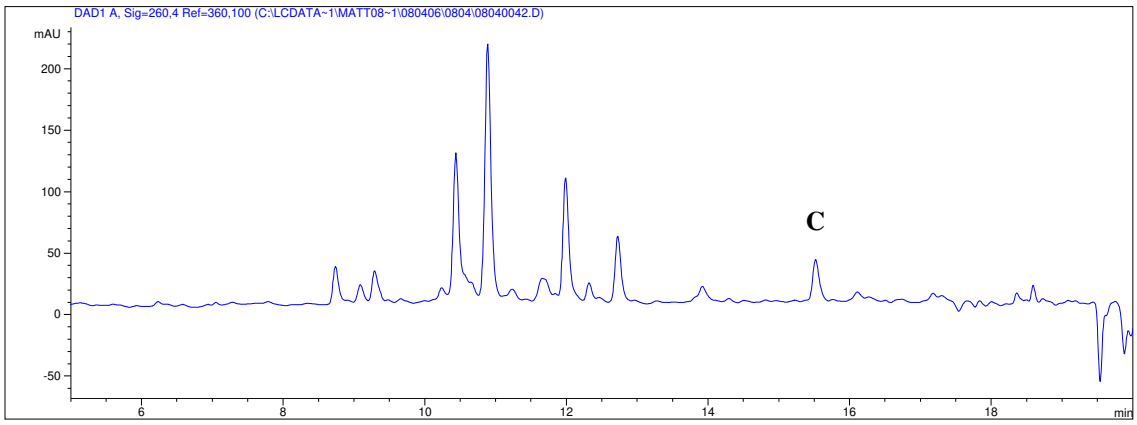


Figure 13F. HPLC chromatogram of methanol extract. The chromatograms show the signal obtained from the methanol extract of *C. globosum* after no treatment (A) or a 1 hour exposure to 50°C (B), 75°C (C), 100°C (D), 125°C (E), 150°C (F) and 175°C (G). The retention times (min) are plotted on the x-axis and the peak sizes (in milli-absorbance units) on the y-axis. Letters indicate the peak corresponding to chaetoglobosin A (A) or chaetoglobosin C (C).

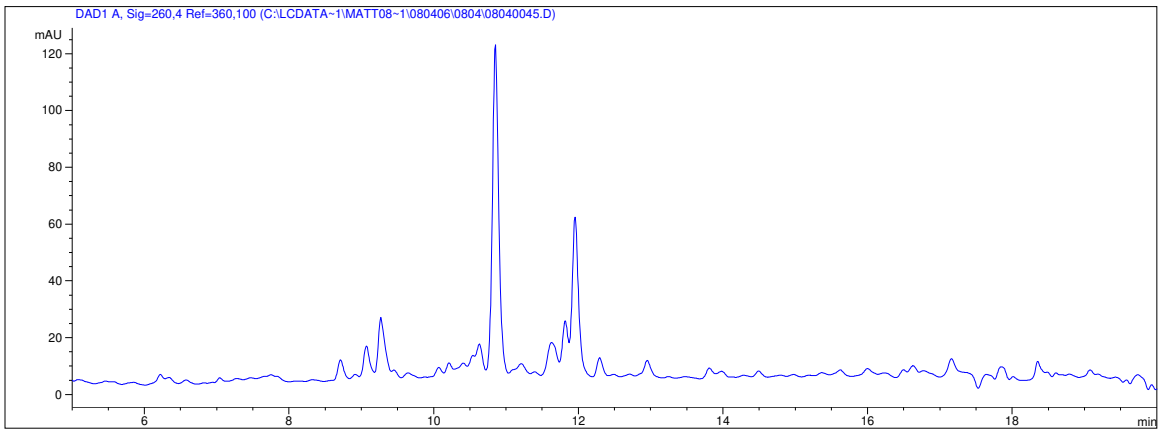


Figure 13G. HPLC chromatogram of methanol extract. The chromatograms show the signal obtained from the methanol extract of *C. globosum* after no treatment (A) or a 1 hour exposure to 50°C (B), 75°C (C), 100°C (D), 125°C (E), 150°C (F) and 175°C (G). The retention times (min) are plotted on the x-axis and the peak sizes (in milli-absorbance units) on the y-axis. Letters indicate the peak corresponding to chaetoglobosin A (A) or chaetoglobosin C (C).

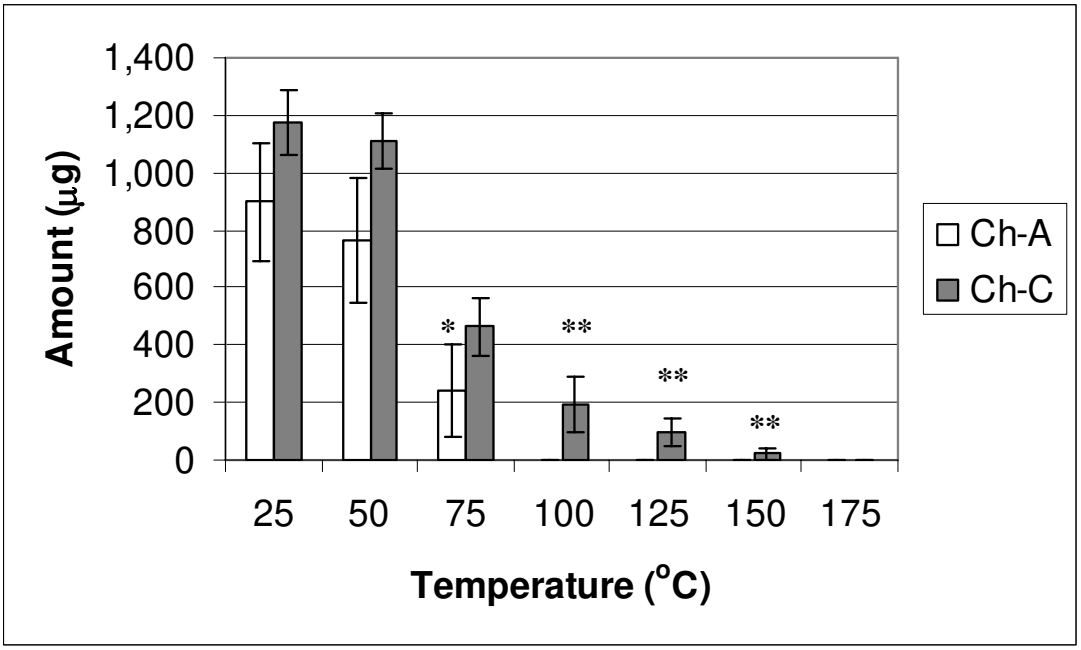


Figure 14. Exposure to various temperatures for 24 hours. The amounts (mean and standard error of the mean) of chaetoglobosin A (Ch-A) and chaetoglobosin C (Ch-C) are shown after no treatment (25°C) or a 24 hour exposure to 50°C, 75°C, 100°C, 125°C, 150°C and 175°C. Asterisks indicate a difference at the $P<0.05$ significance level.

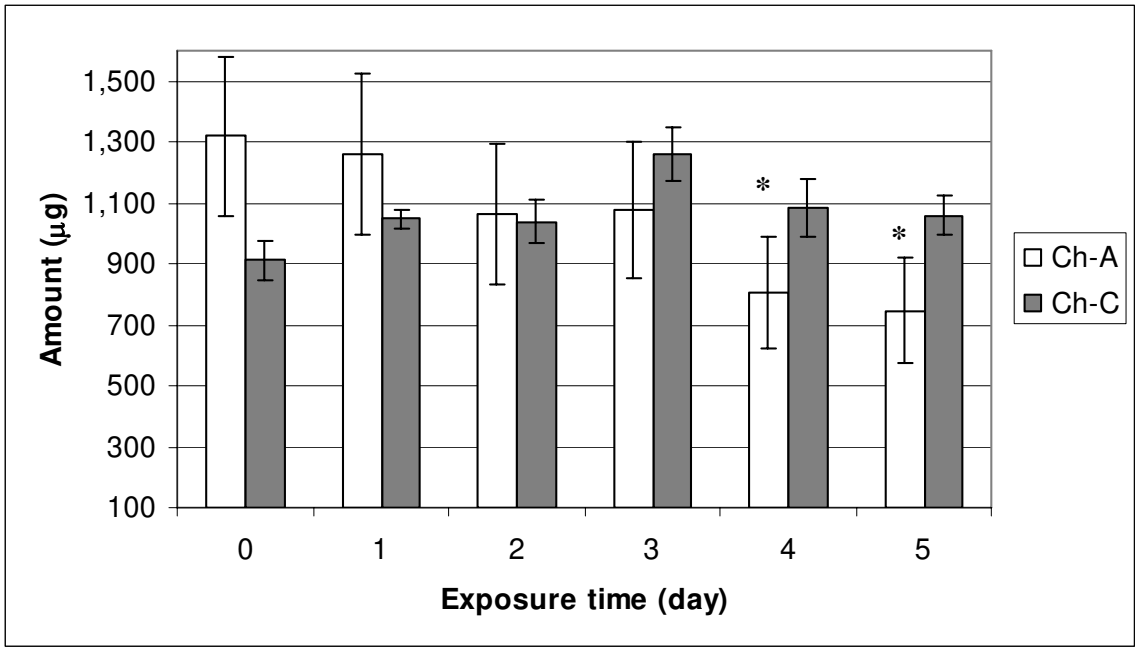


Figure 15. Exposure to 50°C over a 5 day period. The amounts (mean and standard error of the mean) of chaetoglobosin A (Ch-A) and chaetoglobosin C (Ch-C) are shown. Control samples were left at room temperature (0 days). Treated samples were exposed to 50°C over a 1 to 5 day period. Asterisks indicate a difference at the $P<0.05$ significance level.

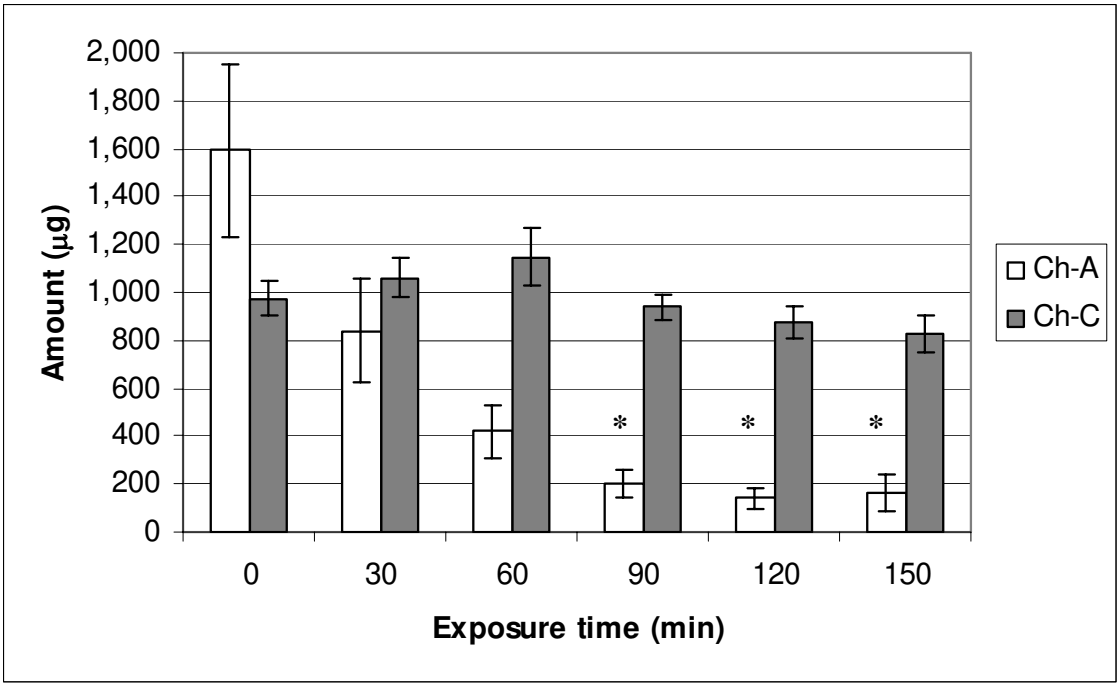


Figure 16. Exposure to 100°C over a 150 minute period. The amounts (mean and standard error of the mean) of chaetoglobosin A (Ch-A) and chaetoglobosin C (Ch-C) are shown. Control samples were left at room temperature (0 minutes). Treated samples were exposed to 100°C over a 30 to 150 minute period. Asterisks indicate a difference at the $P<0.05$ significance level.

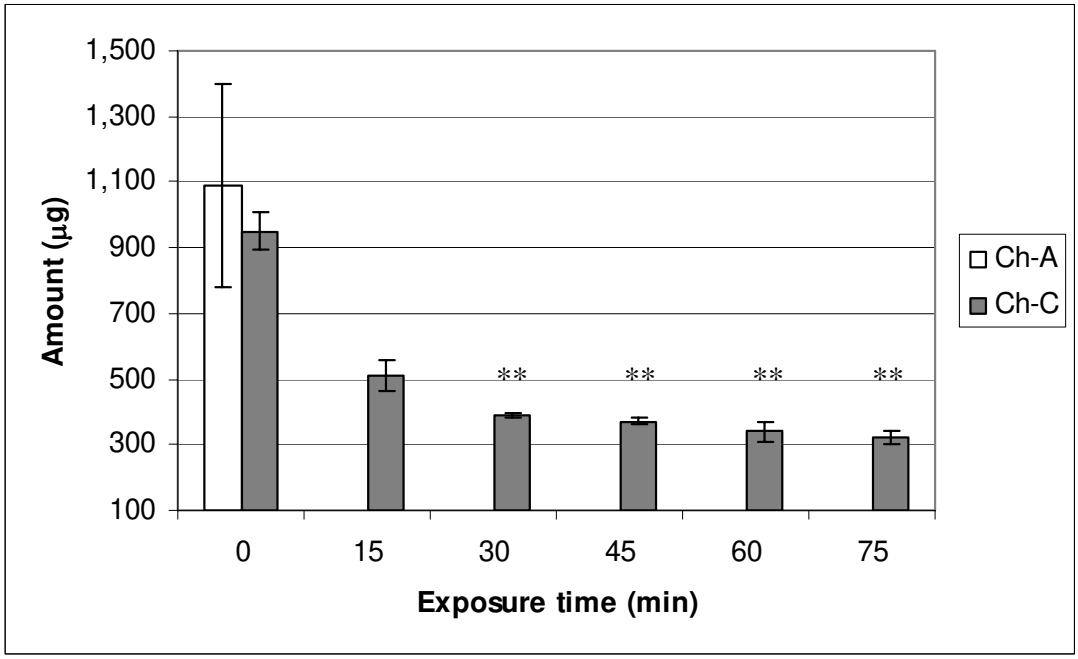


Figure 17. Exposure to 150°C over a 75 minute period. The amounts (mean and standard error of the mean) of chaetoglobosin A (Ch-A) and chaetoglobosin C (Ch-C) are shown. Control samples were left at room temperature (0 minutes). Treated samples were exposed to 150°C over a 15 to 75 minute period. Asterisks indicate a difference at the $P<0.05$ significance level.

CHAPTER IV

EFFECTS OF AMBIENT pH ON GROWTH AND MYCOTOXIN PRODUCTION BY *CHAETOMIUM GLOBOSUM* AND *STACHYBOTRYS CHARTARUM*

Introduction

Sick building syndrome (SBS) is a term commonly used to describe a set of non-specific symptoms resulting from poor indoor air quality (IAQ). These symptoms include: irritation of the eyes, nose and throat, dry skin, fatigue, headache, nausea, dizziness, increased number of respiratory tract infections, hoarseness and wheezing (17). Over the last several years, mounting evidence has shown that fungal contamination within buildings is associated with SBS (27, 58). In 2002, Dr. Stephen Redd presented a report on the behalf of the Centers for Disease Control (CDC) and Prevention to Congress. Dr. Redd stated "While there remain many unresolved scientific questions, we do know that exposure to high levels of mold causes some illnesses in susceptible people. Because molds can be harmful, it is important to maintain buildings, prevent water damage and mold growth, and clean up moldy materials (75)." This point was reiterated recently when Hurricanes Katrina and Rita caused extensive flooding in New Orleans and the surrounding areas. The CDC issued guidelines emphasizing the necessity to limit exposure to mold contamination in water-damaged buildings (2, 25).

The focus of this study is on two fungi; *Chaetomium globosum* and *Stachybotrys chartarum*. Both fungi have been frequently isolated in water-damaged buildings (26, 62). When cultured on building material, *C. globosum* produces chaetoglobosins A and C (63) while *S. chartarum* produces satratoxins G and H (64-66). The presence of either

chaetoglobosins or satratoxins can be lethal to mammalian cells, but due to different modes of action. The 50% lethal doses for the following mycotoxins was determined in mice via intraperitoneal injection: <2.0 mg/kg for chaetoglobosin A (67), 1.23 mg/kg for satratoxin G, and 5.69 mg/kg for satratoxin H (74). Chaetoglobosins A and C are cytochalasins which bind to actin leading to inhibition of cell division, locomotion, and formation of cell surface projections (4, 61). Satratoxins G and H are macrocyclic trichothecenes which bind to the 60S ribosomal subunit resulting in inhibition of protein synthesis (48).

In a previous study, we examined the growth of *C. globosum* on four commercially available media. We found that the medium that supported the best growth also supported the highest production of chaetoglobosins A and C (Chapter 2). Based on these results, we hypothesize that mycotoxin production is directly related to growth. In this study, the influence of ambient pH on fungal growth was examined on an artificial medium. We expect that as growth is reduced under sub-optimal conditions mycotoxin production will also decline. In addition, we examined whether or not altering the pH of building material would inhibit the growth of *C. globosum* and *S. chartarum*.

Materials and Methods

Preparation of buffered PDA

The following buffers were prepared at the predicted pH as described by Gomori (33): citrate-phosphate buffer, pH 3.0, 4.0, 5.0, 6.0 and 7.0; Tris (hydroxymethyl) aminomethane (Tris) buffer, pH 7.2, 8.0, and 9.0; carbonate-bicarbonate buffer, pH 9.2, 10.0 and 10.7; and Tris (hydroxymethyl) aminomethane-maleate (Tris-maleate) buffer,

pH 5.2, 6.0, 7.0, 8.0, and 8.6. Double-strength Difco potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, MD) was mixed with an equal volume of each buffer to obtain the desired concentration of medium as described by Kim et al. (54). The buffers and PDA were autoclaved separately and aseptically mixed during cooling. The buffered medium was poured into Petri dishes (VWR International, Inc., Aurora, CO) and allowed to solidify at room temperature (RT) or 25^{o}C.

Inoculation of PDA plates

Chaetomium globosum American Type Culture Collection 16021 (ATCC, Manassas, VA) and *Stachybotrys chartarum* strain 29-69-16 (52) were used in these experiments. The inocula were prepared as previously described (Chapter 2).

Evaluation of growth and production of chaetoglobosins A and C

Every week, colony diameters were measured at right angles on each agar plate resulting in two readings. The maximum diameter of each plate was 83 mm. For each buffered medium, tape slides were taken from a representative plate to examine sporulation at 4, 6 and 8 weeks post-inoculation. Clear adhesive tape was used to sample fungal growth and placed onto a glass slide containing lactophenol cotton blue (82) . Slides were examined with a BH-2 transmitted light microscope (Olympus, Center Valley, PA) at a magnification of 100X or 400X.

After four weeks, 20 ml of methanol were poured onto each plate and the fungal growth scraped off using a sterile loop. The contents of five agar plates were combined into a single group. The volume of methanol was measured and recorded. The spore concentration of each group was determined using a hemacytometer as previously described (Chapter 2).

For each group, the methanol extract and agar from 5 plates were placed in a 600 ml beaker. The agar was allowed to soak in methanol overnight at RT before removing and discarding. The beakers were placed under a fume hood to evaporate the methanol. The dried contents of each 1 L beaker were resuspended in 20 ml of methanol. The concentrated methanol extract was then transferred into a 20 ml glass scintillation vial. The vial was placed under a fume hood to evaporate the methanol. To recover any residual material left in the beaker, 20 ml of methanol as added to each beaker and then transferred to the corresponding vial. Finally, the beakers were rinsed a second time with 20 ml of methanol and transferred to the corresponding vial.

After allowing to completely dry, each scintillation vial received 2 ml of methanol. The vials were vortexed until the dried residue was dissolved. The extracts were passed through 0.45 μm syringe filters (Fisherbrand, Pittsburg, PA) into 2 ml glass vials (C4000-1W, National Scientific Company, Rockwood, TN). Detection of chaetoglobosins A and C was performed using an 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a UV-visible diode array detector as previously described (Chapter 2).

Measurement of pH

The pH of agar plates was determined by removing a 1 inch circular piece of agar from the center of each agar plate and placing it in a 50 ml polypropylene tube (VWR International Inc., Aurora, CO). Twenty-five ml of water were added to each tube. The agar was allowed to incubate at RT for 1 hour. After removing the agar, the pH of the water was measured with a Model 15 pH meter (Fisher Scientific, Pittsburg, PA) while stirring.

Inoculation of gypsum board

Gypsum board was cut into 6 cm by 6 cm pieces. Each piece was placed facedown in a round Pyrex jar containing 40 ml of either buffer or water for 2 hours at RT. Each piece was turned over and autoclaved at 121^{o}C for 20 minutes. The inoculum was prepared as previously described (Chapter 2). Each piece received 100,000 spores (either *C. globosum* or *S. chartarum*) resuspended in 2 ml of sterile water. Controls received 2 ml sterile water.

Growth was assessed based on visual inspection and spore production. To determine pH, the paper was peeled off the gypsum, placed in a 50 ml polypropylene tube, and soaked in 25 ml of water for 1 hour at RT. One ml of the water samples was collected for spore counts. The number of spores was determined using a hemacytometer as previously described (Chapter 2). The detection limit for these samples was 250,000 spores per piece of gypsum board. The paper was removed and the pH of the water measured with a Model 15 pH meter while stirring.

Statistical analysis

Statistical analysis was performed using the SigmaStat 2.0 software (Systat Software Inc., Richmond, CA). Significance was determined using Kruskal-Wallis analysis of variance on ranks ($P<0.05$) followed by Dunnett's or Tukey's post hoc analysis to determine differences in colony diameter or spore production respectively.

Results

Effect of pH on *C. globosum* cultured on an artificial medium

At the day of inoculation, the pH of sterile unbuffered PDA was 5.63 while the pH of sterile buffered PDA ranged from 3.51 to 9.35 for the *C. globosum* trials. After 4 weeks of incubation at RT, the pH of the sterile agar dropped (as much as 0.19) for the unbuffered, Tris buffered and Tris-maleate buffered PDA, and increased (up to 0.24) on the citrate-phosphate buffered and carbonate-bicarbonate buffered PDA (Table 6).

The colonies on unbuffered PDA (pH 5.63) reached 60 mm in diameter after 4 weeks. When the pH of the PDA was raised with the Tris buffer (pH 6.61, 7.61 and 8.24), the average colony sizes were higher compared to those on unbuffered PDA (Figures 18 and 19). Perithecia were present on unbuffered PDA and all Tris buffered media by 4 weeks. Ascospores were observed after 4 weeks on unbuffered PDA and Tris buffered PDA at pH 6.61 and 7.61, but not at pH 8.24. No ascospores were observed up to 8 weeks post-inoculation on Tris buffered PDA at pH 8.24 (Table 7).

The carbonate-bicarbonate buffer raised the pH of PDA higher than the Tris buffer (pH 9.07, 9.25 and 9.35) (Table 6). The average colony size on each Tris buffered medium was larger compared to unbuffered PDA at 2, 3 and 4 weeks (Figures 18 and 20). No perithecia or ascospores were present at 4, 6 or 8 weeks post-inoculation (Table 7).

A citrate phosphate buffer was used to obtain PDA ranging in pH from 3.51 to 7.01 (Table 6). The colonies cultured at a pH of 7.01 covered the entire plate (83 mm in diameter) 4 weeks post-inoculation. As pH decreased on each medium, colony sizes decreased. After 4 weeks, the colonies grown at a pH of 3.51 only reached an average of

11 mm in diameter (Figures 21 and 22) and no hyphal filaments were observed (data not shown). At a pH of 4.28, 5.17, 6.07 and 7.01, no perithecia were produced 4 weeks post-inoculation, but did eventually form 8 weeks post-inoculation. After 8 weeks, ascospores were present at a pH of 4.28, 5.28 and 7.28 (Table 7).

Tris-maleate buffer resulted in PDA ranging in pH from 5.21 to 7.91 (Table 6). After 2 weeks, the largest colonies were observed at the lowest pH. At 3 and 4 weeks, the colonies with the largest diameter were on PDA with a pH of 7.37 and 7.91 (Figure 23 and 24). At 4 weeks, numerous ascospores were observed at a pH of 5.21 and 5.84 while few or no ascospores were seen on the other Tris-maleate buffered PDA (data not shown). Within 6 weeks, ascospores were produced on each medium (Table 7).

Overall, the largest colonies were obtained at a neutral pH (7.01). By 2 weeks, these colonies were significantly larger compared to every other medium (Figure 25A). By 4 weeks, the average colony size for each medium was significantly smaller except at a pH of 6.07, 7.37, 7.61, 7.91 and 9.25 compared to a pH of 7.01 (Figure 25B). On unbuffered PDA (pH 5.63), *C .globosum* produced approximately 4,240,000 spores per group (i.e. 5 plates) after 4 weeks. Detectable levels of ascospores were present on Tris-maleate buffered PDA at a pH 5.21 and pH 6.53, although neither were significantly different compared to unbuffered PDA (Figure 26). Tape slides revealed the production of ascospores on four other buffered media (Tris buffered PDA at pH of 6.61 and 7.61; Tris-maleate buffered PDA at pH of 8.84 and 7.91) (Table 7) but not above the detection limit of the hemacytometer (10,000 spores/ml). Chaetoglobosin C was obtained from colonies grown at a pH of 7.01, but not from media at any other pH (Figure 27). No chaetoglobosin A was detected in any of the samples.

Effect of pH on *S. chartarum* cultured on an artificial medium

At the day of inoculation, the pH of sterile unbuffered PDA (pH 5.56) and the pH of sterile buffered PDA (ranged from pH of 3.45 to 8.27) for the *S. chartarum* trials (Table 8) was similar to those for the *C. globosum* trials (Table 6). The pH of the sterile unbuffered, Tris buffered, and citrate-phosphate buffered PDA varied slightly 4 weeks post-inoculation whereas the pH of Tris-maleate buffered media dropped between 0.21 and 0.36 (Table 8). *S. chartarum* was not cultured on carbonate-bicarbonate buffered PDA.

After 4 weeks, the average colony diameter on unbuffered PDA (pH 5.56) was 62 mm. On the Tris buffered media, colony sizes were lower at pH 6.74 and higher at pH 7.67 and 8.27 (Figures 28 and 29). Conidia were present 4 weeks post-inoculation on unbuffered PDA and Tris buffered PDA at pH of 6.74 but not at pH of 7.67 and 8.27. Within 6 weeks, conidia were produced on all Tris buffered media (Table 9).

S. chartarum growth was very poor on citrate-phosphate buffered medium. No growth occurred at a pH of 3.45. At 4 weeks, the largest average colony diameter was 27 mm (Figures 30 and 31). The colony morphology on the citrate-phosphate buffered PDA differed from unbuffered medium in that no hyphal filaments were observed and therefore no conidia were produced (data not shown).

The Tris-maleate buffered media ranged in pH from 5.25 to 8.11. The average colony diameters were largest (79 mm) at a pH of 5.25 and smallest (62 mm) at a pH of 5.78 (Figures 32 and 33). Conidia were produced on every medium 4 weeks post-inoculation (Table 9).

Overall, the largest colonies were obtained on Tris-maleate buffered PDA at a pH of 5.25 after 4 weeks. These colonies were significantly larger than all other media except at pH 7.65, 8.11 and 8.27 (Figure 34). Out of these 14 media, 6 produced conidia at levels ranging from 5,350,000 (pH 6.74) to 279,000,000 spores per group (pH 5.78) (Figure 35). Conidia were produced on every medium except the citrate-phosphate buffered PDA within 6 weeks (Table 9). Satratoxins G and H were not detected by HPLC on any of the 14 media tested.

Effect of pH on *C. globosum* and *S. chartarum* cultured on building material

The pH range of the paper obtained from the gypsum board samples used in the *C. globosum* trials was between 5.74 and 8.32 (Table 10). A similar range was observed in the *S. chartarum* trials (Table 11). The pH of sterile gypsum board dropped as much as 0.37 and increased as much as 0.57 after 4 weeks (Tables 10 and 11). Visible growth was present on all samples inoculated with either *C. globosum* (Figure 36) or *S. chartarum* (Figure 37). Ascospores or conidia were present at detectable levels on each set of samples. The average number of ascospores produced per sample by *C. globosum* ranged from 458,000 (pH 6.76) to 4,880,000 (pH 7.77) (Figure 38). Over 76,000,000 conidia were produced on each piece of gypsum board inoculated with *S. chartarum* (Figure 39).

Discussion

Few studies have examined the influence of ambient pH on the growth of either *C. globosum* or *S. chartarum*. The optimal pH range for the growth of *C. globosum* was previously described as 7.1 to 10.4. *S. chartarum* has been reported to grow within a pH

range of 3.6 to 7.7 (30). Our results indicate that both fungi grow over a range of different pH (approximately 4.3 to 8.2). Although *C. globosum* grew at a pH of 3.51, these colonies were small in size and had an abnormal morphology (Figures 21 and 22). The growth of *C. globosum* is optimal at a neutral pH (Figure 25); however, this is not the case with *S. chartarum*. After 4 weeks, the largest *S. chartarum* colonies were obtained on a Tris-maleate buffered PDA at a pH of 5.25 (Figure 34). The lack of any clear pattern between pH and colony diameter suggests the presence of the buffer influenced the growth of *S. chartarum* rather than solely the pH.

The formation of perithecia and ascospores by *C. globosum* appears to be favored in an acidic environment and inhibited under basic conditions on an artificial medium (Table 7 and Figure 26). After 4 weeks, ascospores were present in detectable levels on unbuffered PDA (pH 5.63) and Tris-maleate buffered PDA (pH 5.21 and 6.53) (Figure 26). *C. globosum* eventually produced perthecia and ascospores on citrate-phosphate buffered PDA 8 weeks post-inoculation. No ascospores were produced on Tris buffered PDA at pH 8.24 or on the carbonate-bicarbonate buffered pH at 9.07, 9.25, and 9.35 (Table 7). It is also possible that this inhibition of sporulation at a basic pH is due to the presence of one of the buffer's components, although the mechanism remains unknown at the present time.

Ambient pH does not appear to greatly affect sporulation by *S. chartarum*, but this stage of development may be triggered more quickly in an acidic environment. The highest numbers of conidia were produced at a pH of 5.78 (Tris-maleate buffered PDA) (Figure 35). The delay in the appearance of conidia on the Tris buffered PDA at pH 7.67

and 8.27 was likely due to some unknown effect of the buffer since conidia were present 4 weeks post-inoculation on Tris-maleate buffered PDA at a pH 7.65 and 8.11 (Table 9).

Detectable levels of chaetoglobosin C were only observed on the medium with the largest *C. globosum* colonies (Figures 25 and 27). This finding supports our hypothesis that the production of chaetoglobosins is directly related to growth. It does not appear that pH has any direct influence on the production of chaetoglobosin C. Amibient pH has been shown to influence metabolite production in other filamentous fungi. The best studied regulatory system is in *Aspergillus nidulans* which is controlled by a transcription factor called PacC (69). Under alkaline conditions, PacC activates alkaline-expressed genes such as *acvA* and *ipnA* which are involved in penicillin synthesis and represses acid-expressed genes such as *stcU* which is involved in sterigmatocystin synthesis. Other filamentous fungi with PacC homologs include *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium chrysogenum*, *Acremonium chrysogenum*, *Sclerotinia sclerotiorum* (69), and *Fusarium verticillioides* (31). A hypothetical protein similar to PacC has been located with the *C. globosum* genome (1). Assuming this fungus has a similar regulatory system as in *A. nidulans*, these results suggest that chaetoglobosin production is not under its control.

Sporulation and chaetoglobosin C production do not appear to be directly related (Figures 26 and 27). Results from this study and previous work (Chapter 2) suggest chaetoglobosin C may be carried on hyphae rather than spores. This implication could be important with regard to IAQ within water-damaged buildings. Gorny et al. demonstrated with *Aspergillus versicolor*, *Penicillium melinii* and *Cladosporium cladosporioides* that fungal fragments could be aerosolized in much higher amounts than

spores. It is plausible that these fungal fragments could become airborne within a mold-contaminated building and possibly contribute to the adverse health effects described by the occupants (34). However, whether or not this is the case remains to be determined.

Lowering or raising the pH of gypsum board (approximately 5.6 and 8.3 respectively) was not sufficient to inhibit the growth of either *C. globosum* or *S. chartarum* (Figures 36 and 37). These results on gypsum board are consistent with those obtained on buffered PDA since fungal growth was also not inhibited within this pH range (Figures 25 and 34). The growth of *C. globosum* and *S. chartarum* was reflected by the detection of ascospores or conidia respectively in every set of buffered or unbuffered gypsum board samples (Figures 38 and 39). Future studies should examine whether or not growth by these and other fungi can be inhibited outside this pH range.

Table 6. pH measurements of potato dextrose agar for *C. globosum* trials.

Medium	Predicted pH of buffer	Sterile agar[1]	
		Day 0	Day 28
Unbuffered PDA	n/a	5.63	5.47
Tris buffered PDA	7.2	6.61	6.50
	8.0	7.61	7.59
	9.0	8.24	8.19
Citrate-phosphate buffered PDA	3.0	3.51	3.69
	4.0	4.28	4.49
	5.0	5.17	5.40
	6.0	6.07	6.31
	7.0	7.01	7.23
Carbonate-bicarbonate buffered PDA	9.2	9.07	9.13
	10.0	9.25	9.26
	10.7	9.35	9.39
Tris-maleate buffered PDA	5.2	5.21	5.02
	6.0	5.84	5.75
	7.0	6.53	6.45
	8.0	7.37	7.30
	8.6	7.91	7.83

[1]Average of three agar plates are shown at either 0 or 28 days post-inoculation.

Table 7. Effect of pH on the sporulation of *C globosum*.

Medium	Predicted pH of buffer	Presence of perithecia[1]			Presence of ascospores[1]		
		Week 4	Week 6	Week 8	Week 4	Week 6	Week 8
Unbuffered PDA	n/a	+	+	+	+	+	+
Tris buffered PDA	7.2	+	+	+	+	+	+
	8.0	+	+	+	+	+	+
	9.0	+	+	+	-	-	-
Citrate-phosphate buffered PDA	3.0	-	NT	NT	-	NT	NT
	4.0	-	+	+	-	-	+
	5.0	-	+	+	-	-	+
	6.0	-	-	+	-	-	-
	7.0	-	+	+	-	-	+
Carbonate-bicarbonate buffered PDA	9.2	-	-	-	-	-	-
	10.0	-	-	-	-	-	-
	10.7	-	-	-	-	-	-
Tris-maleate buffered PDA	5.2	-	-	-	+	+	+
	6.0	+	+	+	+	+	+
	7.0	+	+	+	+	+	+
	8.0	+	+	+	-	+	+
	8.6	+	+	+	+	+	+

[1]Tape slides were taken from a single agar plate at 4, 6 or 8 weeks post-inoculation.

Present or absence of perithecia or ascospores is indicated with a "+" or "–" respectively.

Samples not taken are indicated by "NT".

Table 8. pH measurements on potato dextrose agar for *S. chartarum* trials.

Medium	**Predicted pH of buffer**	**Sterile agar[1]**	
		Day 0	**Day 28**
Unbuffered PDA	n/a	5.56	5.50
Tris buffered PDA	7.2	6.74	6.72
	8.0	7.67	7.70
	9.0	8.27	8.27
Citrate-phosphate buffered PDA	3.0	3.45	3.46
	4.0	4.28	4.32
	5.0	5.28	5.33
	6.0	6.30	6.36
	7.0	7.28	7.32
Tris-maleate buffered PDA	5.2	5.25	5.04
	6.0	5.78	5.54
	7.0	6.70	6.45
	8.0	7.65	7.40
	8.6	8.11	7.74

[1]Average of three agar plates are shown at either 0 or 28 days post-inoculation.

Table 9. Effect of pH on the sporulation of *S. chartarum*.

Medium	Predicted pH of buffer	Presence of conidia[1]		
		Week 4	Week 6	Week 8
Unbuffered PDA	n/a	+	+	+
Tris buffered PDA	7.2	+	+	+
	8.0	-	+	+
	9.0	-	+	+
Tris-maleate buffered PDA	5.2	+	NT	NT
	6.0	+	NT	NT
	7.0	+	NT	NT
	8.0	+	NT	NT
	8.6	+	NT	NT

[1]Tape slides were taken from a single agar plate at 4, 6 or 8 weeks post-inoculation. Present or absence of conidia is indicated with a "+" or "–" respectively. Samples not taken are indicated by "NT".

Table 10. pH measurements of gypsum board for *C. globosum* trials.

Buffer	**Predicted pH of buffer**	**Sterile gypsum board**	
		Day 0[1]	**Day 28[2]**
None	n/a	7.97	7.99
Citrate-phosphate buffer	3.0	5.74	6.13
	7.0	6.76	7.33
Tris buffer	7.2	7.77	7.71
	9.0	8.32	7.95

[1]Average of 3 samples is shown at 0 days post-inoculation.

[2]Average of 4 samples is shown at 28 days post-inoculation.

Table 11. pH measurements of gypsum board for *S. chartarum* trials.

Buffer	**Predicted pH of buffer**	**Sterile gypsum board**[1]	
		Day 0	**Day 28**
None	n/a	8.04	8.10
Citrate-phosphate buffer	3.0	5.66	6.23
	7.0	6.99	7.39
Tris buffer	7.2	7.82	7.74
	9.0	8.36	8.03

[1]Average of 4 samples is shown at 0 and 28 days post-inoculation.

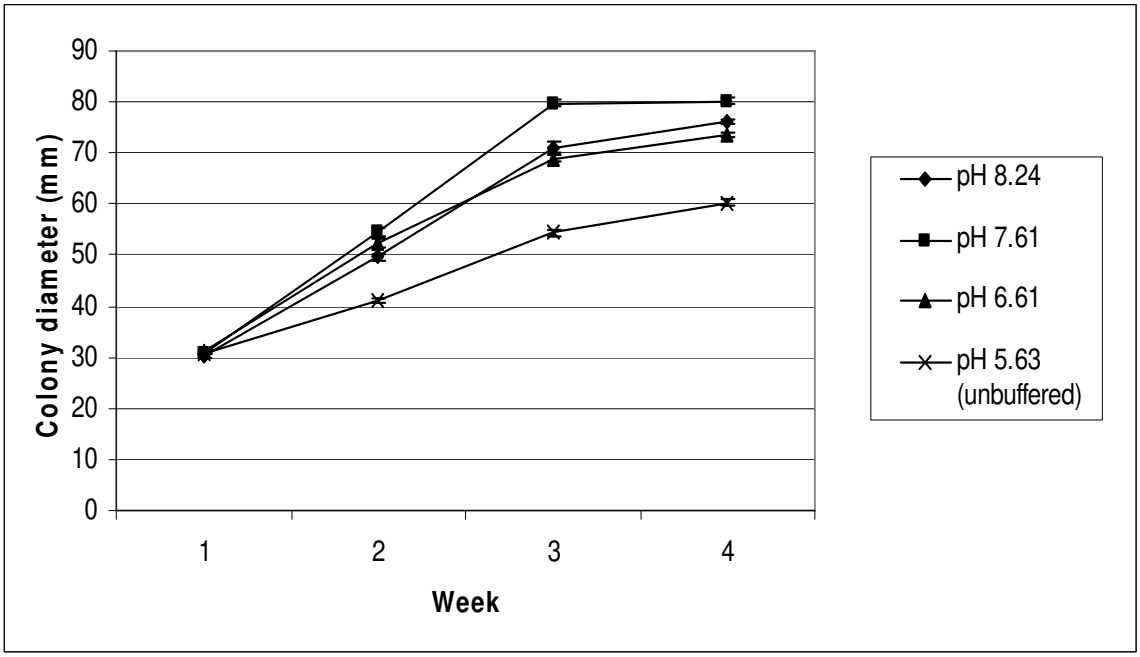


Figure 18. Colony diameters of *C. globosum* over 4 weeks on Tris buffered and unbuffered potato dextrose agar. The center of each agar plate was inoculated with 500 *C. globosum* spores suspended in 20 µl of water. The agar plates were incubated at room temperature. Colony diameters were measured every week. Mean and standard error of the mean are shown (n=15 plates).

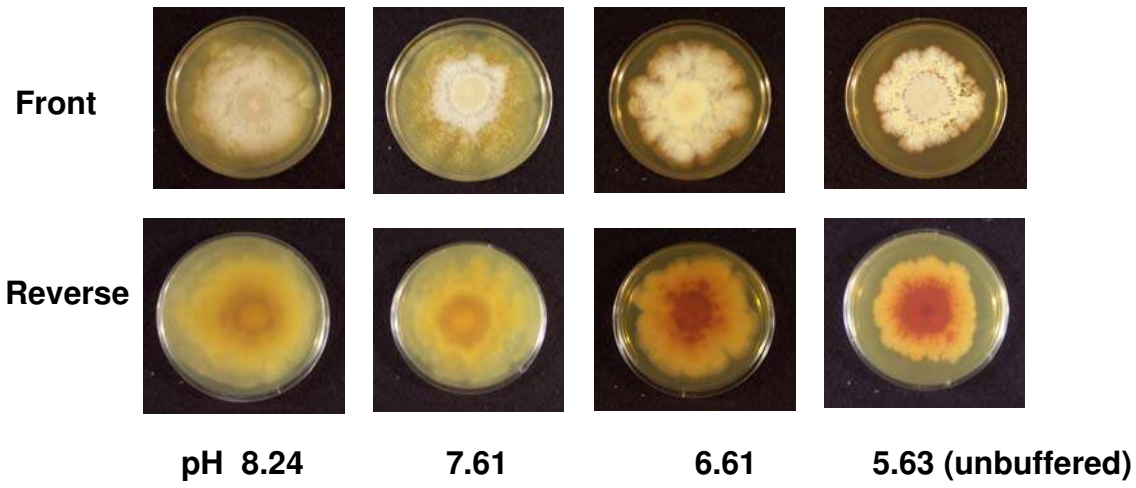


Figure 19. Photographs of *C. globosum* colonies at 4 weeks on Tris buffered and unbuffered potato dextrose agar. The center of each agar plate was inoculated with 500 *C. globosum* spores suspended in 20 µl of water. These photographs depict the front and reverse sides of agar plates with *C. globosum* colonies after four weeks of incubation at room temperature.

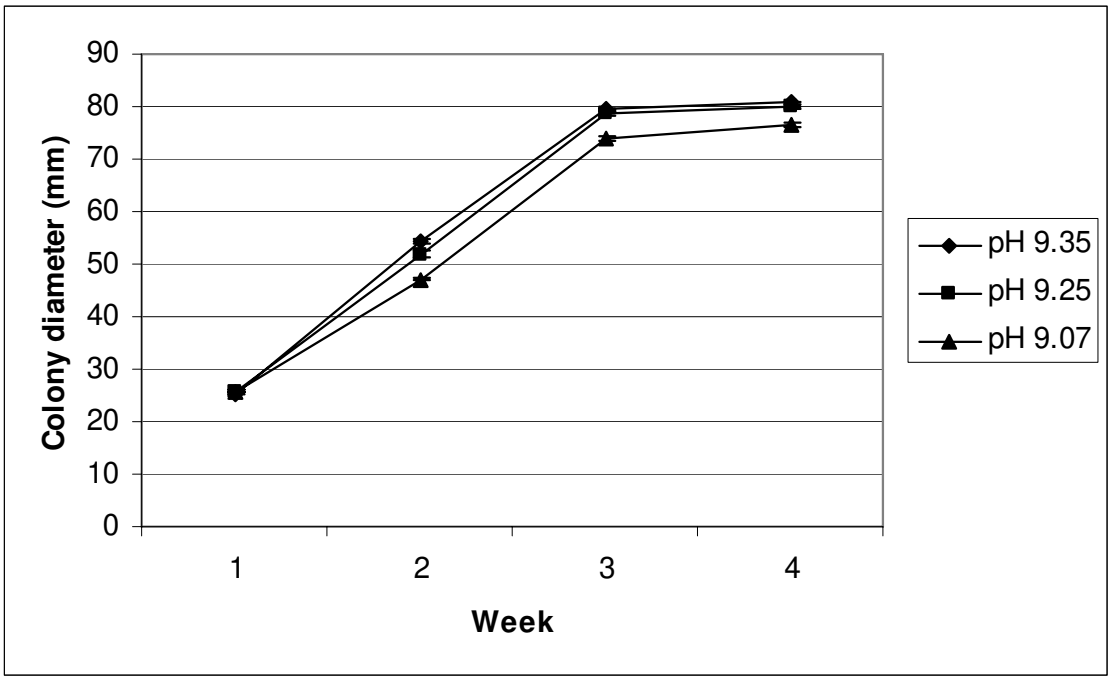


Figure 20. Colony diameters of *C. globosum* over 4 weeks on carbonate-bicarbonate buffered potato dextrose agar. The center of each agar plate was inoculated with 500 *C. globosum* spores suspended in 20 µl of water. The agar plates were incubated at room temperature. Colony diameters were measured every week. Mean and standard error of the mean are shown (n=15 plates).

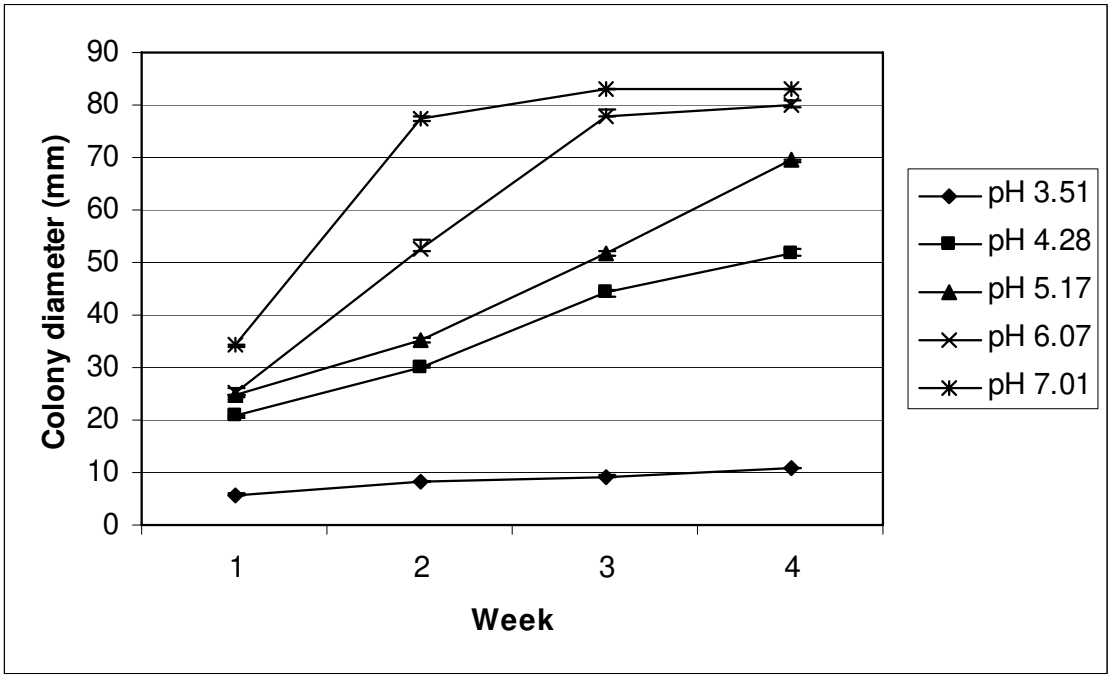


Figure 21. Colony diameters of *C. globosum* over 4 weeks on citrate-phosphate buffered potato dextrose agar. The center of each agar plate was inoculated with 500 *C. globosum* spores suspended in 20 µl of water. The agar plates were incubated at room temperature. Colony diameters were measured every week. Mean and standard error of the mean are shown (n=15 plates).

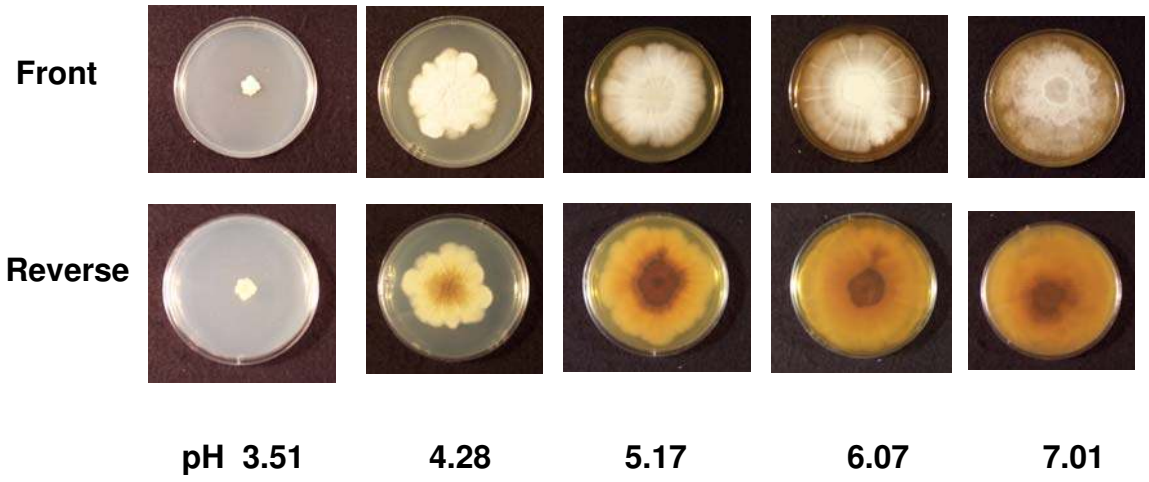


Figure 22. Photographs of *C. globosum* colonies at 4 weeks on citrate-phosphate buffered potato dextrose agar. The center of each agar plate was inoculated with 500 *C. globosum* spores suspended in 20 µl of water. These photographs depict the front and reverse sides of agar plates with *C. globosum* colonies after four weeks of incubation at room temperature.

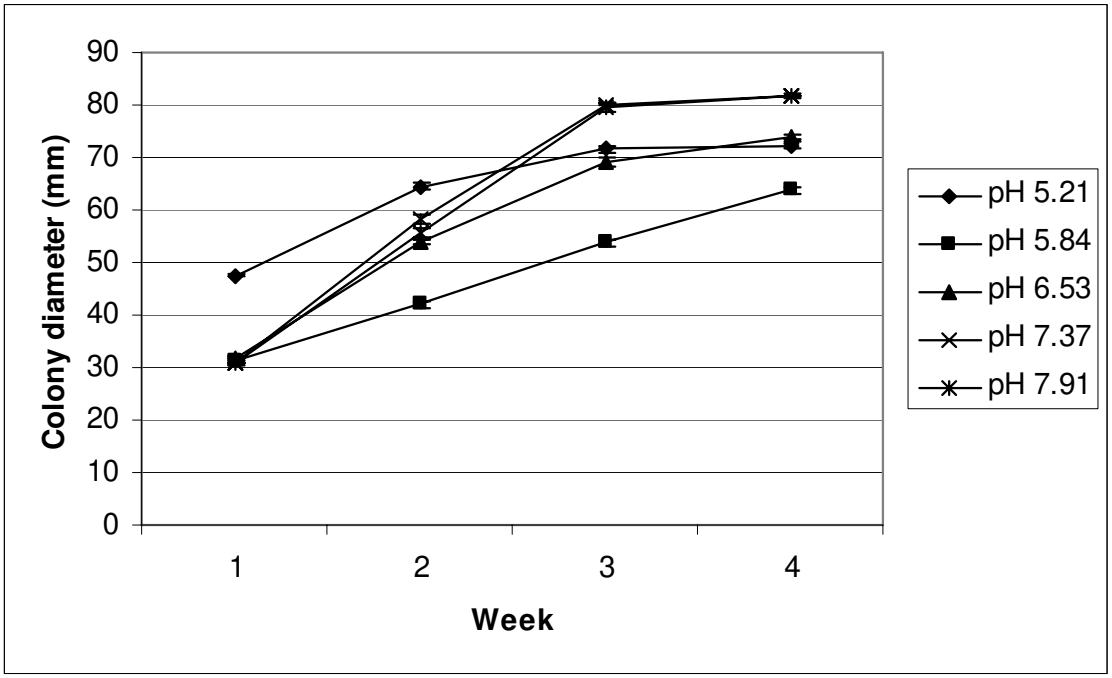


Figure 23. Colony diameters of *C. globosum* over 4 weeks on Tris-maleate buffered potato dextrose agar. The center of each agar plate was inoculated with 500 *C. globosum* spores suspended in 20 µl of water. The agar plates were incubated at room temperature. Colony diameters were measured every week. Mean and standard error of the mean are shown (n=15 plates).

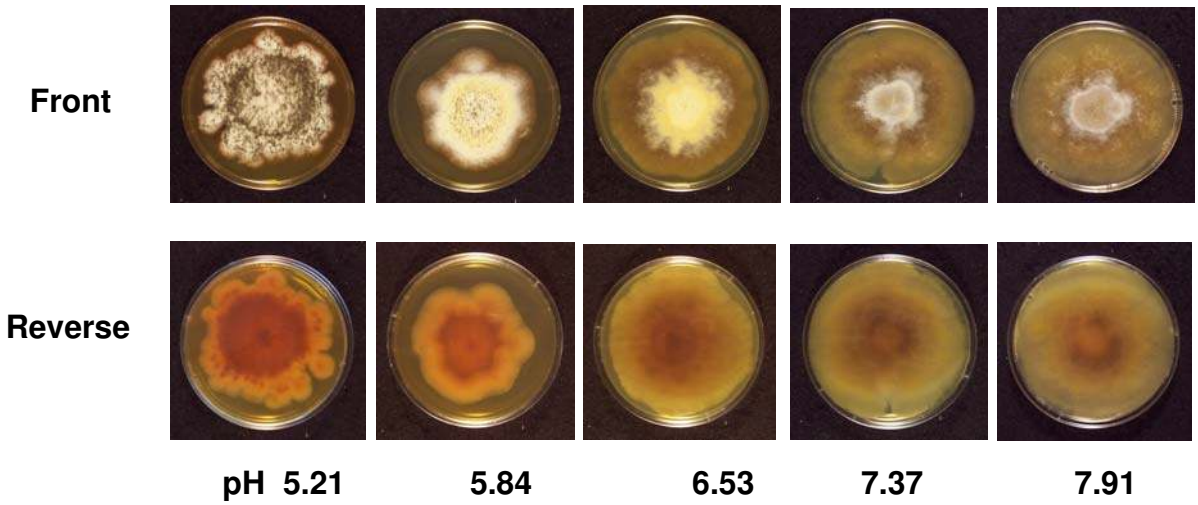


Figure 24. Photographs of *C. globosum* colonies at 4 weeks on Tris-maleate buffered potato dextrose agar. The center of each agar plate was inoculated with 500 *C. globosum* spores suspended in 20 µl of water. These photographs depict the front and reverse sides of agar plates with *C. globosum* colonies after four weeks of incubation at room temperature.

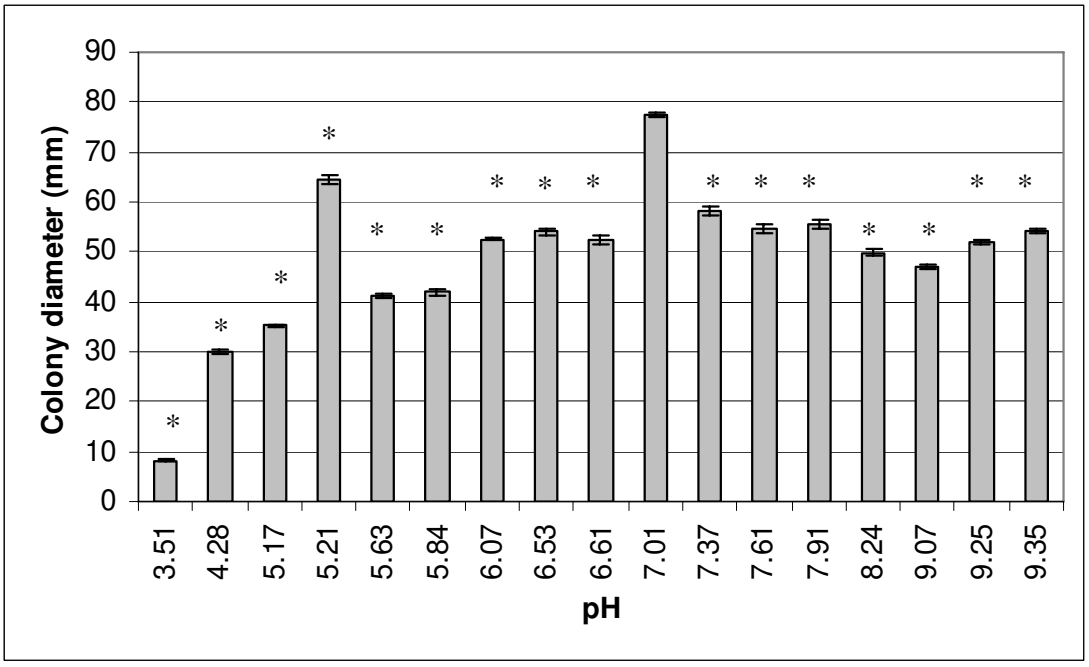


Figure 25A. Colony diameters of *C. globosum* at 2 and 4 weeks on buffered and unbuffered potato dextrose agar. Colony diameters were measured 2 weeks (A) and 4 weeks (B) post-inoculation. Mean and standard error of the mean are shown (n = 15 plates). Asterisks indicate a significantly lower diameter compared to the colony size at a pH 7.01.

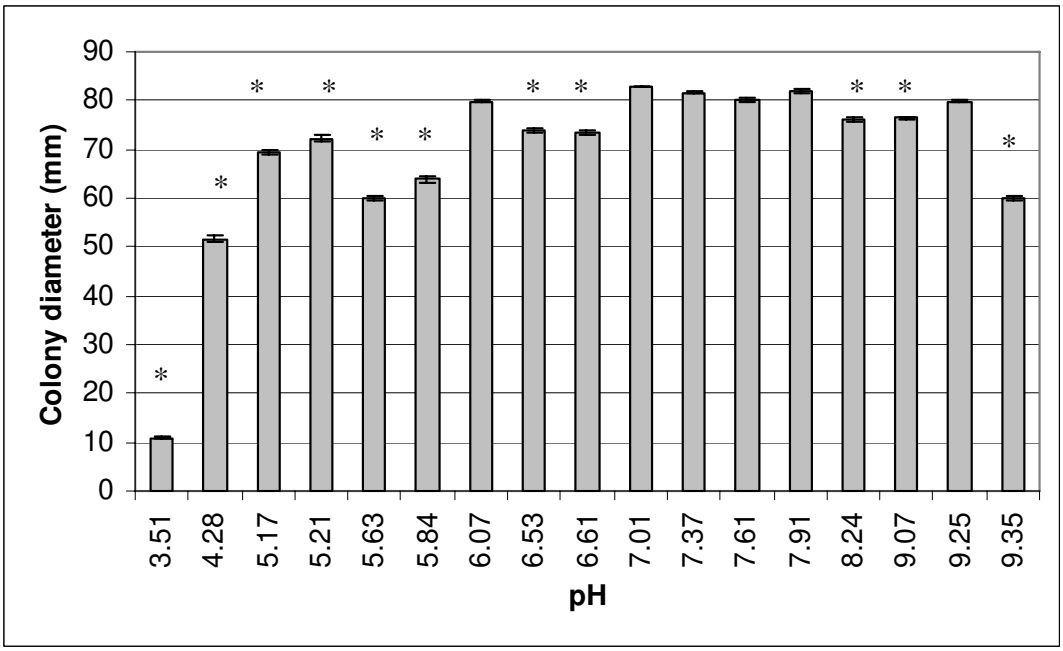


Figure 25B. Colony diameters of *C. globosum* at 2 and 4 weeks on buffered and unbuffered potato dextrose agar. The center of each agar plate was inoculated with 500 *C. globosum* spores suspended in 20 µl of water. Colony diameters were measured 2 weeks (A) and 4 weeks (B) post-inoculation. Mean and standard error of the mean are shown (n = 15 plates). Asterisks indicate a significantly lower diameter compared to the colony size at a pH 7.01.

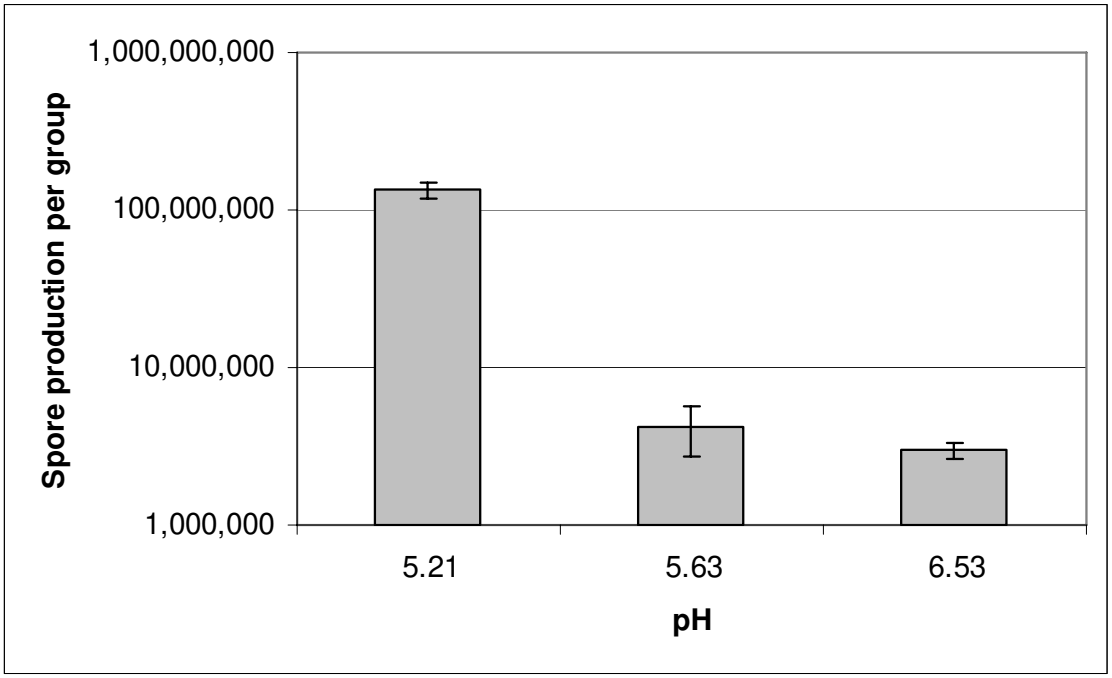


Figure 26. Spore production by *C. globosum* at 4 weeks on unbuffered and Tris-maleate buffered potato dextrose agar. The center of each agar plate was inoculated with 500 *C. globosum* spores suspended in 20 µl of water. The number of spores (mean and standard error of the mean) produced by *C. globosum* on five agar plates is shown (n = 3 groups).

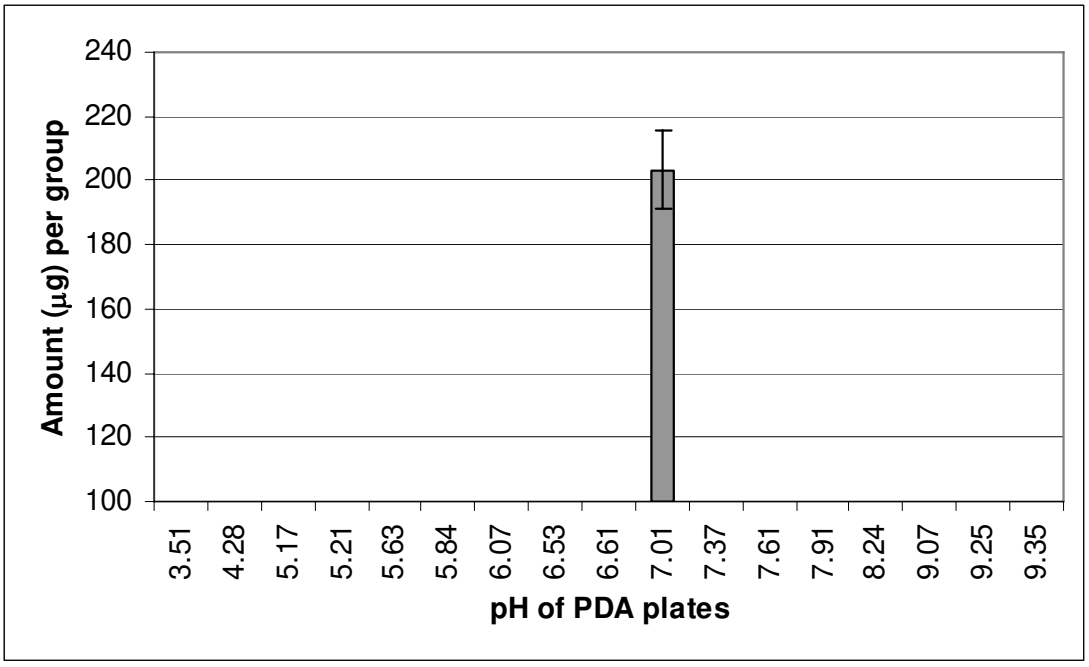


Figure 27. Production of chaetoglobosin C by *C. globosum* at 4 weeks on unbuffered and buffered potato dextrose agar. The amount (mean and standard error of the mean) of chaetoglobosins C produced by *C. globosum* on five agar plates is shown (n = 3 groups). The agar plates were inoculated with 500 spores of *C. globosum* and incubated at room temperature for 4 weeks.

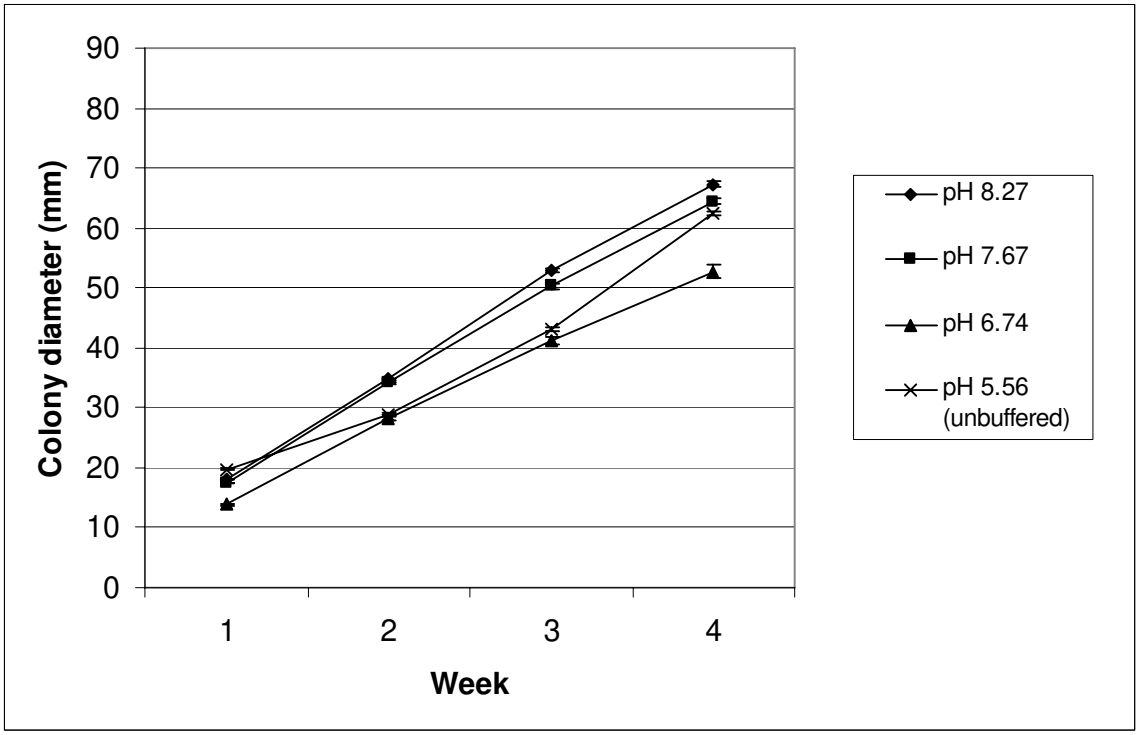


Figure 28. Colony diameters of *S. chartarum* over 4 weeks on Tris buffered and unbuffered potato dextrose agar. The center of each agar plate was inoculated with 500 *S. chartarum* spores suspended in 20 µl of water. The agar plates were incubated at room temperature. Colony diameters were measured every week. Mean and standard error of the mean are shown (n=15 plates).

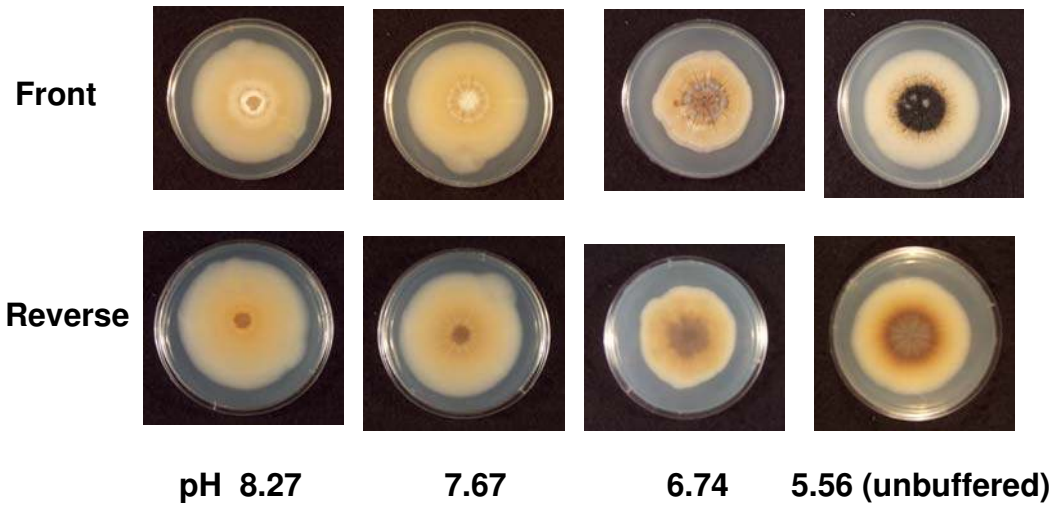


Figure 29. Photographs of *S. chartarum* colonies at 4 weeks on Tris buffered and unbuffered potato dextrose agar. The center of each agar plate was inoculated with 500 *S. chartarum* spores suspended in 20 µl of water. These photographs depict the front and reverse sides of agar plates with *S. chartarum* colonies after four weeks of incubation at room temperature.

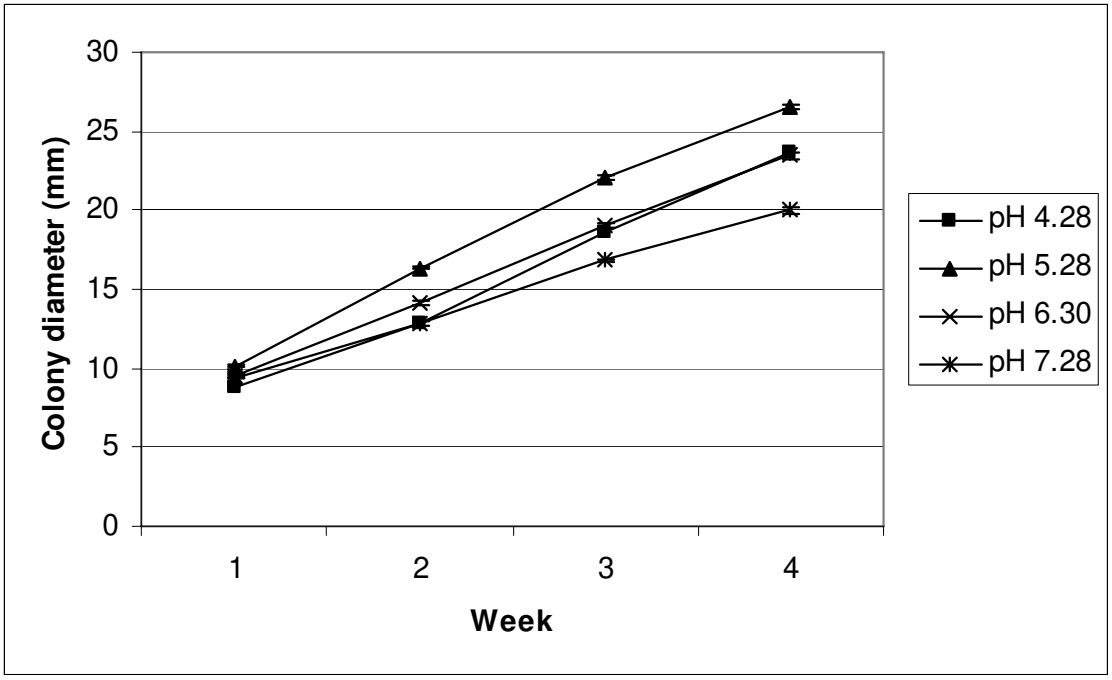


Figure 30. Colony diameters of *S. chartarum* over 4 weeks on citrate-phosphate buffered potato dextrose agar. The center of each agar plate was inoculated with 500 *S. chartarum* spores suspended in 20 µl of water. The agar plates were incubated at room temperature. Colony diameters were measured every week. Mean and standard error of the mean are shown (n=15 plates).

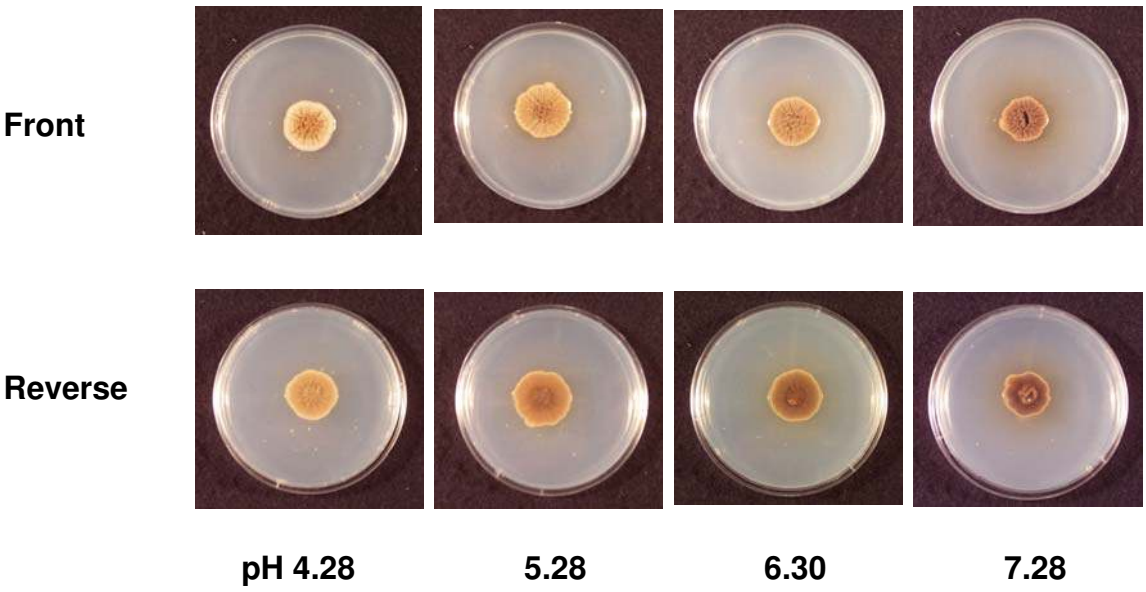


Figure 31. Photographs of *S. chartarum* colonies at 4 weeks on citrate-phosphate buffered potato dextrose agar. The center of each agar plate was inoculated with 500 *S. chartarum* spores suspended in 20 µl of water. These photographs depict the front and reverse sides of agar plates with *S. chartarum* colonies after four weeks of incubation at room temperature.

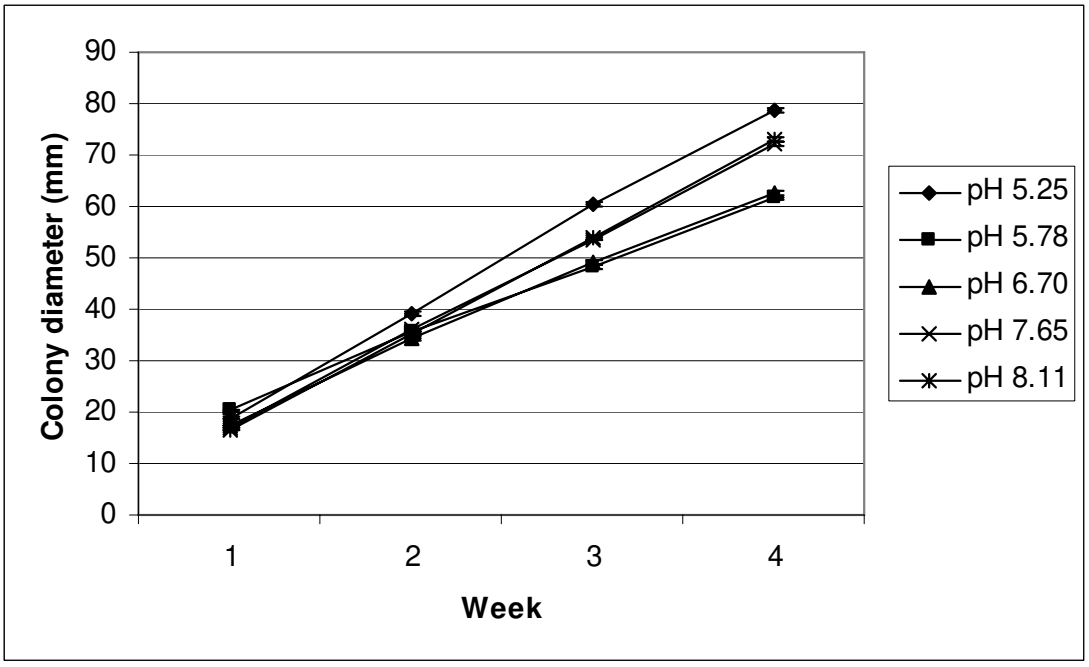


Figure 32. Colony diameters of *S. chartarum* over 4 weeks on Tris-maleate buffered potato dextrose agar. The center of each agar plate was inoculated with 500 *S. chartarum* spores suspended in 20 µl of water. The agar plates were incubated at room temperature. Colony diameters were measured every week. Mean and standard error of the mean are shown (n=15 plates).

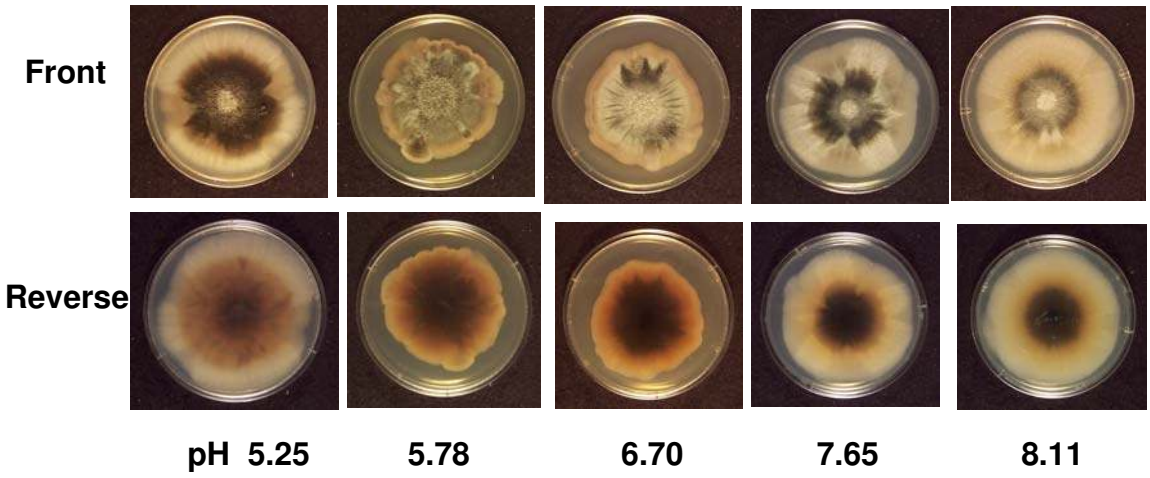


Figure 33. Photographs of *S. chartarum* colonies at 4 weeks on Tris-maleate buffered potato dextrose agar. The center of each agar plate was inoculated with 500 *S. chartarum* spores suspended in 20 µl of water. These photographs depict the front and reverse sides of agar plates with *S. chartarum* colonies after four weeks of incubation at room temperature.

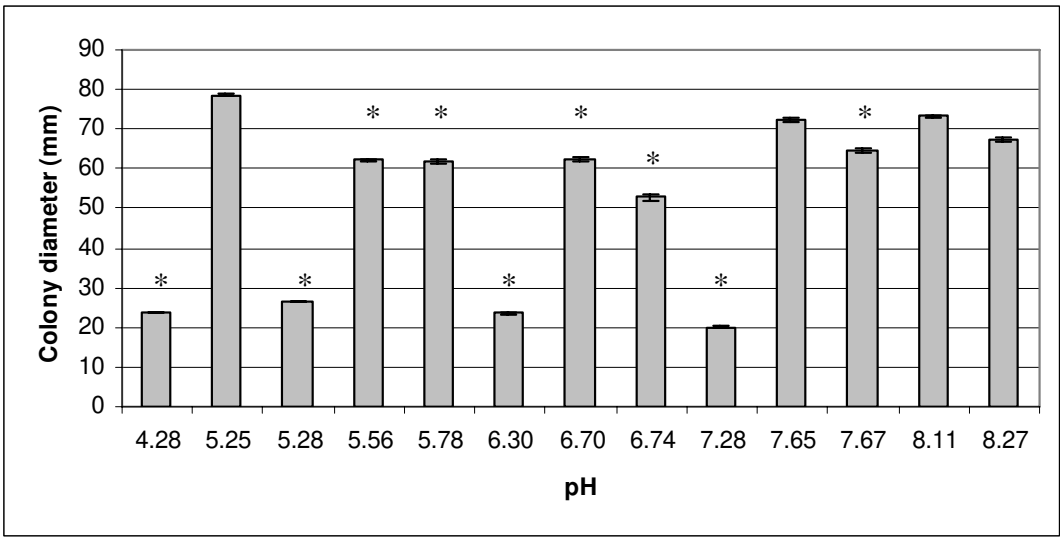


Figure 34. Colony diameters of *S. chartarum* at 4 weeks on buffered and unbuffered potato dextrose agar. The center of each agar plate was inoculated with 500 *S. chartarum* spores suspended in 20 µl of water. Colony diameters were measured 4 weeks post-inoculation. Mean and standard error of the mean are shown (n = 15 plates). Asterisks indicate a significantly lower diameter compared to the colony size at a pH 5.25.

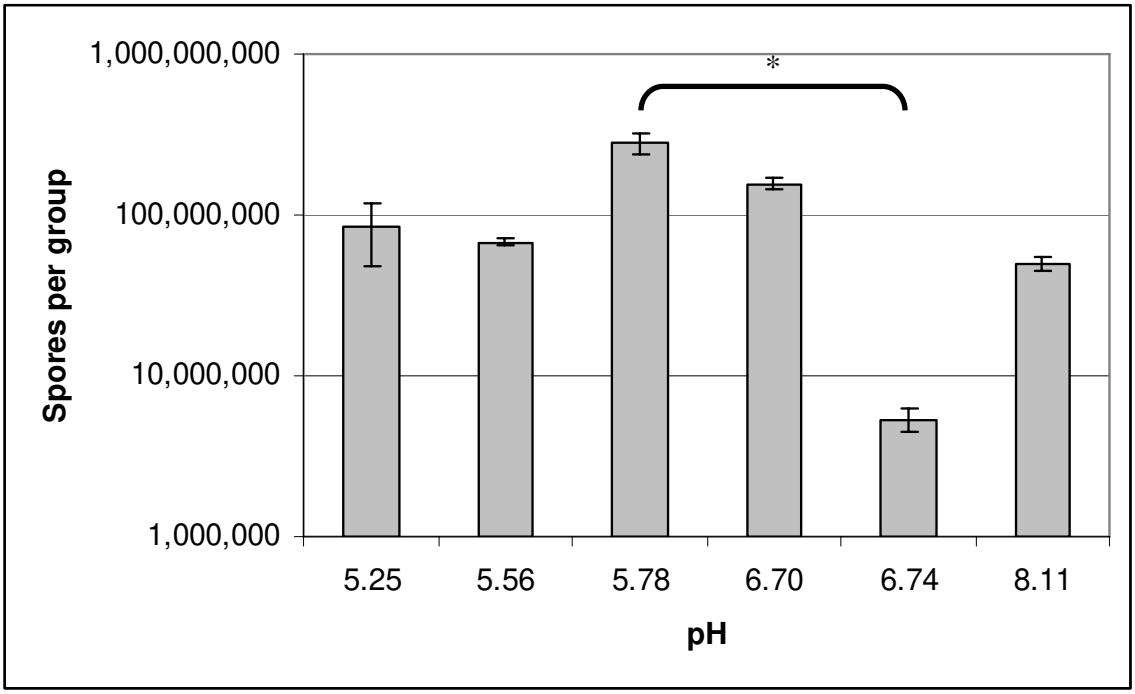


Figure 35. Spore production by *S. chartarum* at 4 weeks on unbuffered, Tris buffered, and Tris-maleate buffered potato dextrose agar. The center of each agar plate was inoculated with 500 *S. chartarum* spores suspended in 20 µl of water. The number of spores (mean and standard error of the mean) produced by *S. chartarum* on five agar plates is shown (n = 3 groups). Asterisk indicates a significant difference at the $P<0.05$ level.

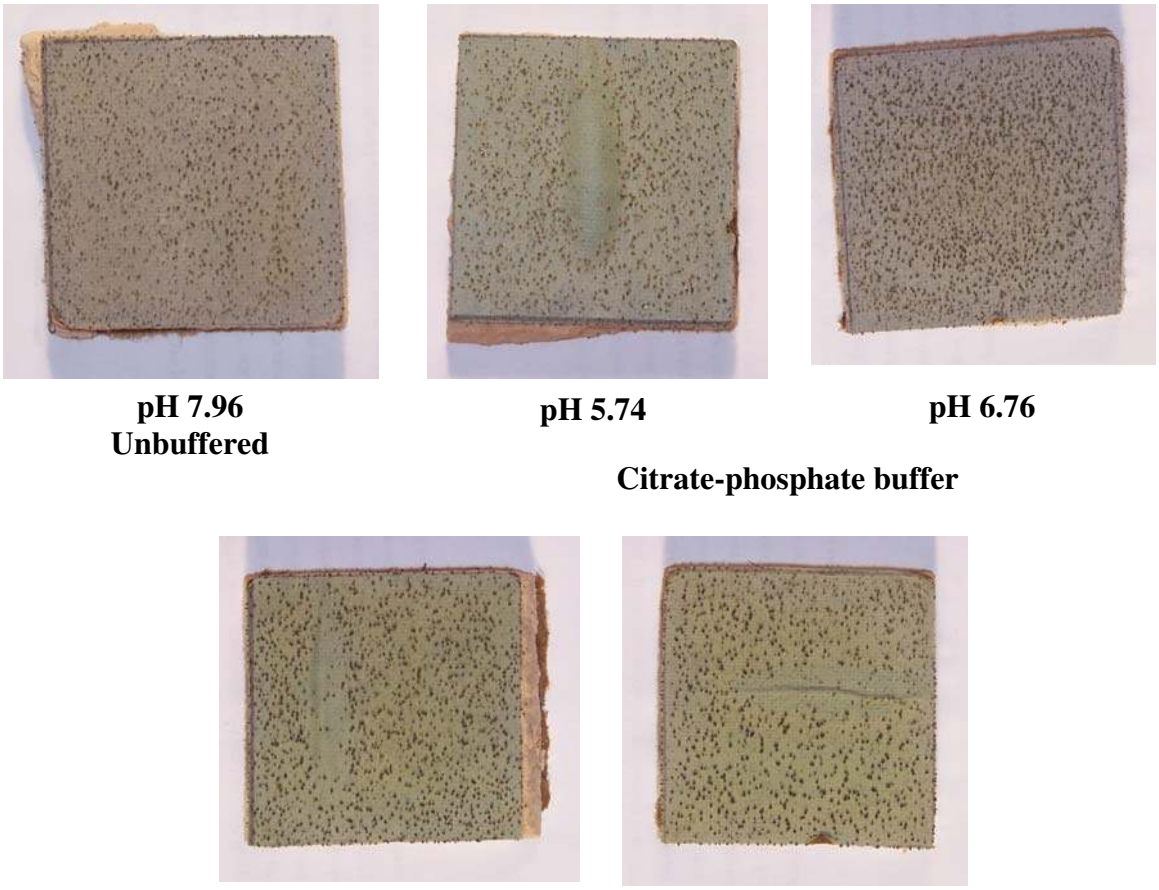


Figure 36. Photographs of *C. globosum* on gypsum board 4 weeks post-inoculation. Each 6 cm by 6 cm piece of gypsum board was soaked facedown in 40 ml of water or buffer for 2 hours and then turned over. After autoclaving, each piece received 100,000 *C. globosum* spores resuspended in 2 ml of sterile water. Samples were incubated at room temperature for 4 weeks.

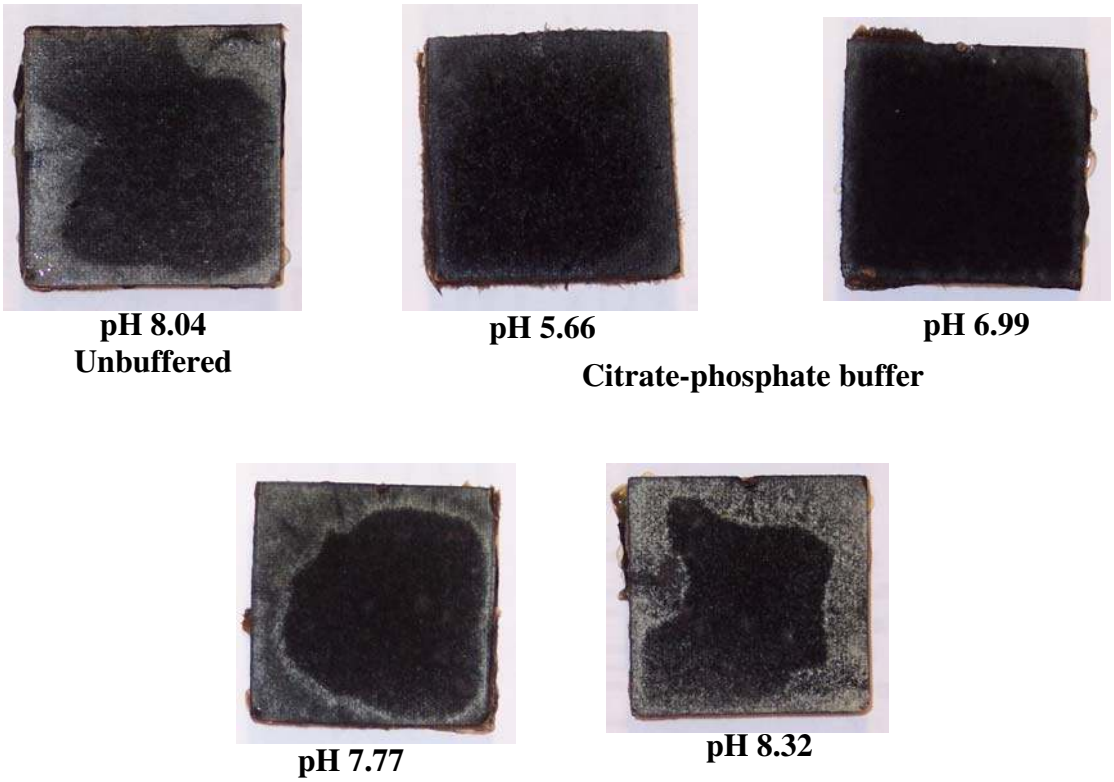


Figure 37. Photographs of *S. chartarum* on gypsum board 4 weeks post-inoculation. Each 6 cm by 6 cm piece of gypsum board was soaked facedown in 40 ml of water or buffer for 2 hours and then turned over. After autoclaving, each piece received 100,000 *S. chartarum* spores resuspended in 2 ml of sterile water. Samples were incubated at room temperature for 4 weeks.

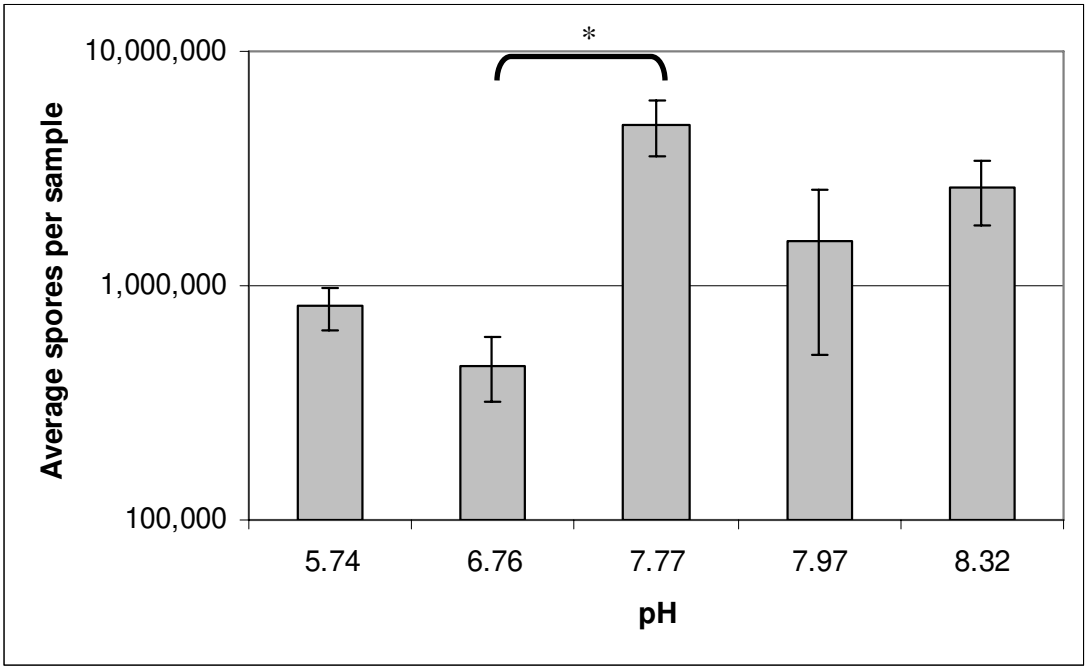


Figure 38. Spore production of *C. globosum* on gypsum board 4 weeks post-inoculation. Each 6 cm by 6 cm piece of gypsum board was soaked facedown in 40 ml of water or buffer for 2 hours and then turned over. After autoclaving, each piece received 100,000 *C. globosum* spores resuspended in 2 ml of sterile water. Samples were incubated at room temperature for 4 weeks. The paper from each piece was removed and placed in 25 ml of water for 1 hour. Mean and standard error of the mean are shown (n = 6). Asterisk indicates a difference at the $P<0.05$ level.

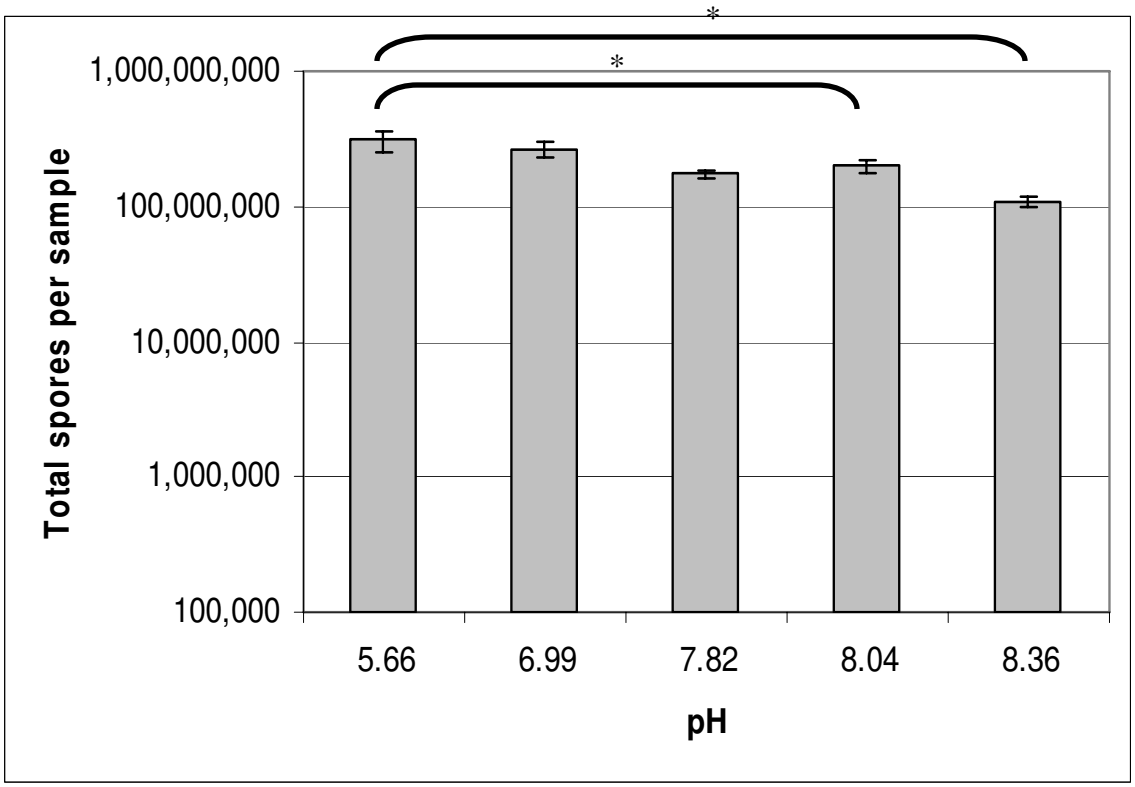


Figure 39. Spore production of *S. chartarum* on gypsum board 4 weeks post-inoculation. Each 6 cm by 6 cm piece of gypsum board was soaked facedown in 40 ml of water or buffer for 2 hours and then turned over. After autoclaving, each piece received 100,000 *S. chartarum* spores resuspended in 2 ml of sterile water. Samples were incubated at room temperature for 4 weeks. The paper from each piece was removed and placed in 25 ml of water for 1 hour. Mean and standard error of the mean are shown (n = 6). Asterisks indicate a difference at the $P<0.05$ level.

CHAPTER V

GENERAL DISCUSSION

Sick building syndrome (SBS) is a term commonly used to describe a set of non-specific symptoms resulting from poor indoor air quality (IAQ). These symptoms include: irritation of the eyes, nose and throat, dry skin, fatigue, headache, nausea, dizziness, increased number of respiratory tract infections, hoarseness, and wheezing (17). Over the last several years, mounting evidence has shown that fungal contamination within buildings is associated with SBS (27, 58). Our results demonstrate that *Chaetomium* species are commonly encountered in water-damaged buildings. The most common species within this genus is *C. globosum* (85). Previous work has shown that this fungus can produce two mycotoxins called chaetoglobosins A and C when cultured on building material (63). When incubated with various mammalian cell lines, both metabolites caused lethal changes at relatively low doses (89). Based on this information, it is possible that the production of chaetoglobosins A and C may contribute to the adverse health effects described by building occupants exposed to *C. globosum*.

The results of this study show that all 30 isolates of *C. globosum* produce detectable levels of chaetoglobosin C and 16 out of 30 isolates produced chaetoglobosin A when cultured on artificial media (Figures 10 and 11). It appears that the production of these metabolites is conserved among *C. globosum* isolates; but the selective advantage within its natural environment is currently unknown. Possible roles for other mycotoxins within an evolutionary context have been reviewed by Jarvis (49). These roles include (but are not limited to) growth inhibition of competing organisms, growth stimulation of

other organisms antagonistic to the growth of competitors, and protection from insect predators.

Chaetomium species were isolated less often in the air than in samples collected on surfaces in buildings (Table 5). This trend most likely occurs due to the tendency of the relatively large spores to settle out of the air more quickly than smaller spores (35). Another explanation for the low recovery of *C. globosum* from air samples is the use of non-specific media (6). A third possibility may result from *C. globosum's* preference for a substrate with high water activity for its optimal growth (62, 63). This saturated environment could prevent spores from being easily aerosolized.

Although *Chaetomium* species spores are not detected in air samples at a high frequency, the presence of *C. globosum* contamination within a water-damaged building should not be overlooked. Our results suggest that chaetoglobosins A and C may be carried on hyphae rather than spores. Although spores were not produced by *C. globosum* on malt extract agar 4 weeks post-inoculation, chaetoglobosins A and C were still detected (Figures 4 and 7). When cultured on molasses broth, *C. globosum* produced chaetoglobosins A and C, even though sporulation did not occur in the liquid medium. It is plausible that these fungal fragments could become airborne within a *C. globosum*-contaminated building and possibly contribute to the adverse health effects described by the occupants. However, it is not known if this occurs.

C. globosum was capable of growing on an artificial medium over a wide pH range (approximately 4.3 to 9.3) with optimal growth occurring at a neutral pH (Figure 25). The pH of gypsum board had an approximate pH of 8.0. When the pH of the gypsum board was raised or lowered (approximately 5.7 and 8.3 respectively), the growth

of *C. globosum* was not inhibited (Figure 36) which was consistent with the findings on the artificial media. Future studies should examine whether or not growth by these and other fungi can be inhibited outside this pH range. Altering the pH may provide a practical way to prevent mold growth on wet building material.

Remediation of mold-contaminated buildings is an important issue. Our results concerning the heat stability of chaetoglobosins A and C provide a basis for predicting the effectiveness of various remediation efforts. Chaetoglobosins A and C were relatively stable when exposed to 50^{o}C for up to 3 days (Figure 15). After 4 and 5 days, the overall amount decreased compared to the control sample suggesting that these compounds are not stable when exposed to this temperature for long periods of time. Exposure to 75^{o}C and higher temperatures resulted in the rapid breakdown of chaetoglobosins A and C (Figures 12, 14, 16, and 17). Neither compound was detected after heating samples to 175^{o}C for 1 hour (Figure 12). Heating *C. globosum* contaminated buildings materials may be a practical method for inactivating chaetoglobosins A and C; however, specific methods for remediation will require further testing to ensure their effectiveness.

Karunasena et al. (52) suggested that solubility in water could allow these mycotoxins to be easily spread throughout the indoor environment away from the fungal growth site and distributed to other materials during a watering event. Our results indicate that chaetoglobosins A and C are poorly soluble in water. Thus, these mycotoxins are less likely to be distributed throughout a building in this manner. As a result, other mycotoxins that are more soluble in water (such as satratoxins G and H) compared to chaetoglobosins A and C, may pose a more significant risk to human health.

Future work should focus on developing a more sensitive assay than high performance liquid chromatography (such as an enzyme linked immunosorbent assay) for detecting chaetoglobosins A and C. This technology would allow IAQ researchers to answer two important questions. Are chaetoglobosins A and C present in the air of *C. globosum*-contaminated buildings? Are these mycotoxins present in the sera (or other bodily fluids) of occupants suffering from SBS? These questions have been answered with another fungus associated with SBS. Despite the fact that *Stachybotrys* species spores are also not commonly found in air samples, previous studies have detected the macrocyclic trichothecenes in the air of *S. chartarum* contaminated buildings (14, 24) as well as the sera of occupants exposed to this organism (12, 96).

LITERATURE CITED

1. January 10, 2007, posting date. CHGG_05804: hypothetical protein similar to transcription factor PacC. Broad Institute. Cambridge, MA. http://www.broad.mit.edu/annotation/genome/chaetomium_globosum/GeneDetails.html?sp=S7000000317914914

2. Centers for Disease Control and Prevention. Mold prevention strategies and possible health effects in the aftermath of hurricanes and major floods. MMWR 2006; 55 (No. RR-8): 1-27.

3. **Abbott, S. P., L. Sigler, R. McAleer, D. A. McGough, M. G. Rinaldi, and G. Mizell.** 1995. Fatal cerebral mycoses caused by the ascomycete *Chaetomium strumarium*. J Clin Microbiol **33:**2692-2698.

4. **Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter.** 2002. Chapter 16. The cytoskeleton., Molecular biology of the cell., Fourth ed. Garland Science, New York.

5. **Anandi, V., T. J. John, A. Walter, J. C. M. Shastry, M. K. Lalitha, A. A. Padhye, L. Ajello, and F. W. Chandler.** 1989. Cerebral phaeohyphomycosis caused by *Chaetomium globosum* in a renal transplant recipient. J Clin Microbiol **27:**2226-2229.

6. **Andersen, B., and A. T. Nissen.** 2000. Evaluation of media for detection of *Stachybotrys* and *Chaetomium* species associated with water-damaged buildings. Int Biodeterior Biodegradation **46:**111-116.

7. **Aribandi, M., C. Bazan III, and M. G. Rinaldi.** 2005. Magnetic resonance imaging findings in fatal primary cerebral infection due to *Chaetomium strumarium*. Australas Radiol **49:**166-169.

8. **Atlas, R. M.** 2004. Handbook of Microbiological Media., 3rd ed. CRC Press, New York.

9. **Barron, M. A., D. A. Sutton, R. Veve, J. Guarro, M. G. Rinaldi, E. Thompson, P. J. Cagnoni, K. Moultney, and N. E. Madinger.** 2003. Invasive mycotic infections caused by *Chaetomium perlucidum*, a new agent of cerebral phaeohyphomycosis. J Clin Microbiol **41:**5302-5307.

10. **Basu, S. N.** 1951. Significance of calcium in the fruiting of *Chaetomium* species, particularly *Chaetomium globosum*. J Gen Microbiol **5:**231-238.

11. **Basu, S. N., and R. G. Bose.** 1950. Factors affecting the fruiting of *Chaetomium* species. J Gen Micro **4:**132-140.

12. **Brasel, T. L., A. W. Campbell, R. E. Demers, B. S. Ferguson, J. Fink, A. Vojdani, S. C. Wilson, and D. C. Straus.** 2004. Detection of trichothecene mycotoxins in sera from individuals exposed to *Stachybotrys chartarum* in indoor environments. Arch Environ Health **59:**317-323.

13. **Brasel, T. L., D. R. Douglas, S. C. Wilson, and D. C. Straus.** 2005. Detection of airborne *Stachybotrys chartarum* macrocyclic trichothecene mycotoxins on particles smaller than conidia. Appl Environ Microbiol **71:**114-122.

14. **Brasel, T. L., J. D. Martin, C. G. Carriker, S. C. Wilson, and D. C. Straus.** 2005. Detection of airborne *Stachybotrys chartarum* macrocyclic trichothecene mycotoxins in the indoor environment. Appl Environ Microbiol **71:**7376-7388.

15. **Brewer, D., and A. Taylor.** 1978. The production of toxic metabolites by *Chaetomium* spp. isolated from soils of permanent pasture. Can J Microbiol **24:**1082-1086.

16. **Bright, R. K., and J. D. Lewis.** 2003. Long-term culture of normal and malignant human prostate epithelial cells., p. 125-144. *In* R. Pfragner and R. I. Freshney (ed.), Culture of human tumor cells. Wiley-Liss, Inc.

17. **Brightman, H. S., and N. Moss.** 2000. Sick building syndrome studies and the compilation of normative and comparative values., p. 3.1-3.32. *In* J. D. Spengler, J. M. Samet, and J. F. McCarthy (ed.), Indoor air quality handbook. McGraw-Hill, Washington D.C.

18. **Burge, H. A., and J. A. Otten.** 1999. Chapter 19. Fungi. *In* J. Macher (ed.), Bioaerosols: Assessment and control. ACGIH Worldwide, Cincinnati, OH.

19. **Buston, H. W., and S. N. Basu.** 1948. Some factors affecting the growth and sporulation of *Chaetomium globosum* and *Memnoniella echinata*. J Gen Microbiol **2:**162-172.

20. **Buston, H. W., and A. H. Khan.** 1956. The influence of certain micro-organisms on the formation of perithecia by *Chaetomium globosum*. J Gen Microbiol **14:**655-660.

21. **Buston, H. W., M. O. Moss, and C. Tyrrell.** 1966. The influence of carbon dioxide on growth and sporulation of *Chaetomium globosum*. Trans Br Mycol Soc **49:**387-396.

22. **Buston, H. W., and B. Rickard.** 1956. The effect of a physical barrier on sporulation of *Chaetomium globosum*. J Gen Microbiol **15:**194-197.

23. **Calvo, A. M., R. A. Wilson, J. W. Bok, and N. P. Keller.** 2002. Relationship between secondary metabolism and fungal development. Microbiol Mol Biol Rev **66:**447-459.

24. **Charpin-Kadouch, C., G. Maurel, R. Felipo, J. Queralt, M. Ramadour, H. Dumon, M. Garans, A. Botta, and D. Charpin.** 2006. Mycotoxin identification in moldy dwellings. J Appl Toxicol **26:**475-479.

25. **Chew, G. L., J. Wilson, F. A. Rabito, F. Grismsley, S. Iqbal, T. Reponen, M. L. Muilenberg, P. S. Thorne, D. G. Dearborn, and R. L. Morley.** 2006. Mold and endotoxin levels in the aftermath of Hurricane Katrina: A pilot project of homes in New Orleans undergoing renovation. Environ Hlth Perspect **114:**1883-1889.

26. **Cooley, J. D., W. C. Wong, C. A. Jumper, and D. C. Straus.** 1998. Correlation between the prevelance of certain fungi and sick building syndrome. Occup Environ Med **55:**579-584.

27. **Cooley, J. D., W. C. Wong, C. A. Jumper, and D. C. Straus.** 2004. Fungi and the indoor environment: Their impact on human health., p. 3-30. *In* D. C. Straus (ed.), Sick Building Syndrome., vol. 55. Elsevier Academic Press, San Diego.

28. **Costa, A. R., E. Porto, S. Lacaz, N. T. de Melo, J. F. Calux, and N. Y. S. Valente.** 1988. Cutaneous and ungual phaeohyphomycosis caused by species of *Chaetomium* Kunze (1817) ex Fresenius, 1829. J Med Vet Mycol **26:**261-268.

29. **Deacon, J.** 2006. Fungal biology., 4th ed. Blackwell Publishing., Malden, MA.

30. **Domsch, K. H., W. Gams, and T. Anderson.** 1993. Compendium of soil fungi., vol. 1. IHW-Verlag.

31. **Flaherty, J. E., A. M. Pirttila, B. H. Bluhm, and C. P. Woloshuk.** 2003. PAC1, a pH-regulatory gene from *Fusarium verticillioides*. Appl Environ Microbiol **69:**5222-5227.

32. **Glinsukon, T., R. Shank, G. Wogan, and P. Newberne.** 1975. Acute and subacute toxicity of cytochalasin E in the rat. Toxicol Appl Pharmacol **32:**135-146.

33. **Gomori, G.** 1955. Preparation of buffers for use in enzyme studies., p. 138-146. *In* S. P. Colowick and N. O. Caplan (ed.), Methods of enzymology. Academic Press, New York.

34. **Gorny, R. L., T. Reponen, K. Willeke, D. Schmechel, E. Robine, M. Boissier, and S. A. Grinshpun.** 2002. Fungal fragments as indoor air biocontaminants. Appl Environ Microbiol **68:**3522-3531.

35. **Gravesen, S.** 1978. Identification and prevalence of culturable mesophilic microfungi in house dust from 100 Danish homes. Allergy **33:**268-272.

36. **Gravesen, S., L. Larsen, F. Gyntelberg, and P. Skov.** 1986. Demonstration of microorganisms and dust in schools and offices. Allergy **41:**520-525.

37. **Gregory, L., J. J. Pestka, D. G. Dearborn, and T. G. Rand.** 2004. Localization of satratoxin-G in *Stachybotrys chartarum* spores and spore-impacted mouse lung using immunocytochemistry. Toxicol Pathol **32:**26-34.

38. **Guarro, J., J. Gene, and A. M. Stchigel.** 1999. Developments in fungal taxonomy. Clin Microbiol Rev **12:**454-500.

39. **Guarro, J., L. Soler, and M. G. Rinaldi.** 1995. Pathogenicity and antifungal susceptibility of *Chaetomium* species. Eur J Clin Microbiol Infect Dis **14:**613-618.

40. **Guppy, K. H., C. Thomas, K. Thomas, and D. Anderson.** 1998. Cerebral fungal infections in the immunocompromised host: A literature review and a new pathogen - *Chaetomium atrobrunneum*: Case report. Neurosurgery **43:**1463-1469.

41. **Hardin, B. D., B. J. Kelman, and A. Saxon.** 2003. Adverse human health effects associated with molds in the indoor environment. J Occup Environ Med **45:**470-478.

42. **Harrach, B., M. Nummi, M. Niku-Paavola, C. J. Mirocha, and M. Palyusik.** 1982. Identification of "water-soluble" toxins produced by a *Stachybotrys atra* strain from Finland. Appl Environ Microbiol **44:**494-495.

43. **Haselwandter, K., and M. R. Ebner.** 1994. Microorganisms surviving for 5,300 years. FEMS Microbiol Letters **116:**189-194.

44. **Hattori, N., M. Adachi, T. Kaneko, M. Shimozuma, M. Ichinohe, and K. Iozumi.** 2000. Case report. Onychomycosis due to *Chaetomium globosum* successfully treated with itraconazole. Mycoses **43:**89-92.

45. **Hinkley, S. F., and B. B. Jarvis.** 2001. Chromatographic method for *Stachybotrys* toxins., p. 173-194. *In* M. W. Trucksess and A. W. Pohland (ed.), Methods in molecular biology. Volume 157: Mycotoxin protocols. Humana Press, Totowa, NJ.

46. **Hoppin, E. C., E. L. McCoy, and M. G. Rinaldi.** 1983. Opportunistic mycotic infection caused by *Chaetomium* in a patient with acute leukemia. Cancer **52:**555-556.

47. **Jarvis, B. B.** 2003. Analysis for mycotoxins: The chemist's perspective. Arch Environ Health **58:**479-483.

48. **Jarvis, B. B.** 1991. Chapter 14. Macrocyclic trichothecenes., p. 361-421. *In* R. P. Sharma and D. K. Salunkhe (ed.), Mycotoxins and phytoalexins. CRC Press, Boca Raton.

49. **Jarvis, B. B.** 1995. Secondary metabolites and their role in evolution. An Acad bras Ci **67:**329-345.

50. **Jarvis, B. B., and J. D. Miller.** 2005. Mycotoxins as harmful indoor air contaminants. Appl Environ Biotechnol **66:**367-372.

51. **Jarvis, B. B., W. G. Sorenson, E. Hintikka, M. Nikulin, Y. Zhou, J. Jiang, S. Wang, S. Hinkley, R. A. Etzel, and D. G. Dearborn.** 1998. Study of toxin production by isolates of *Stachybotrys chartarum* and *Memnoniella echinata* isolated during a study of pulmonary hemosiderosis in infants. Appl Environ Microbiol **64:**3620-3625.

52. **Karunasena, E., J. D. Cooley, D. R. Douglas, and D. C. Straus.** 2004. Protein translation inhibition by *Stachybotrys chartarum* conidia with and without the mycotoxin containing polysaccharide matrix. Mycopathologia **158:**87-97.

53. **Kendrick, B.** 2000. The Fifth Kingdom., Third ed. Focus publishing, Newburyport, MA.

54. **Kim, Y. K., C. L. Xiao, and J. D. Rogers.** 2005. Influence of culture media and environmental factors on mycelial growth and pycnidial production of *Sphaeropsis pyriputrescens*. Mycologia **97:**25-32.

55. **Kirk, P. M., P. F. Cannon, J. C. David, and J. A. Stalpers.** 2001. Dictionary of the fungi., 9th ed. CABI Publishing, Wallingford, Oxon, United Kingdom.

56. **Kozak, P. P., J. Gallup, L. H. Cummins, and S. A. Gillman.** 1979. Factors of importance in determining the prevalence of indoor molds. Ann Allergy **43:**88-94.

57. **Lesire, V., E. Hazouard, P. F. Dequin, M. Delain, M. Therizol-Ferly, and A. Legras.** 1999. Possible role of *Chaetomium globosum* in infection after autologous bone marrow transplantation. Intensive Care Med **25:**124-125.

58. **Li, D., and C. S. Yang.** 2004. Fungal contamination as a major contributor to sick building syndrome., p. 31-112. *In* D. C. Straus (ed.), Sick Building Syndrome., vol. 55. Elsevier, San Diego.

59. **Lodha, B. C.** 1987. Pleomorphy in Sordariales., p. 57-77. *In* Sugiyama (ed.), Pleomorphic fungi: The diversity and its taxonomic implications. Alsevier, Tokyo.

60. **Naidu, J., S. M. Singh, and M. Pouranik.** 1991. Onychomycosis caused by *Chaetomium globosum* Kunze. Mycopathologia **113:**31-34.

61. **Natori, S.** 1977. Toxic cytochalasins. *In* J. V. Rodricks, C. W. Hesseltine, and M. A. Mehlman (ed.), Mycotoxins in human and animal health. Pathotox Publishers, Park Forest South, Ilinois.

62. **Nielsen, K. F.** 2003. Mycotoxin production by indoor molds. Fungal Genet Biol **39:**103-117.

63. **Nielsen, K. F., S. Gravesen, P. A. Nielsen, B. Andersen, U. Thrane, and J. C. Frisvad.** 1999. Production of mycotoxins on artificially and naturally infested building materials. Mycopathologia **145:**43-56.

64. **Nielsen, K. F., M. O. Hansen, T. O. Larsen, and U. Thrane.** 1998. Production of trichothecene mycotoxins on water damaged gypsum boards in Danish buildings. International Biodeterioration and Biodegradation **42:**1-7.

65. **Nielsen, K. F., U. Thrane, T. O. Larsen, P. A. Nielsen, and S. Gravesen.** 1998. Production of mycotoxins on artificially inoculated building materials. International Biodeterioration and Biodegradation **42:**9-16.

66. **Nikulin, M., A. Pasanen, S. Berg, and E. Hintikka.** 1994. *Stachybotrys atra* growth and toxin production in some building materials and fodder under different relative humidities. Appl Environ Microbiol **60:**3421-3424.

67. **Ohtsubo, K., M. Saito, S. Sekita, K. Yoshihira, and S. Natori.** 1978. Acute toxic effects of chaetoglobosin A, a new cytochalasan compound produced by *Chaetomium globosum*, on mice and rats. Jap J Exp Med **48:**105-110.

68. **Page, W. M.** 1951. Viability of spores of some coprophilious species of *Sodaria* and *Chaetomium*. Trans Br Mycol Soc **34:**539.

69. **Penalva, M. A., and J. Arst, H N.** 2002. Regulation of gene expression by ambient pH in filamentous fungi and yeasts. Microbiol Mol Biol Rev **66:**426-446.

70. **Power, D. A., and M. J. Zimbro.** 2003. Difco & BBL Manual of Microbiological Culture Media., 1st ed. BD Diagnostic Systems.

71. **Probst, A., and C. Tamm.** 1981. 19-O-acetylchaetoglobosin B and 19-O-acetylchaetoglobosin D, two new metabolites of *Chaetomium globosum*. Helvetica Chimica Acta **64:**2056-2064.

72. **Probst, A., and C. Tamm.** 1981. Biosynthesis of the cytochalasans. Biosynthetic studies on chaetoglobosin A and 19-O-acetylchaetoglobosin A. Helvetica Chimica Acta **64:**2065-2077.

73. **Pujol, I., J. Guarro, C. LLop, L. Soler, and J. Fernandez-Ballart.** 1996. Comparison study of broth macrodilution and microdilution antifungal

susceptibility tests for the filamentous fungi. Antimicrob Ag Chemother **40:**2106-2110.

74. **Rao, C. Y.** 2000. Chaper 46. Toxigenic fungi in the indoor environment., p. 46.1-46.19. *In* J. D. Spengler, J. M. Samet, and J. F. McCarthy (ed.), Indoor Air Quality Handbook. McGraw-Hill, New York, NY.

75. **Redd, S. C.** 2002. State of the science on molds and human health., p. 1-12, Subcommittee on Oversight and Investigations and Housing and Community Opportunity. Committee on Financial Services.

76. **Sekita, S., K. Yoshihira, and S. Natori.** 1976. Structures of chaetoglobosins C, D, E, F, cytotoxic indol-yl-[13] cytochalasans from *Chaetomium globosum*. Tetrahedron Letters **17:**1351-1354.

77. **Sekita, S., K. Yoshihira, S. Natori, S. Udagawa, T. Murol, Y. Sugiyama, H. Kurata, and M. Umeda.** 1981. Mycotoxin production by *Chaetomium* spp. and related fungi. Can J Microbiol **27:**766-772.

78. **Serena, C., M. Ortoneda, J. Capilla, F. J. Pastor, D. A. Sutton, M. G. Rinaldi, and J. Guarro.** 2003. In vitro activities of new antifungal agents against *Chaetomium* spp. and inoculum standardization. Antimicrob Ag Chemother **47:**3161-3164.

79. **Shelton, B. G., K. H. Kirkland, W. D. Flanders, and G. K. Morris.** 2002. Profiles of airborne fungi in buildings and outdoor environments in the United States. Appl Environ Microbiol **68:**1743-1753.

80. **Sorenson, W. G.** 1999. Fungal spores: Hazardous to health? Environ Hlth Perspect **107:**469-472.

81. **Spengler, J. D., and K. Sexton.** 1983. Indoor air pollution: A public health perspective. Science **221:**9-17.

82. **St. Germain, G., and R. C. Summerbell.** 1996. Identifying filamentous fungi: A clinical laboratory handbook. Star Publishing Company, Belmont, CA.

83. **Stiller, M. J., S. Rosenthal, R. C. Summerbell, J. Pollack, and A. Chan.** 1992. Onchomycosis of the toenails caused by *Chaetomium globosum*. J Amer Acad Dermatol **26:**775-776.

84. **Straus, D. C., J. D. Cooley, W. C. Wong, and C. A. Jumper.** 2003. Studies on the role of fungi in sick building syndrome. Arch Environ Hlth **58:**475-478.

85. **Sutton, D. A., A. Fothergill, and M. G. Rinaldi.** 1998. Guide to clinically significant fungi. Williams and Wilkins, Baltimore.

86. **Teixeira, A. B. A., P. Trabasso, M. L. Moretti-Branchini, F. H. Aoki, A. C. Vigorito, M. Miyaji, Y. Mikami, M. Takada, and A. Z. Schreiber.** 2003. Phaeohyphomycosis caused by *Chaetomium globosum* in an allogeneic bone marrow transplant recipient. Mycopathologia **156:**309-312.

87. **Thomas, C., D. Mileusnic, R. B. Carey, M. Kampert, and D. Anderson.** 1999. Fatal *Chaetomium* cerebritis in a bone marrow transplant patient. Hum Pathol **30:**874-879.

88. **Udagawa, S., T. Murol, H. Kurata, S. Sekita, K. Yoshihira, S. Natori, and M. Umeda.** 1979. The production of chaetoglobosins, sterigmatocystin, O-methylsterigmatocystin, and chaetocin by *Chaetomium* spp. and related fungi. Can J Microbiol **25:**170-177.

89. **Umeda, M.** 1977. Cytotoxicity of mycotoxins. *In* J. V. Rodricks, C. W. Hesseltine, and M. A. Mehlman (ed.), Mycotoxins in human and animal health. Pathotox Publishers, Park Forest South, Illinois.

90. **Vesper, S. J., C. McKinstry, C. Yang, R. A. Haughland, C. M. Kercsmar, I. Yike, M. D. Schluchter, H. L. Kirchner, J. Sobelewski, T. M. Allan, and D. G. Dearborn.** 2006. Specific molds associated with asthma in water-damaged homes. J Occup Environ Med **48:**852-858.

91. **Volz, P. A., and S. L. Parent.** 1998. Space flight micro-fungi after 27 years storage in water and in continuous culture. Microbios **96:**111-125.

92. **Watanabe, T.** 2002. Pictorial atlas of soil and seed fungi. Morphologies of cultured fungi and key to species., Second ed. CRC Press, Boca Raton, FL.

93. **Wilson, S. C., C. G. Carriker, T. Brasel, E. Karunasena, D. R. Douglas, C. Wu, L. A. Andriychuk, M. R. Fogle, J. D. Martin, and D. C. Straus.** 2004. Culturability and toxicity of sick building syndrome-related fungi over time. J Occup Environ Hyg **1:**500-504.

94. **Woods, G. L., J. C. Davis, and W. P. Vaughan.** 1988. Failure of the sterile air-flow component of a protected environment detected by demonstration of *Chaetomium* species colonization of four consecutive immunosuppressed occupants. Infect Contr Hosp Epidemiol **9:**451-456.

95. **Yeghen, T., L. Fenelon, C. K. Campbell, D. W. Warnok, A. V. Hoffbrand, H. G. Prentice, and C. C. Kibbler.** 1996. *Chaetomium* pneumonia in patient with acute myeloid leukaemia. J Clin Pathol **49:**184-186.

96. **Yike, I., A. M. Distler, A. G. Ziady, and D. G. Dearborn.** 2006. Mycotoxin adducts on human serum albumin: Biomarkers of exposure to *Stachybotrys chartarum*. Environ Hlth Perspect **114:**1221-1226.

97. **Yu, J., S. Yang, and R. Li.** 2006. A case of subcutaneous phaeohyphomycosis caused by *Chaetomium globosum* and the sequences analysis of *C. globosum*. Med Mycol **44:**541-545.

APPENDIX

Table 12. Concentration of each component in the buffered medium.

Medium	Predicted pH	Component A[1]		Component B[1]	
		Amount (g/L)	Concentration (M)	Amount (g/L)	Concentration (M)
Tris buffered PDA		THAM		HCL	
	7.2	6.06	0.05	1.61	0.0442
	8.0	6.06	0.05	0.98	0.0268
	9.0	6.06	0.05	0.18	0.0050
Citrate-phosphate buffered PDA		Citric acid		Sodium phosphate	
	3.0	7.65	0.0398	2.90	0.0204
	4.0	5.90	0.0307	5.48	0.0386
	5.0	4.67	0.0243	7.30	0.0514
	6.0	3.44	0.0179	9.11	0.0642
	7.0	1.25	0.0065	12.38	0.0872
Carbonate-bicarbonate buffered PDA		Sodium carbonate		Sodium bicarbonate	
	9.2	0.42	0.0040	3.86	0.0460
	10.0	2.93	0.0276	1.88	0.0224
	10.7	4.79	0.0452	0.42	0.0050
Tris-maleate buffered PDA		Tris-maleate		NaOH	
	5.2	11.86	0.05	0.28	0.0070
	6.0	11.86	0.05	1.04	0.0260
	7.0	11.86	0.05	1.92	0.0480
	8.0	11.86	0.05	2.76	0.0690
	8.6	11.86	0.05	3.46	0.0865

[1]Each buffer consists of two components. The amount (g/L) and concentration (M) of each component is listed for each buffer.

Table 13. Effect of different volumes of water on the pH measurements of sterile PDA.

Medium	Predicted pH of buffer	pH[1]		
		12.5 ml	25 ml	50 ml
Unbuffered PDA	n/a	5.28	5.18	4.98
Tris buffered PDA	7.2	6.62	6.54	6.33
	8.0	7.63	7.60	7.54
	9.0	8.15	8.11	8.04
Citrate phosphate buffered PDA	3.0	3.41	3.47	3.55
	4.0	4.29	4.29	4.33
	5.0	5.28	5.30	5.28
	6.0	6.29	6.33	6.32
	7.0	7.23	7.22	7.21
Carbonate-bicarbonate buffered PDA	9.2	8.98	8.93	8.78
	10.0	9.04	8.99	8.85
	10.7	9.20	9.15	9.06
Tris-maleate buffered PDA	5.2	5.17	5.13	5.09
	6.0	5.77	5.75	5.71
	7.0	6.43	6.40	6.35
	8.0	7.28	7.22	7.14
	8.6	7.84	7.73	7.67

[1]Average of 2 plates is shown. One inch circular piece of agar soaked in either 12.5, 25 or 50 ml of water for 1 hour at room temperature. Agar removed and pH of water measured while stirring.

Table 14. Comparison of indirect and direct methods for measuring pH on sterile PDA.

Medium	Predicted pH of buffer	pH Indirect method[1]	pH Direct method[2]
Unbuffered PDA	n/a	5.18	5.14
Tris buffered PDA	7.2	6.54	6.41
	8.0	7.60	7.39
	9.0	8.11	7.91
Citrate phosphate buffered PDA	3.0	3.47	3.33
	4.0	4.29	4.16
	5.0	5.30	5.00
	6.0	6.33	5.94
	7.0	7.22	6.87
Carbonate-bicarbonate buffered PDA	9.2	8.93	8.73
	10.0	8.99	8.75
	10.7	9.15	8.90
Tris-maleate buffered PDA	5.2	5.13	5.02
	6.0	5.75	5.60
	7.0	6.40	6.23
	8.0	7.22	7.19
	8.6	7.73	7.71

[1]Indirect method – One inch circular piece of agar soaked in 25 ml of water for 1 hour at room temperature. Agar removed and pH of water measured using a Model 15 pH meter (Fisher Scientific, Pittsburg, PA) while stirring. An average of 2 plates is shown.

[2]Direct method – The pH of the surface of the agar plates were measured using an SA520 pH meter (Orion Research Inc., Boston, MA). Three measurements were taken on each plate. An average of 2 plates is shown.