DISTRIBUTION OF MYCOTOXINS IN FUNGAL STRUCTURES

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Dedication

This dissertation is dedicated to my mother, Marion Errede, who always encouraged her family to be productive and to do their best, and who did her best to stay with us as long as she could.

Abstract

Exposure to mycotoxins associated with small fungal fragments may cause adverse health effects. Current air sampling methods may not detect such fragments and may cause researchers to underestimate fungal exposures. Understanding how time and substrate affect the distribution of toxins and their release into the environment can strengthen estimates of potential risks of exposure to these toxin-carrying particles.

This research investigated the influence of time and growth substrate on the amount and distribution of mycotoxins in fungal structures and particles released from the fungal colonies. A common, toxin-producing, indoor mold, *Aspergillus versicolor*, was inoculated on two different agars: Malt Extract (MEA) and Wallpaper Paste (WPA). Fungal material was collected both by coring the plates, and by washing the plates with sterile water and glass beads at weekly intervals from one to six weeks. The wash water suspensions were filtered through 20- and 1-micron filters and lyophilized. The filters and the lyophilized wash water samples were extracted with methanol and analyzed for both sterigmatocystin and ergosterol (a marker of fungal growth).

Results indicate that overall growth and toxin levels are higher on MEA than on WPA, but when standardized against ergosterol, sterigmatocystin levels on WPA are higher. Measurable amounts of ergosterol and sterigmatocystin are detectable in the lyophilized wash water. Ergosterol and sterigmatocystin increase steadily over six weeks on WPA, but peak around 3-4 weeks on MEA. Our conclusions are that small fungal fragments can carry toxins at levels that could deliver biologically significant doses if the fragments were inhaled into the deep lung.

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Chapter 1 – Introduction

The hypothesis for this research is that time and growth substrate affect the amount and distribution of mycotoxins in particles released from fungal colonies. This information is important because exposure to mycotoxins associated with small fungal fragments may cause adverse health effects. Understanding how time and substrate affect the distribution of toxins and their release into the environment can help us better estimate potential risks of exposure to these toxin-carrying particles. This thesis includes a review and discussion of background information regarding this topic (Chapter 1), studies of the toxicity of *Aspergillus versicolor* extracts to fetal rat lung cells (Chapter 2), studies to determine the amount and distribution of sterigmatocystin, a mycotoxin produced by *A. versicolor*, in fungal structures and release of particles with this toxin (Chapter 3), a discussion of whether detected sterigmatocystin could be dissolved or particulate-associated (Chapter 4), and a summary and discussion of findings and recommendations for future research (Chapter 5).

1.1 Mold classification, structures and metabolites

Fungi are eukaryotic, multicellular organisms that are classified in their own kingdom, as they are neither plants nor animals (Deacon 2006). They obtain energy by decomposing organic material, and play a vital role in recycling nutrients in the environment. Formerly, fungi were divided into four phyla: Zygomycota, Ascomycota, Basidiomycota, and Deuteromycota (also known as Fungi Imperfecti) (Columbia University Press 2008). Basidiomycota are typically found outdoors growing on dead

trees and plant material and include mushrooms, shelf fungi and puffballs. Zygomycota are characterized by nonseptate mycelium, while Ascomycota and Deuteromycota have septate mycelium. The Deuteromycota produce only asexual spores called conidia, while the Ascomycota produce sexual as well as asexual spores (Pitt and Hocking 1999). Recently, the kingdom has been divided more finely (Hibbett and others 2007) and the Deuteromycota have been incorporated into either the Ascomycota and Basidiomycota (Deacon 2006)(Hibbett and others 2007). Current thinking is that the sexual stage of former Deuteromycota species either remain to be discovered, or in some cases may have been abandoned. Under this system, indoor molds are generally part of the Ascomycota.

With organic material, oxygen and water, mold grows vegetatively, sending out branched, filamentous hyphae that expand apically and collectively form a rope-like structure called the mycelium (Eduard 2009). Primary metabolites involved in this growth include the cell wall components, chitins (a long-chain polymer of a *N*-acetylglucosamine with β-1,4 linkages) and glucans (glucose polymers with mostly β-1,3 and β-1,6 linkages), and ergosterol, a fungal sterol found in the cell membrane (Deacon 2006). As fungal glucans can produce nonspecific inflammatory reactions (Douwes and others 1996) and may also have immuno-modulating effects (Seo and others 2008), exposure to fungal particles carries some potential for health risks.

In addition to vegetative growth, molds can reproduce through production of spores and conidia, which can be widely dispersed. Specialized hyphae that produce conidia are called conidiophores (Samson and others 1996). As fungal colonies mature and produce conidia, the conidia may be released to the environment through active or

passive means (Meredith 1973). In some molds, dropping humidity causes fungal structures to twist and scatter conidia in all directions. In others, increasing humidity causes structures to swell, burst and release conidia. In still others, movement of wind, water or animals may passively move conidia to new growth substrates.

Intuitively, it would seem that conidia, which can be removed from the fungal colony and distributed in the environment, would pose more of an exposure hazard than hyphae and mycelia, which are typically associated with the substrate. However, fragments released from fungal colonies may outnumber larger intact conidia by orders of magnitude (Górny and others 2002), and it is unclear whether these fragments originate from hyphae or conidia.

To investigate release of fragments, ceiling tiles and agar plates with Malt Extract Agar were inoculated separately with *Aspergillus versicolor*, *Cladosporium cladosporiodides*, and *Penicillium melinii*, and incubated to achieve abundant fungal growth (Górny and others 2002). The plates were then exposed to moving air (from 0.3 meters per second (m/s), typical of indoor air, to 29.1 m/s, typical of a ventilation duct) for 30 minutes, and the size and number of particles released from the substrate were measured.

Results showed that up to 320 times more fragments (<1.6 µm) than intact particles (≥ 1.6 µm) were released over a 30-minute test period (Górny and others 2002). Higher air velocities did not increase the numbers of spores or fragments released from substrates inoculated with *A. versicolor*. In contrast, more intact spores were released at the highest air velocity from substrates inoculated with *C. cladosporiodides* and *P.*

melinii, but like *A. versicolor*, numbers of fragments were unaffected. Even at the highest velocity however, fragments outnumbered spores by one to two orders of magnitude. Interestingly, about 80% of the particles were released in the first 10 minutes of the 30-minute test period (Górny and others 2001), indicating that particles tend to be released in bursts, and not at a steady rate.

The fungal origin of the fragments was confirmed by testing the immunological reactivity of both the spores and fragments of *A.versicolor* and *P.melinii* with monoclonal antibodies (Mab) in an enzyme-linked immunosorbent assay (Górny and others 2002). The Mabs were produced against *A. versicolor*, *P. brevicompactum* and *P. melinii*, but all three cross-reacted with *P. melinii*, and the last two Mabs cross reacted with *A. versicolor*. The cross-reactivities are not unexpected, as *Aspergillus* and *Penicillium* species share multiple antigens (Schmechel and others 2003). Both spores and fragments showed significant immunological reactivity, compared to blank filter samples (Górny and others 2002). However, the reactivity of the fragments was 2-5 times greater than the reactivity of the spores. This finding supports the hypothesis that fungal fragments may pose more of a health hazards than intact particles.

Time and the type of substrate can affect growth by a given fungus. Malt Extract Agar (MEA), cellulose ceiling tiles and wall-papered gypsum board were inoculated with *A. versicolor* and *Stachybotrys chartarum*, separately, and incubated from one to six months (Seo and others 2008). The researchers measured β-1,3-D-glucan to determine the amount of fungal growth on the surface of the substrate. Results showed the most growth for *A. versicolor* on MEA, followed by gypsum board and ceiling tile. For *S.*

Chartarum, growth was heaviest on the gypsum board, followed by the ceiling tile and MEA. Over the six months, fungal material on the ceiling tiles increased for both species but held steady on gypsum board. On MEA, levels of measured β-1,3-D-glucan decreased for *A. versicolor* and held steady for *S. chartarum.*

Aerosolization of fungal particles was also affected by the type of substrate (Seo and others 2008). When air was blown onto the inoculated samples, aerosolized particles were collected and analyzed for β-1,3-D-glucan. Results showed that for both species approximately 10% of the fungal material aerosolized, except for *S. chartarum* on MEA when only between 0.01% and 0.1% of the material aerosolized. Time did not affect aerosolization of fungal material, as β-1,3-D-glucan levels at 1 month and at 6 months were statistically the same for both species.

Under certain conditions, often during conidiogenesis, fungi may also produce secondary metabolites including mycotoxins (Guzmán-de-peña, Aguirre, Ruiz-Herrera 1998). Unlike primary metabolites, secondary metabolites are not generally essential for growth or normal metabolism, and are often specific to a certain genus, species or even strain (Deacon 2006). Some of these mycotoxins, such as aflatoxins from *Aspergillus flavus*, are toxic and carcinogenic (WHO-IARC 1976) with even greater potential for health effects than the primary metabolites.

Toxins may be extracellular or intracellular. Aflatoxins are extracellular metabolites, meaning they may diffuse into the growth substrate (Filtenborg, Frisvad, Svendsen 1983). Others, such as sterigmatocystin from *Aspergillus versicolor*, are intracellular and do not typically diffuse into the growth substrate. *A. versicolor* is

commonly found in water-damaged indoor environments (Nielsen 2003). In addition to producing sterigmatocystin, which is structurally related to aflatoxin and is itself toxic and carcinogenic (Purchase and Van Der Watt, J. J. 1970), *A. versicolor* also produces other secondary metabolites, including 5-methoxysterigmatocystin (Gravesen 2000), 6-methoxysterigmatocystin, aversin, averufin, averythrin and three closely related pigments, versicolorin A, B and C (Hamasaki and others 1967). Although sterigmatocystin is an intracellular metabolite, tests for ciliostatic activity of fungal extracts indicated that *A. versicolor* metabolites could be extracellular (Piecková and Kunová 2002). The location of the toxins is important because toxins that partition into fungal material with the potential to fragment into small airborne particles are more likely to impact people and pose greater health risks.

The type of growth substrate can affect toxin production. After inoculation with *A. versicolor* and 10 days of incubation, building materials were assessed for ergosterol, a marker of fungal growth, and sterigmatocystin content (Moularat and Robine 2008). Interestingly, glass fabric (fiberglass wallpaper) produced ergosterol but no sterigmatocystin. There was much less fungal growth on compressed fiberglass filters, but sterigmatocystin was produced in approximately a 4/1 ratio with ergosterol. Traditional paper-based wallpaper produced even more sterigmatocystin, but the ratio compared to ergosterol was approximately 6/17. Vinyl wallpaper produced the most toxin overall, and the ratio with ergosterol was approximately 8/5. These results show that fungal growth does not correlate directly with toxin production, and that substrate can have dramatic influence over toxin production.

Mycotoxins are not necessarily distributed uniformly thoughout the fungal structure. Rice inoculated with four different species of *Aspergillus* (*parasiticus, parasiticus* mutant, *niger*,and *fumigatus*), and *Penicillium oxalicum* was analyzed for mycotoxin distribution (Palmgren and Lee 1986). After seven days of incubation and three days of oven drying, air was pulled through the sample to remove conidia from the mycelia on the rice, although not all were removed. The results showed species variability, as toxin from both strains of *A. parasiticus* and *P. oxalicum* remained mostly in the rice-hyphae matrix, whereas the conidia contained most of the toxin from *A. niger* and *A. fumigatus*. This work supported earlier findings that *A. fumigatus*, when grown in liquid medium, produced toxin in the conidia, but not in the mycelia (Parker and Jenner 1968).

Recent studies confirm that fragments released from fungal colonies can contain mycotoxins. Mycotoxins were detected in fragments collected from air pulled over ceiling tiles inoculated with *Stachybotrys chartarum* (Brasel and others 2005a). In another study, samplers collected inhalable (<100 µm), thoracic (<10 µm) and alveolar fractions (< 4.25 µm) of airborne particulates generated from *A. versicolor*- inoculated vinyl wallpaper (Moularat and Robine 2008). Results showed comparable levels of sterigmatocystin in all three fractions.

1.2 Mold in the environment

Mold is an integral part of our indoor and outdoor environments. Depending on our geographical location and the season, whenever we go outdoors we breathe in

particles of several different molds. Recent findings of fungal DNA in house dust have shown that fungal species indoors are more diverse in temperate than tropical regions, and are more strongly influenced by fungal composition of the outdoor environment, than by building design or construction (Amend and others 2010).

Typically, air samples collected for viable fungal organisms indicate that colony counts are higher outdoors than indoors (Flannigan and Miller 1994). In mechanically ventilated buildings, air handling systems filter the air that is drawn into our homes and workplaces, removing some of the fungal particles and reducing indoor concentrations compared to outdoor exposures. In naturally ventilated buildings, air movement slows down and particles have time to settle out of the air and deposit on surfaces, which may lead to lower airborne counts.

Although fungal counts may be lower indoors, the types of fungal species in air samples from contaminated buildings may be very different from species in outdoor controls (Miller, Haisley, Reinhardt 2000). Indoor samples may be dominated by a species that has found an indoor environment conducive to germination, growth and reproduction. Because this typically occurs when the building has sustained some kind of water damage, preventing water damage is critical for preventing indoor mold growth. If water leaks in through roofs, around windows, or condenses on air conditioned surfaces, dormant mold particles can germinate and colonize the substrate. Even surfaces, such as fiberglass, that would seem to lack the organic matter to support mold growth can become colonized under certain conditions. For example, analysis of fiberglass used in heating and air conditioning systems in buildings with moldy odors revealed heavy

colonization with *Aspergillus versicolor* (Ezeonu and others 1994). The fiberglass trapped dust and dirt, providing the organic fuel that, when wetted, supported germination and growth of an adaptable species such as *A. versicolor*.

Sources contributing to indoor mold exposures may or may not be obvious. Moldy ceiling tiles or sheetrock are often clearly visible. Contaminated ventilation ductwork can release particles directly into occupied building space. Sometimes, though, water damage is hidden inside walls or above false ceilings, and would seem unlikely to contribute to mold exposures in occupied spaces. However, particles from more inaccessible sources can move through even small openings around lights or electrical outlets. In laboratory studies, particles have been shown to migrate through slits between compartments (Mosley and others 2001). Using pressure differences ranging from 2 – 20 Pascals, typical of pressures within an indoor residence, particles moved from one compartment to another. With increasing pressure, higher percentages of smaller particles migrated than larger particles.

Regardless of the source, indoor fungal particles and associated toxins may deposit as dust inside homes. Activity within the home may resuspend particles accumulated on surfaces or in carpeting, leading to inhalation exposures for building occupants (Buttner and Stetzenbach 1993). To investigate this potential hazard, settled dust samples were collected from homes and workplaces with indoor air problems. The samples were cultured, and isolates of *Penicillium* and *Aspergillus* were tested for their ability to produce mycotoxins (Frisvad and Gravesen 1994). Almost all of the tested strains produced mycotoxins, indicating a potential for exposure to these compounds. A

confounding factor, however, was the choice of growth substrate, which was found to influence toxin production.

Mycotoxins have been detected in airborne particles from homes and workplaces. Particles and fragments collected in homes contaminated with *S. chartarum* were tested directly for mycotoxins (Brasel and others 2005b). Results showed that toxin levels were significantly elevated above control homes, confirming the potential for inhalation exposure to mycotoxins. In Germany, air samples were collected from three different composting facilities to investigate exposure to toxins from *A. fumigatus* in compost workers. Interestingly, total dust measurements (mg dust/m^3 air) did not correlate with airborne colony forming units (cfu) of *A. fumigatus* (cfu/m^3). However, where cfu's were highest ($3.2x10^7$ cfu/m^3), the researchers also detected the mycotoxins tryptoquivaline, a tremorigen, and trypacidin.

Primary metabolites in these particles may also pose exposure risks. As noted in the introduction, fungal glucans can produce nonspecific inflammatory reactions (Douwes and others 1996) and may also have immuno-modulating effects (Seo and others 2008). The fungal polysaccharide β-1,3-D-glucan was detected in air samples collected during vacuuming of homes (Salares, Hinde, Miller 2009). Particles in the air samples were separated into eight size fractions ranging from 18 μm to 0.18 μm, and approximately equal amounts of glucans were found in each, illustrating the potential for fine fragments as well as larger particles to contribute to fungal exposure.

1.3 Particle deposition and clearance from the lungs

If inhaled, particles such as those described above could cause adverse health effects. Fragments may present more of a health hazard than intact spores or larger particles because they are more likely to deposit in the alveoli of the lungs (Figure 1-1). Where and how particles deposit in the lungs depends in large part on the size and shape of the particle, but also on a person's breathing pattern or flow rate (Cho and others 2005).

Mechanisms of deposition include interception, impaction, sedimentation or diffusion (Witschi and others 2008). Particles that are less spherical and more fiber-shaped are more likely to deposit by interception. Coarse particles (5-10 µm) are most likely to deposit through impaction in the upper respiratory portion of the lung (nasal, pharyngeal, laryngeal) (Hatch 1961). However, Figure 1-1 shows that during nasal breathing, some of the smallest particles (<0.01 µm) are also efficiently deposited in this NPL region, due to diffusion (Witschi and others 2008).

In general, as particles get smaller, they are more likely to reach lower areas of the lung where they may be harder to clear (Witschi and others 2008). As particles move beyond the upper airways into the finer branches of the bronchial region, air velocity decreases and particles greater than 0.5 µm in diameter tend to deposit by sedimentation. Sedimentation is no longer effective on particles with diameters smaller

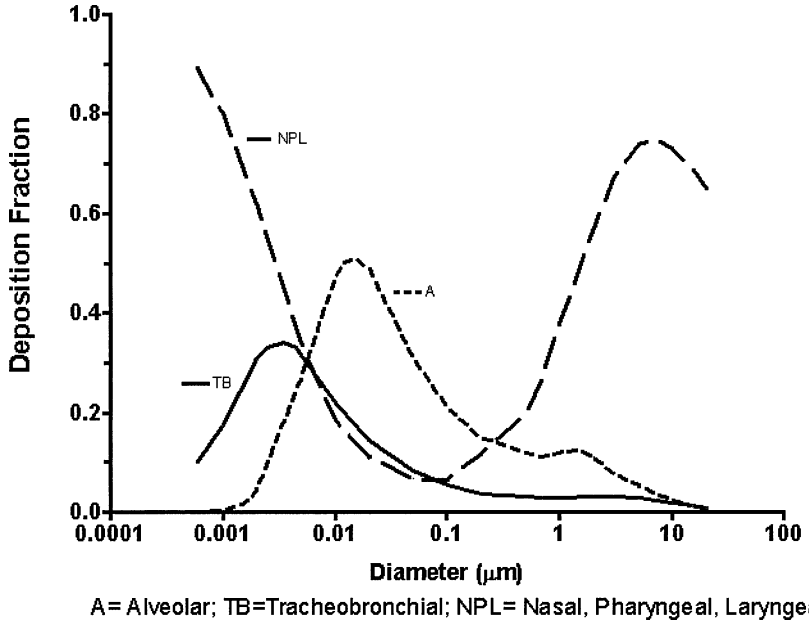


Figure 1-1. **Particle Deposition in the Respiratory Tract** International Commission on Radiological Protection (1994) model of fractional depositions of inhaled particles ranging from 0.5 nm to 20 µm in the nasal, pharyngeal, laryngeal region, the tracheobronchial region and the alveolar region of the human respiratory tract during nasal breathing (Oberdörster and others 2004).

than 0.5 µm, and the particles will tend to follow air streamlines through the ever-finer branching airways to the alveoli. Air velocity is lowest in the alveoli and diffusion becomes the primary force for deposition of the smallest particles. As shown in Figure 1-1, fungal fragments <0.5 µm would have a good chance of depositing in the alveoli. However, respiratory deposition models predict that, due to breathing pattern differences, deposition rates in the lower airways of children may be 4-5 time greater than deposition rates in adults (Cho and others 2005).

Once deposited, particle-associated toxins could affect surrounding tissues, but clearance mechanisms reduce some of the potential for toxin exposure. The mechanism

of particle removal varies partly with the site of deposition (Witschi and others 2008). Coughing and nose blowing remove particles entirely from the body, but other lung clearance mechanisms simply move the particles to the stomach and gastrointestinal tract, the lymphatics and lymph nodes, or the pulmonary vasculature. Cilia under the mucous in the tracheobronchial region move in concert to bring particles up to where they can be swallowed. In the alveolar region, however, macrophages may phagocytize the particles. The macrophages can then be cleared by either lymphatic drainage or movement on the alveolar fluid layer to the mucociliary escalator.

1.4 Limitations of current exposure sampling methods

The previous sections show that mold and its primary and secondary metabolites are present in the environment, and that inhalation could lead to exposure through deposition in the respiratory system. However, studies showing a causal relationship between exposure to these compounds and health effects are lacking (IOM 2004). This may be due in part to limitations in current exposure sampling methods, which generally include collection of viable samples or collection of total particles for standard microscopic analysis. For example, field studies do not always find elevated airborne fungal concentrations in moldy spaces compared to non-moldy spaces (Cho and others 2005). Out of 58 apartments sampled for viable fungal particles, there was no detectable difference in numbers of colony forming units between the 15 apartments with the most fungal contamination (assessed visually), and the 15 with the least (Miller, Haisley, Reinhardt 2000).

Viable sampling results may skew our perceptions of 'typical' indoor fungal species for a number of reasons (Amend and others 2010). First, only culturable fungal particles are counted. Other fungal particles in the sample, too small, damaged or desiccated during the collection process, may fail to germinate and therefore not be counted. Some conidia such as those from *Aspergillus* or *Penicillium* species are more resistant to dessication, but the viability of others such as *Botrytis cinerea* or *Stachybotrys atra* can decline rapidly (Flannigan and Miller 1994). Species susceptible to desiccation would tend to be under-reported by viable counts.

Another limitation of viable sampling is that the selected collection media may favor the growth of certain species over others. Slow growers may be over-run by faster growing species and, therefore, be under-counted. The sampling time, depth of impaction in the collection agar and desiccation of the particles all affect results obtained from viable samplers (Zhen and others 2009). Particles that land too shallowly or deeply in the agar may fail to germinate. Fungal particles that are simply too small or fragmented may fail to germinate and will not be counted by this method, even though they may still contain toxins or allergens which may affect exposed individuals.

Because of the drawbacks of viable sampling, researchers sometime prefer to sample for total fungal particles, viable or non-viable. One device designed for total particle collection is the Air-O-Cell sampler (Zefon International, Inc., Ocala FL). When connected to a sampling pump, air samples are pulled into an Air-O-Cell cassette where particles deposit onto a glass slide coated with adhesive. All the particles, viable or not, are later examined under a research-grade microscope. The microscopist determines if

the particles are fungal and, if so, counts them and identifies them to the genus level if possible.

However, sampling for total fungal particles also has drawbacks. Like the viable sample study described previously, fungal samples collected over one hour with the Air-O-Cell in homes with and without water damage showed no relationship to the extent of area with visible mold in the home (Foto and others 2005). Other disadvantages are that it can be difficult to identify the observed fungal particles to the species or sometimes even the genus level. Also, fungal fragments smaller than the resolution capabilities of the microscope will not be detected. This is important because, like non-viable fungal particles, fragments may contain biologically active compounds that could affect exposed individuals.

Both the viable and non-viable methods collect relatively short-term samples that give researchers a 'snapshot' view of particle concentrations. However, particles may be released from mold colonies only intermittently, for example when disturbed by an external force such as wind or vibration. Such particles, therefore, may not be detectable at the time of sampling. Ten years of air sampling within a hospital showed that *Aspergillus spp.* were typically present and culturable, but approximately 3% of the samples were identified as transient spikes or bursts, as they were at least three standard deviations higher than the mean (Falvey and Streifel 2007). Disturbance of fungal-contaminated surfaces or water supplies may have contributed to these spikes. Other researchers also have noted considerable variation in spore concentrations, as much as a factor of 1,000 in samples collected at weekly intervals in residential homes, and up to

10,000 times in samples collected at hourly intervals over one day (Flannigan and Miller 1994).

Long-term sampling for airborne particles can better capture transient spikes in fungal particle release. Although spikes would be integrated into the average, particles collected over several days on filters can be analyzed for fungal markers such as (1→3)β-D-glucan or ergosterol. Samples collected over 5-7 days in 110 homes in Prince Edward Island, Canada, were analyzed for these compounds (Foto and others 2005). Unlike viable colony forming units or total spore counts, these measures did in fact correlate with area of visible mold damage in the homes. Ergosterol was better correlated to area of mold damage than (1→3)β-D-glucan. Because normal activity within the home may resuspend dust again and again, this research demonstrates that ergosterol concentrations in settled dust can give a better long-term picture of potential fungal exposures.

Although it is more intrusive than ambient air monitoring, biomonitoring can be a more accurate way to assess actual exposures. Mycotoxins were detected in sera from individuals with documented indoor *S. chartarum* exposure, using an enzyme-linked immunosorbent assay highly specific for mycotoxins from *S. chartarum* (Brasel and others 2004). Only 25% of serum samples from control individuals were above detection limits, whereas 50% of serum samples from individuals with documented exposures were above detection limits, and 30% of samples from individuals with reported but undocumented exposures were above detection limits. In this study, ambient exposures were assessed by investigation of the building by consultants and collection of air, surface and bulk samples. Ambient exposures were not quantified, but only qualitatively

expressed as ‘documented’, ‘reported but undocumented’ or ‘controls’ with no known exposures. Linking ambient exposures and biomarker data would increase the probability of detecting a causal relationship between fungal exposures and adverse health effects.

1.5 Toxicity of fungal particles

Difficulties with exposure characterization and lack of biological markers in exposed individuals have made it challenging for researchers to document health effects of exposure to molds in indoor environments. Fungal exposure cannot be avoided, but as described above, moisture can amplify the growth of certain molds in buildings, leading to exposures that are not typical of background and may pose health risks. Molds have been shown to be infectious and allergenic in humans. Toxicity has been documented through ingestion of moldy food. However, toxicity through inhalation exposure is much more controversial (Terr 2009). Fungal infections such as aspergillosis or histoplasmosis are possible, especially in immune-compromised individuals, but otherwise are rare (Robbins and others 2000) and will not be discussed further in this review. The following sections address the health effects of particle and mold exposures documented from *in vitro*, animal and human studies.

1.5.1 Particle effects

As described above, fungal particles released to the environment may be very small and, as such, may pose a hazard simply due to their size. Although the Occupational Health and Safety Administration regulates exposure to dust based on mass (mg dust / m^3 air), the critical measurement with small particles may be particle number

or particle surface area (Witschi and others 2008). A given mass of fine particles (0.1 μm < diameter < 2.5 μm) has 100-1000 times more surface area than an equal mass of coarse particles (2.5 μm < diameter < 10 μm) (Geller and others 2002). As the surface area of deposited particles increases, there is more potential for any toxins or allergens associated with those particles to affect surrounding tissue.

According to ASTM International, the term 'nanoparticle' refers to "a sub-classification of ultrafine particle with lengths in two or three dimensions greater than 0.001 micrometer (1 nanometer) and smaller than about 0.1 micrometer (100 nanometers) and which may or may not exhibit a size-related intensive property"(ASTM International 2006). Surface area for a given mass of ultrafine or nanoparticles (0.001 μm – 0.1 μm) is approximately 10^5 times more than an equal mass of coarse particles which may significantly affect their relative toxicity (Geller and others 2002).

In research on nanoparticles, the large cumulative surface area and their unique physicochemical properties may pose health risks that extend beyond those posed by the parent material. For example, mice exposed intratracheally to a single dose of single-wall carbon nanotubes (SWCNTs) showed dose-dependent lung lesions and pulmonary granulomas (Lam and others 2003). The researchers concluded that SWCNTs can be more toxic than quartz, a compound with documented health hazards.

Another study of rats exposed to SWCNTs through intratracheal instillation noted the formation of granulomatous lesions without evidence of pulmonary inflammation, cellular proliferation or cytotoxicity typical of exposure to toxic dusts such as quartz or silica (Warheit and others 2003). The researchers hypothesized that this was a potentially

new mechanism of pulmonary toxicity and injury, with the granulomatous lesions forming as the respiratory system activated its immune response to try to remove a not-easily-degradable foreign substance. These studies highlight that, although particles may be small, their size may allow them to deposit deep and the lung and exposure may result in adverse health effects.

Of even more concern is evidence than nanoparticles may translocate from the site of contact to other parts of the body. Ultrafine polymer fumes of polytetrafluroethylene, inhaled by rats, appeared rapidly in epithelial, interstitial and endothelial sites (Oberdörster 2000). Later studies with rats that inhaled ultrafine carbon particles indicated that the particles translocated to the central nervous system via the olfactory nerve (Oberdörster and others 2004). These findings indicate that ultrafine particles can circumvent the blood-brain barrier, and may be capable of causing neurotoxic effects.

1.5.2 *In vitro* studies

Fungal extracts can be cytotoxic to cells. In tests with a human diploid embryonic fibroblast lung cell line, MRC-5, 47 of 119 fungal isolates (39%) collected from the air of damp Scottish homes showed cytotoxic properties in an MTT assay (Anderson, Lewis, Smith 1994). After incubating the fungi on grain samples (wheat or rice), conidia were removed with air, collected on filters, extracted with distilled water and tested on the MRC-5 cells. This assay selected for water-soluble metabolites, but many mycotoxins are only negligibly soluble in water. If samples had been extracted with a solvent such as

chloroform or methanol, even more than 39% of the isolates may have shown cytotoxic properties.

In another study, molds were extracted first with methanol and then dichloromethane before being tested on four different cell lines (Bünger and others 2004). Four different species of *Aspergillus* and a *Penicillium* were chosen to represent the common genera collected from a composting plant. They were cultured for two weeks on Yeast Extract Sucrose agar before being extracted for mycotoxins. The extracts and purified mycotoxins were tested on a human lung cancer cell line, an epithelium-like cell line from human hepatocellular carcinoma, murine fibroblasts and a neuronal cell line from a murine neuroblastoma. Using the Neutral Red Uptake assay, the extracts had serious adverse effects on all cell lines. Because only part of the effects could be explained by the identified toxins in the extracts, the researchers hypothesized other toxins were also present.

Other experiments have also assessed the effects of purified mycotoxins in various cell lines. Exposure of porcine intestinal epithelial cells to fumonisin B1, a mycotoxin produced by *Fusarium verticilliodides*, decreased the growth and impaired the barrier function of these cells (Bouhet and others 2004). The authors hypothesized that by modifying the barrier function, the mycotoxins could potentially be absorbed from the intestines in vivo at higher than normal rates. It is possible that inhaled mycotoxins could produce similar modifications in the lungs, leading to higher than expected absorption rates and systemic exposure.

Enzymes can metabolize mycotoxins to produce compounds that are sometimes less, but sometimes more toxic than the parent compound. Aflatoxin B_1 (AFB_1), a mycotoxin that is a known human carcinogen, is produced by some strains of *Aspergillus flavus* and *Aspergillus parasitacus* (WHO-IARC 1976). *In vitro,* human lung microsomes activate aflatoxin B_1 (AFB_1) to form the highly reactive *exo*-AFB_1-8,9-epoxide (Kelly and others 1997). The epoxide can be detoxified by conjugation with glutathione, catalyzed by the enzyme glutathione-*S*- transferase (Massey, Smith, Tam 2000). If not repaired, however, the epoxide can bind to DNA, preferentially at the N7 position of guanine residues. In turn these DNA adducts, if not repaired, may lead to mutations or cancer.

Several enzymes can bioactivate AFB_1 to the epoxide. For example, mouse liver catalase incubated *in vitro* with calf thymus DNA and [^{3}H]AFB_1 resulted in conversion of [^{3}H]AFB_1 to a DNA-binding form and formation of DNA adducts (Guindon and others 2008). In humans, cytochrome P450 enzymes (CYPs) of the 3A subfamily may be responsible for bioactivation of this mycotoxin (Kelly and others 1997). Many microsomal CYPs have been detected in human lung, including CYP 3A4 and 3A5. Most of these enzymes are expressed in the lung at levels much lower than in liver, but several, including 3A5 are preferentially expressed in the lung (Ding and Kaminsky 2003.). However, other enzymes such as prostaglandin H-synthase (PHS) and lipoxygenases (LOXs) also can activate AFB_1 to the epoxide (Donnelly and Donnelly 1996). PHS and LOXs are found at high levels in human lung microsomes and cytosol, respectively, and may play a larger role in human bioactivation of AFB_1 than P450.

Sterigmatocystin is synthesized by several fungal species from norsolorinic acid and is a close biological precursor of aflatoxin (Brown, Adams, Keller 1996). Like aflatoxin, sterigmatocystin may also form DNA adducts (McConnell and Garner 1994). As noted above, sterigmatocystin is one of several mycotoxins produced by the common indoor mold, *A. versicolor* (Gravesen 2000), (Hamasaki and others 1967). When incubated with calf thymus DNA, phenobarbital-induced rat liver microsomes and an NADPH-generating system, sterigmatocystin formed the electrophilic exo-ST-1,2-oxide (Essigmann and others 1979). After metabolic activation to the epoxide, sterigmatocystin, like AFB_1, targeted the N^7 atom of guanine and formed adducts with this residue. Activated sterigmatocystin has been shown to be a frame-shift mutagen in a bacterial assay (Ames and others 1973). These studies point to the potential for this compound to be carcinogenic in humans.

Fungal extracts also may interfere with movement of cilia in the lungs. Sterigmatocystin has been shown to inhibit the movement of cilia in an *in vitro* chick trachea model (Jesenska and Bernat 1994). Extracts from cultured *A. versicolor*, which are likely to include other secondary metabolites in addition to sterigmatocystin, have shown similar effects (Piecková and Kunová 2002). If cilia movement is diminished, clearance of toxic particles from the lungs may be slowed or even prevented, enhancing the potential for toxic effects from exposure.

Mycotoxins may also be detoxified by enzymes in the body. When sterigmatocystin was incubated with recombinant P450 1A1, three metabolites were detected: monohydroxy-sterigmatocystin, dihydroxy-sterigmatocystin, and one

glutathione adduct, after formation of a transient epoxide (Slominski and others 2005). Incubation with P450 1A2 produced monohydroxy-sterigmatocystin and the glutathione adduct, and incubation with P450 3A4 produced just the glutathione adduct. Incubation of sterigmatocystin with porcine tracheal epithelial cells (PTEC) produced a glucuronide conjugate. If the cells were first incubated with ß-naphthoflavone, two other products were detected: a sulfo conjugate and another glucuronide conjugate. These conjugates make toxins easier to excrete from the body, and may help to mitigate potential adverse effects.

Finally, ergosterol, a primary metabolite present in all fungal cell membranes, also can be metabolized by P450 enzymes and one of the metabolites has antiproliferative properties (Slominski and others 2005). Incubation of ergosterol with P450 side-chain-cleavage enzymes (P45011a1) yielded dihydroxyergosterol as the major product, and hydroxyergosterol as a minor product. The dihydroxy-product (17α,24-dihydroxyergosterol) also appeared when ergosterol was incubated with adrenal mitochondria. Interestingly, this product inhibited DNA synthesis in human epidermal HaCaT keratinocytes, reducing proliferation of these skin cells.

Overall, metabolism of ergosterol and detoxification of mycotoxins may mitigate some of the adverse effects of mycotoxin exposure. However, many adverse effects also have been documented in these *in vitro* studies. How the protective effects balance out against the adverse effects can only be determined by research in more sophisticated *in vivo* systems.

1.5.3 Animal studies

Studies conducted *in vivo* indicate that ingestion of many mycotoxins is hazardous. For example, dairy cattle exposed to sterigmatocystin in their feed (7.75 μg / g of feed) experienced bloody diarrhea, loss of milk production and, in some cases, death (Vesonder and Horn 1985). Less well documented are effects of exposure through inhalation, but inflammation has been reported in several studies. Instillation of *Stachybotrys* spores in rats resulted in pulmonary inflammation, but instillation of spores washed with methanol, which reduced the mycotoxin concentration, did not (Rao, Brain, Burge 2000). Mice instilled once intratracheally with *A. versicolor* spores isolated from a building with water damage experienced acute inflammation (Jussila and others 2002). Increased levels of the proinflammatory cytokines, tumor necrosis factor alpha and interleukin-6, were dose- and time-dependent, but cytotoxicity was apparent only at the highest dose.

Inhalation of purified mycotoxins also increases markers of inflammation. Mice were exposed intratracheally to several different mycotoxins at doses comparable to human exposure levels (Miller and others 2010). Several mycotoxins produced on wet building materials were tested, including sterigmatocystin. Four hours after exposure to a single dose (4×10^{-5} mole / kg lung wt) of mycotoxin, macrophage inflammatory protein-2 and tumor necrosis factor-alpha were detected in respiratory bronchiolar epithelia, alveolar macrophages, and alveolar type II cells. Mucus in bronchiolar spaces, along with alveolar macrophages with red-stained cytoplasm was observed in lungs from treated animals but not controls. In addition, array analysis indicated that 82 of 83

inflammation-associated genes were significantly altered. At 12 hours, 75 of the 83 genes were significantly altered. Such inflammation can contribute to non-allergenic respiratory health effects.

Exposure to mycotoxins can be carcinogenic and toxic. Tumor formation rates increased in female A/J mice exposed intraperitoneally to a single dose of AFB_1 (Guindon and others 2008). Approximately seven months after dosing, treated mice exhibited increased numbers and larger size of lung tumors, 90% of which were adenomas, compared to controls. Rats exposed to sterigmatocystin daily for a year either through ingestion or by gavage developed hepatocellular carcinomas (Purchase and Van Der Watt, J. J. 1970). There were also fibrotic changes in the noncancerous portions of the livers of sterigmatocystin-treated rats, but not in control animals.

1.5.4 Human studies

Occupants of water-damaged buildings often report eye, nose and throat irritation (Andersson and others 1997), known colloquially as 'sick building syndrome'. In 2004, the Institute of Medicine of the National Academies issued a report titled "Damp Indoor Spaces and Health." Although the authors did not find sufficient evidence to claim a causal relationship, they noted "sufficient evidence of an association between exposure to damp indoor environments and some respiratory health outcomes: upper respiratory tract (nasal and throat) symptoms, cough, wheeze, and asthma symptoms in sensitized asthmatic persons" (Institute of Medicine of the National Academies, Committee on Damp Indoor Spaces and Health, Board on Health Promotion and Disease Prevention

2004). Lack of reliable fungal exposure data is part of the reason the authors can only report an association and not a causal relationship.

In humans, exposure to fungal particulates may exacerbate asthma or bronchitis, cause allergic reactions in sensitive individuals (Anderson, Lewis, Smith 1994) or lead to chronic sinusitis (Landers 2003). Although it is not clear what exactly is causing such symptoms, researchers have investigated the role of a number of fungal components, including β-1,3-glucans, extracellular polysaccharides and mycotoxins. As components of the fungal cell wall that are relatively resistant to degradation in the environment, β-1,3-glucans have been measured as a marker of indoor fungal load. They can also produce nonspecific inflammatory reactions (Douwes and others 1996)and, therefore, have been suggested as a potential cause of sick-building complaints.

Inflammation resulting from exposure to fungal metabolites may be caused by mycotoxins. A variety of fungi have been found in nasal secretions of both healthy individuals and those with sinusitis (Ponikau and others 1999). However, eosinophils, a type of white blood cell involved in inflammatory response, were elevated in the nasal mucous of the sinusitis sufferers, but nearly absent in controls. On recognizing fungal material, eosinophils respond by degranulating and releasing inflammatory mediators (Inoue and others 2005), which can cause collateral damage (sinusitis) in surrounding tissue. In fact, indicators of inflammation, such as elevated cytokines and humoral immune responses, have been detected in response to noninvasive and nonpathogenic fungi (Shin and others 2004).

Besides inflammation, workers exposed to high levels of agricultural dusts sometimes experience flu-like symptoms including fever, chills, body aches, headaches, breathing difficulties and/or nausea. The dusts may originate from handling a variety of moldy organic material such as grain, wood chips, hay or garbage (Seifert and others 2003). The condition, named Organic Dust Toxic Syndrome (ODTS), is distinguished from hypersensitivity pneumonitis in that its cause is non-immunologic and may be related to fungal spores or mycotoxins.

Researchers in Sweden investigated potential causes of wood-trimmers' disease (Land and others 1987). This disease, like ODTS, is characterized by fever, shivering, cough and shortness of breath. Symptoms typically occur 4-8 hours after exposure and usually disappear after 24 hours. As *Aspergillus fumigatus* is commonly detected in the air at Swedish sawmills, eight different strains of *A. fumigatus* were cultured from wood dried in kilns from five different sawmills. The cultures were extracted for toxins and rats were exposed to these toxins through intragastric administration of the extracts in peanut oil. Extracts from four of the eight strains produced mild tremors, and one produced very strong tremors that occurred spontaneously. The researchers noted such toxins could contribute to the cause of the wood-trimmers' symptoms. However, because this study did not include any exposure monitoring, it is difficult to conclude any more than a potential association between the presence of *A. fumigatus* and wood-trimmers' disease.

Because mycotoxins such as aflatoxin and sterigmatocystin are carcinogenic, there is a concern that inhalation exposure could lead to cancer. After an excess of new

cases of liver cancer had been noted among animal-feed plant workers in Denmark, researchers investigated inhalation exposures to AFB_1 at the plant by measuring AFB_1-protein adducts in blood (Autrup, Schmidt, Autrup 1993). These adducts are cleared more slowly than DNA adducts, which may be repaired in less than 24 hours. Since there is no known repair pathways for blood protein adducts, they represent cumulative exposure over the half-life of the blood proteins. Of nine workers sampled, serum albumin adducts were nondetectable in seven of them after at least two weeks of vacation. After returning to work, six of the nine workers had detectable adducts after four weeks, ranging from 44 to 100 pg AFB_1/ mg albumin. Again however, this study did not have a strong exposure assessment component. Exposure was evaluated by noting hours worked in a given location at the plant.

1.6 Proposed research

As shown in this review of the literature, occupants of moldy buildings often report adverse health effects, but conclusive research supporting a causal relationship is lacking. Research has demonstrated that fungal particles carry irritants, allergens and toxins, that these particles can be released to the indoor environment, and that a large proportion of these particles are sub-micron fragments. These particles and associated compounds have biological effects in cell culture and animals, and also have the potential to deposit in the alveoli of the lungs, especially in children. Evidence of adverse health effects in humans, however, is less convincing, due at least in part to a lack of reliable fungal exposure data.

What is not known is how time and growth substrate affect toxin production and distribution within mold structures. To investigate these effects, we have chosen to work with *A. versicolor,* a toxin-producing mold commonly found in indoor environments with water damage (Nielsen 2003). *A. versicolor* is a useful model for these experiments because it produces toxins including sterigmatocystin, which can be purchased in pure form to create a standard curve. In this research, sterigmatocystin is a marker for the various potentially toxic secondary metabolites produced by this organism. Experiments will enable us to test our hypothesis that time and growth substrate affect the amount and distribution of mycotoxins in particles released from colonies of *A. versicolor*. The results and conclusions from these experiments will help researchers design future studies to more accurately characterize the toxic potential of fungal fragments.

Chapter 2 – Toxicity of *Aspergillus versicolor* extracts

2.1 Introduction

As noted in the previous chapter, exposure to mycotoxins associated with small fungal fragments may cause adverse health effects. Understanding how time and substrate affect the distribution of toxins and the release of particles from the mold can help us better estimate potential risks of exposure to these toxin-carrying particles. The objectives of this set of experiments were to verify that our strain of *A. versicolor* produced sterigmatocystin, to test whether the toxin was intracellular or extracellular, to determine the effects of time and substrate on production of that mycotoxin, and to assess whether extracts of this mold were toxic in an *in vitro* system.

Although older references indicated that secondary metabolites from *A. versicolor* were primarily intracellular (Filtenborg, Frisvad, Svendsen 1983), more recent studies on the ciliostatic activity of fungal extracts indicated *A. versicolor* metabolites could be extracellular (Piecková and Kunová 2002). This determination is important, because if *A. versicolor* secreted secondary metabolites into the substrate, the particles released from that colony could contain less toxin than if the secondary metabolites were all held intracellularly. Because toxin-rich particles could pose greater health risks to exposed individuals, an accurate risk assessment depends on a reliable characterization of the distribution of mycotoxins produced by the mold.

2.2 Materials and methods

2.2.1 Starting inoculum

A wild strain of *A. versicolor* was isolated from a building with water damage and stored in sterile buffered distilled water at 4^{o}C. When cultured on several different types of agar, there was abundant growth on Malt Extract Agar (MEA) and Czapek Yeast Agar (CYA). These substrates were selected for further investigation with this organism.

A sterile 150 X 15 mm polystyrene Petri dish (BD Biosciences, San Jose, CA) containing Malt Extract Agar (MEA) (Remel Inc., Lenexa KS) was inoculated inside a biosafety cabinet (NuAire Class II Type A) with the *A. versicolor* conidia. A sterile swab was inserted into the conidia suspension and then spread over the MEA-containing culture plate, moving the swab up and down, then left to right to ensure confluent growth. The plate was then placed in a plastic bag sealed with a twist tie and incubated in the dark at 25 ^{o}C for 2 weeks.

2.2.2 Inoculating filters

After the plate achieved confluent growth at approximately 2 weeks, the plate was flooded with approximately 10 mL of sterile buffered distilled water (SBDW) containing 0.05 % Tween 80 (Sigma, St. Louis MO) to separate the conidia. Two sterile cotton swabs were then rubbed lightly up and down, then left to right over the entire colony surface to dislodge the conidia. The suspension was then removed with a sterile 5 mL pipette and placed in a sterile plastic 50 mL screw capped tube.

After vortexing for 15 seconds, half of the fungal suspension was vacuum filtered through a sterile funnel assembly equipped with a 1-micron pore-size Binder-Free Glass Fiber Filter (Pall Life Sciences Type A/B, 47 mm Extra Thick). The filtrate was then discarded and the inoculated glass fiber filter was removed from the funnel assembly with flame-sterilized tweezers. The filter was placed, inoculated side up, on a sterile 100 X 15 mm polystyrene petri dish (BD Biosciences, San Jose, CA) containing Malt Extract Agar (MEA). The remaining fungal suspension was then vacuum filtered through a second Type A/B Glass Fiber Filter in a new sterile funnel, and the filter was then removed and placed on a sterile 100 X 15 mm culture plate containing Czapek Yeast-extract Agar (CYA). The plates were then placed in plastic bags, sealed with twist ties and incubated in the dark at 25 ^{o}C for 19 days.

Extracellular metabolites would be expected to accumulate in the agar. Therefore, the glass-fiber filters inoculated with *A. versicolor* were incubated on the agars and later removed for analysis. As removal of the filter cleanly separated visible fungal material from the agar, intracellular metabolites detected on the filters could be tested and analyzed separately from any extracellular metabolites secreted into the agar below.

2.2.3 Sample processing

After three days, the filters had heavy fungal growth. At five days, the MEA and CYA plates were moved temporarily into a biosafety cabinet for processing. Each filter was cut in half with a sterile scalpel. Using flame-sterilized tweezers, one half of each filter was placed into a glass screw-capped tube with 5 mL of high-performance liquid chromatography (HPLC)-grade methanol. An uninoculated filter half was placed in a

third tube with methanol as a control. Two cores from the agar directly below the removed half-filters were collected from each agar plate using a flame-sterilized 9-mm diameter metal cork borer and consolidated in a glass screw-capped tube with 5 mL of HPLC-grade methanol. Control samples of uninoculated MEA and CYA were also collected for a total of seven samples. Extraction of the fungal material with a solvent such as methanol releases intracellular toxins by denaturing the proteins in membranes and breaking the protein-lipid bonds (Filtenborg, Frisvad, Svendsen 1983). The remaining halves of the inoculated filters were left on the agar plates and incubated further in the dark at 25^{o}C.

The five-day old samples and controls were sonicated in a Transsonic TS540 sonicator (Elma, Singen, Germany) for 20 minutes and then vortexed for 15 seconds. Each 5 mL extract was then filtered through grade 515 18.5 cm diameter fluted filter paper (Eaton-Dikeman Co., Mount Holly Springs, PA) and the filtrate collected in two 4 mL amber glass vials. The amber glass vials were placed on a speed vac and taken half way to dryness. At that point, remaining solvent was pulled up in a 1 mL syringe (BD tuberculin slip tip), and filtered through a 13 mm diameter, 0.45 micron syringe filter (Millex-HV, Billerica, MA) into a single new clean 4 mL amber glass vial. These final vials were taken to dryness on the speed vac, and the residue brought up in 100 µL of tissue grade dimethyl sulfoxide (DMSO) so that the samples could be used for HPLC analysis and cell toxicity tests. After a total of 19 days incubation, samples and controls from the remaining halves of the filters on agars were collected and processed as described above.

2.2.4 HPLC toxin analysis

As sterigmatocystin is toxic and carcinogenic (Purchase and Van Der Watt, J. J. 1970), a labcoat, eye protection, and nitrile gloves were worn whenever handling this material in solid form or in solution. Serial dilutions of a 10 mM stock solution of sterigmatocystin (Aldrich Chemical Co, Milwaukee WI) were prepared for a standard curve and for spiking duplicate samples. Amber glass 4 mL vials with 300 µL glass inserts were prepared for loading into the HPLC's autosampler. Five µL aliquots from the samples described in Section 2.2.3, dissolved in 100 µL of tissue grade DMSO, were diluted in 95 µL of HPLC-grade methanol (Fisher Scientific, Pittsburg PA). This 100 µL mixture was loaded into the inserts for HPLC analysis. Duplicates of the 19-day samples were each spiked with sterigmatocystin to confirm peak identity at a concentration expected to yield 1 nanomole (nmol) in the analyzed sample.

A Waters high-performance liquid-chromatograph (Mildford, MA) with a diode array detector was used to analyze fungal extracts. The HPLC system was equipped with a Waters 600E pump, Waters 996 PDA detector, and a Waters 717 autosampler. Ten µL of each sample and control were separated over a reverse-phase Luna C18 (2) column (250 x 4 mm, 5 µm particle size, 100A pore size) (Phenomenex, Torrance, CA) at a flow rate of 0.4 mL/min. With Buffer A as 10 mM aqueous ammonium acetate and Buffer B as 95% methanol with 5% millipure water, the concentration of Buffer B was initially 20% held for 4 min. It was raised linearly to 70% in 4 min, held at 70% for 18 min, raised to 90% in 1 min, held at 90% for 38 min, and finally decreased to 20% in 5

minutes. The column was conditioned with the final solvent composition for 10 minutes before the next sample was run with the autosampler.

2.2.5 Cytotoxicity assay optimization and analysis

In order to better determine the toxic potential of fungal fragments, extracts of *Aspergillus versicolor* colonies were tested for toxicity in MP48s, an immortalized pre-type II alveolar epithelial cell line in which cell proliferation is contact inhibited (Mallampalli, Floerchinger, Hunninghake 1992). These cells, which are derived from fetal rat lung, exhibit a normal karyotype and were chosen because lung cells are an appropriate model to use to measure the effects of exposure to mycotoxins through inhalation. The cells were the gift of Dr. Betsy Wattenberg (Division of Environmental Health Sciences, University of Minnesota – School of Public Health) via Dr. David Ingbar (Department of Medicine, University of Minnesota).

Cytotoxicity was determined using the Cell Titer 96 Non-Radioactive Cell Proliferation Assay according to the protocol recommended by Promega (Madison, WI). In this assay, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) is reduced to insoluble, purple formazan by enzymes in living cells. A 'stop/solubilization solution' added after two hours dissolves the formazan, producing a colored solution. The amount of formazan produced (corresponding to the viability of the cells) can be determined by measuring the absorbance of the solution at a certain wavelength with a spectrophotometer.

However, the cytoxicity assay underwent a series of optimizations, due to problems that occurred with the test. First, sterigmatocystin in DMSO formed a precipitate when added to the cell culture media. The toxin had to be kept in solution in order to expose the cells to a known concentration. Initially, glycerol was used to keep sterigmatocystin in solution when treating the cells, but was found to be somewhat toxic to the MP48 cells. By switching to serum-free media, sterigmatocystin stayed in solution. Fortunately, the two-hour treatment in serum-free media had only a slight effect on cell viability.

Next, it was determined that sterigmatocystin was not toxic to MP48 cells unless supplemented with an activation system. Sterigmatocystin exhibited measurable toxicity when incubated with S9, the supernatant fraction obtained after spinning rat liver homogenate for 20 minutes at 9000g, supplemented with NADPH-generating cofactors (10 mM $NADP^+$, 50 mM glucose-6-phosphate, and 50 mM magnesium chloride). The S9 fraction is often used as an activation system as it contains soluble enzymes from the cytosol and membrane-bound P450 enzymes. The system was tested with varying concentrations (mg protein / mL of solution) of S9. The optimal amount was determined to be 1 mg/mL.

Finally, it was determined that the cofactors in the activation system were reacting with the phenol red in the Waymouth's media, and turning the system acidic. Phosphate buffer neutralized the mixture, but killed the cells. Since Waymouth's was not available without phenol red, the system was tested using Minimum Essential Media (MEM)

without phenol red. As this change resolved the problem, MEM was used for treatments involving the cofactor-containing activation system.

Toxicity was also tested using microsomes with $NADP^+$/glucose-6-phosphate/$MgCl_2$ both with and without glucose-6-phosphate dehydrogenase. As a subfraction of S9, microsomes contain membrane-bound P450 enzymes without the soluble enzymes from the cytosol. However, the microsome-activating systems were no more effective than the S9 system tested previously at activating sterigmatocystin.

Ultimately, the supplemented S9 was chosen for the rest of the tests and the analysis was completed as described below. Both fungal extracts and serial dilutions of sterigmatocystin were tested for toxicity in MP48 cells. The fungal extracts were those prepared for HPLC (Section 2.2.3), dissolved in 100 µL of tissue grade DMSO, but further diluted 1/100 in MEM before treating the cells. The sterigmatocystin dilutions were also prepared in DMSO and used to create a standard curve.

The MP48 cells were plated at a density of 2×10^4 cells/well in 96-well plates one day prior to each experiment, and grown overnight in a gassed (5% CO_2), humidified incubator at 37 °C in Waymouth's medium supplemented with 10% fetal bovine serum (FBS). After 24 hours, confluent cells were then incubated for two hours in MEM with S9 and cofactors (10 mM $NADP^+$, 50 mM glucose-6-phosphate, and 50 mM magnesium chloride) in the presence or absence of mold extracts or serial dilutions of sterigmatocystin standard. Each sample extract or serial dilution of sterigmatocystin was tested in triplicate.

After the two-hour treatment, the cells were washed twice with phosphate-buffered saline (PBS) and then incubated in fresh Waymouth's media with FBS. After another 24 hours, 15 µL of room temperature MTT dye was added to each well. After incubating at 37°C for two hours, the assay was stopped by adding 100 µL of the Stop/Solubilization solution to each well. In order to allow for solubilization, the plate was incubated overnight at room temperature in a sealed Ziploc bag with a damp paper towel to prevent evaporation of the incubation fluid. If overnight incubation was not feasible, the plate was incubated for one hour at 37°C before absorbencies were measured.

Treatment effects were assessed by measuring absorbencies of all wells at both 570 and 650 nm. Absorbencies were corrected by subtracting the difference in the absorbance of the blanks (media only – no cells) from the difference in the absorbance of the samples at the two wavelengths, 570 and 650 nm. Cell viabilities (%V) were calculated with the following equation:

$$\%V = 100 \times ([E_{A570} - E_{A650}] - [B_{A570} - B_{A650}]) / ([M_{A570} - M_{A650}] - [B_{A570} - B_{A650}])$$

where A570 and A650 are the absorbencies at 570 and 650 nm; E is the toxin- or extract-exposed cell; B is the blank; and M is the media control (serum-free media with no S9 fraction and no cofactors).

2.2.6 Data analysis

Only a single injection of each sample was analyzed by HPLC, and therefore averages and standard deviations were not calculated for these numbers. In the

cytotoxicity assay, each sample extract or serial dilution of sterigmatocystin was tested in triplicate. The means and standard deviations were calculated and presented in the figures in Section 2.3.

2.3 Results

The sections below detail results from the HPLC analysis for sterigmatocystin in the agar and filter samples, and the cytotoxicity results for these same samples.

2.3.1 HPLC results for sterigmatocystin

Sterigmatocystin levels in 5-day, 19-day and 19-day spiked samples are presented in Table 2-1. The results show the nanomoles of sterigmatocystin detected in the 10 µL sample injected onto the HPLC column. No sterigmatocystin was detected in the agar or filter blanks, or the agar underneath the inoculated filter. After five days, 0.11 nmol and 0.085 nmol of sterigmatocystin were detected in the fungal material from the inoculated filters on both MEA and CYA, respectively. At 19 days of growth, sterigmatocystin on the MEA filter increased by a factor of four to 0.46 nmol, but toxin from the filter on CYA increased by a factor of 10 to 0.89 nmol, almost double that detected on the MEA filter. Duplicates of the 19 day samples were spiked with sterigmatocystin, expecting to yield 1 nmol in addition to any toxin detected in the samples. Spike recoveries were 88% or better of the expected amount.

Table 2-1. **Sterigmatocystin measured in 10 µL of a 1/200-dilution of extracted agar and filter samples**. Sample number, n = 1; ND – Not detected (<0.01 nmol); 19 day samples spiked with 1 nmol sterigmatocystin

Sample Type	5 day (nmol)	19 day (nmol)	19 day spiked (nmol)
MEA agar blank	ND	--	1.09
MEA agar under filter	ND	ND	0.99
inoculated filter on MEA	0.11	0.46	1.47
CYA agar blank	ND	ND	0.94
CYA agar under filter	ND	ND	1.00
inoculated filter on CYA	0.085	0.89	1.65

As discussed in the introduction, *A. versicolor* can produce several secondary metabolites. In this analysis, sterigmatocystin is a marker for those other compounds. The HPLC chromatograms of the extracts of the inoculated filters in Figure 2-1 show several peaks in addition to the sterigmatocystin peak, both after 5-days of growth, and after 19 days of growth. These peaks may indicate the presence of other secondary metabolites. On both CYA and MEA, these peaks increase with time. The traces support the data presented in Table 2-1, showing that although sterigmatocystin peaks are roughly comparable at day 5, the sterigmatocystin peak at day 19 on MEA is approximately half the size of the sterigmatocystin peak on CYA at day 19. It is also clear that there are no peaks in the HPLC chromatograms of the agar beneath the inoculated filters after 19-days. No sterigmatocystin peaks were detected in agar under filters at 5-days of growth either (data not shown).

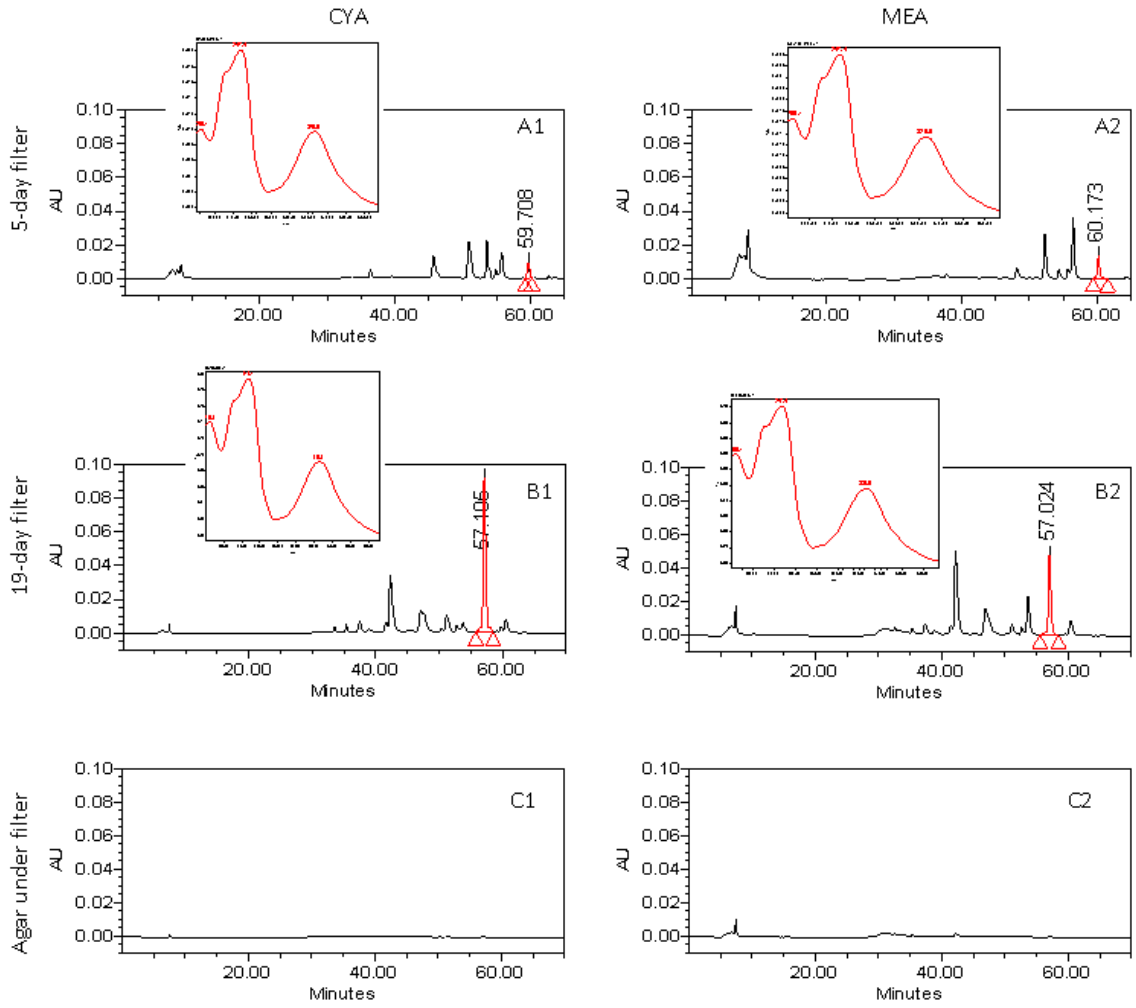


Figure 2-1. HPLC chromatograms and UV traces of samples on CYA (A1, B1, C1) and MEA (A2, B2, C2). Chromatograms were extracted at 325 nm. UV traces of sterigmatocystin peak (λ max at 247 nm and 325 nm). A1-A2: 5-day inoculated filters; B1-B2: 19-day inoculated filters; C1-C2: agar under 19-day inoculated filters. Sterigmatocystin peak is time-marked.

2.3.2 Cytotoxicity Results

In order to develop a standard dose-response curve, MP48 cells were incubated with various concentrations of ST with or without S9 and cofactors (Figure 2-2).

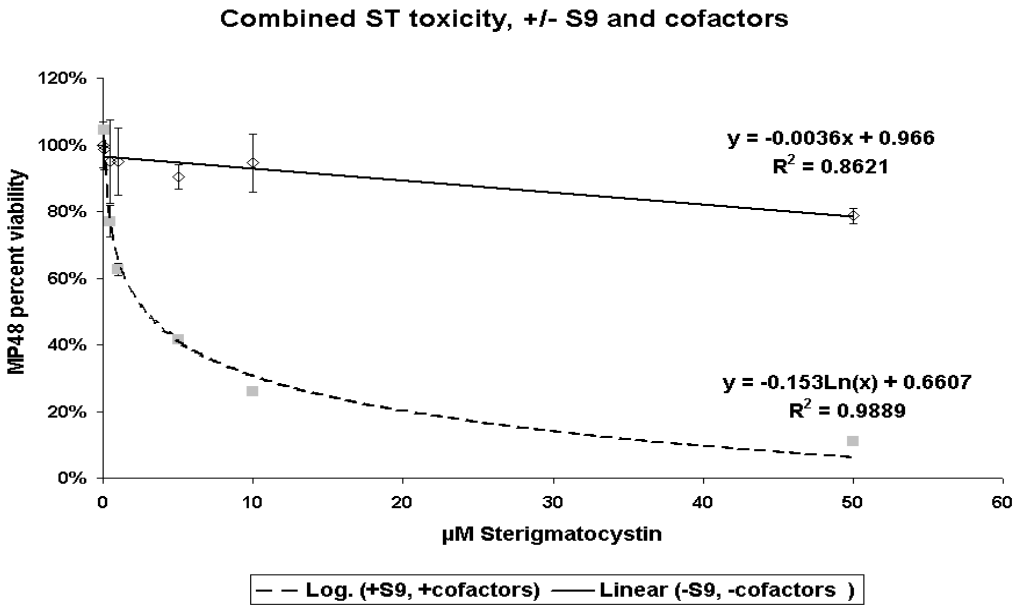


Figure 2-2. **Concentration-response curves for sterigmatocystin-induced cytotoxicity**. MP48 cells were incubated for 2 hr with various concentrations of sterigmatocystin, with (squares) and without (diamonds) S9 and cofactors. Cell viability was determined in triplicate for each data point. Error bars represent the standard deviation. Trendlines fit to the data with linear (-S9,-co-factors) and logarithmic (+S9, +co-factors) curves.

Without the S9, the percent viability never dropped below 79%, even at the highest dose of 50 µM sterigmatocystin. With the activating system, sterigmatocystin decreased viability in a logarithmic fashion to approximately 50% at 1.5 µM, and as low as 10.8% at 50 µM. The trendlines were fitted to the data using Excel software, and chosen based on a visual observation of best fit as well as by which curve gave the highest R^2 value.

Fungal extracts were tested on the MP48 cells in the same manner. Table 2-2 compares the sterigmatocystin levels detected by HPLC (1/200 dilutions of original

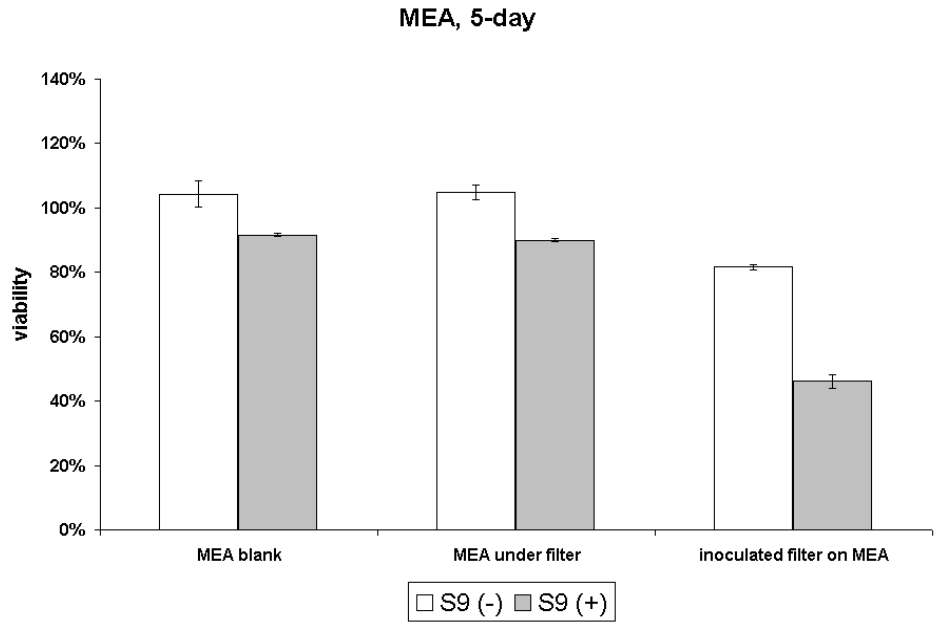
Table 2-2. Comparison of sterigmatocystin levels analyzed by HPLC (nmol), and concentrations used to treat MP48 cells (µM)

Sample Type	**5 day**	**19 day**
nmol in 10 µL MEA sample for HPLC analysis	0.11 nmol	0.46 nmol
µM in MEA sample for cytotoxicity assay	2.2 µM	9.1 µM
nmol in 10 µL CYA sample for HPLC analysis	0.085 nmol	0.89 nmol
µM in CYA sample for cytotoxicity assay	1.7 µM	17.8 µM

DMSO extracts), with concentrations in the 1/100 dilutions of those same extracts used to treat the MP48 cells at both 5- and 19-days of incubation.

Figure 2-3 shows cytotoxicity results for cells treated with the 5-day old mold extracts detailed in Table 2-2. The extracts contained fungal metabolites including sterigmatocystin at concentrations of 2.2 µM and 1.7 µM for MEA and CYA, respectively. The percent survival was calculated using results from 'cells in serum-free media + DMSO' without S9 and cofactors as the media control. As noted with pure sterigmatocystin, extracts of the mold cultures were slightly toxic without the S9 and cofactors. Without the activating system, cells exposed to the MEA filter extracts survival dropped 22% from 104% (MEA blank) to 82% (inoculated filter on MEA). With the activating system cell viability dropped 50%, from 92% to 46%. Toxicity was more than twice as great as without the activating system. On MEA, there was no significant difference in toxicity between the agar blank and the agar under the filter.

A



B

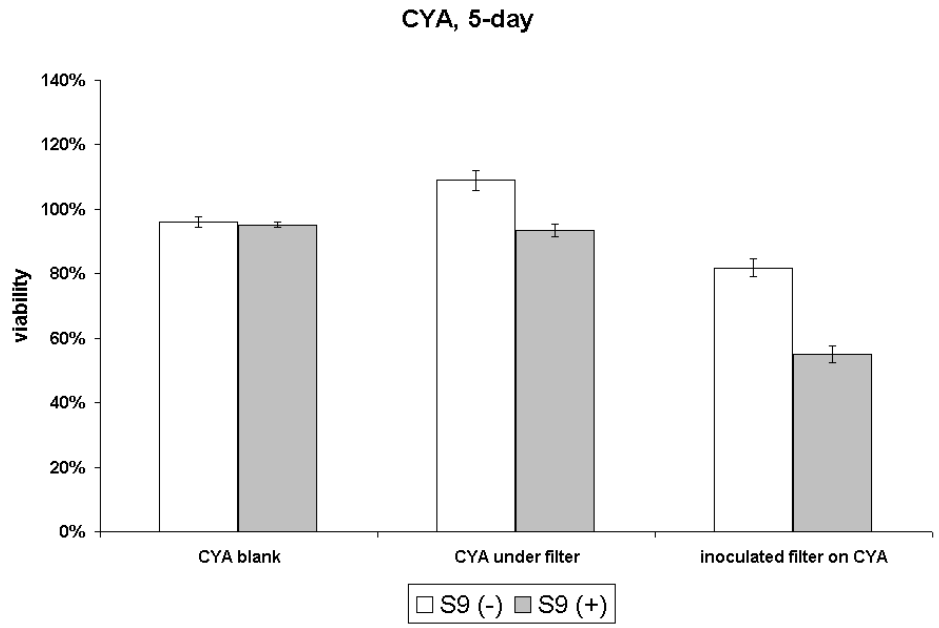


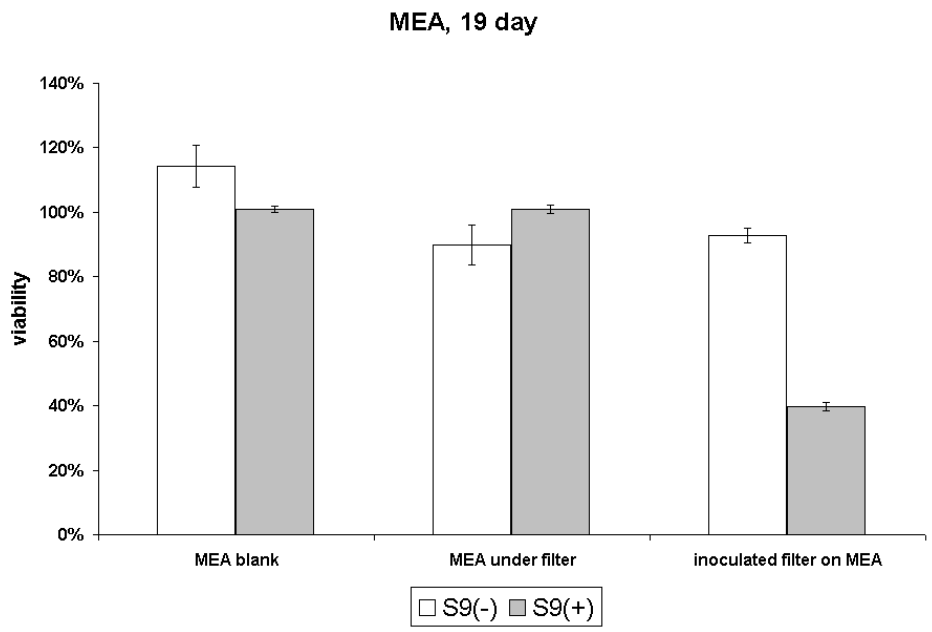
Figure 2-3. Cytotoxicity of extracts of 5-day old cultures of *A. versicolor* to MP48 fetal rat lung cells. A – MEA extracts tested with and without S9 and cofactors. B – CYA extracts tested with and without S9 and cofactors. Columns represent arithmetic means with associated standard deviations of n=3 replicates of a single sample. Results from 'cells in serum-free media + DMSO' were used as the media control for viability calculations.

Figure 2-3 also shows the viability of the MP48 cells exposed to 5-day old CYA filter extracts (Figure 2-3 B). Without the activating system, the CYA extracts were only slightly toxic. Survival dropped 15% from 96% (CYA blank) to 82% (inoculated filter on CYA). With the activating system (B), the extracts were much more toxic. Survival dropped 42% from 95% to 55%, for an increase in toxicity almost three times greater than without the activating system. On CYA, there was also no significant difference in toxicity between the agar blank and the agar under the filter (95% and 93%, respectively) when the activating system was used.

Figure 2-4 shows viability results for 19-day old mold extracts. MEA extracts without the activating system were only slightly toxic. The survival of the MP48 cells dropped from 18% from 114% (MEA blank) to 93% (inoculated filter on MEA). With the activating system, survival dropped 60% from 101% to 40%, more than three times as toxic as without the activating system. On MEA at 19 days with the activating system, there was still no significant difference in toxicity between the agar blank, and the agar under the filter.

Figure 2-4 also shows the survival of the MP48 cells exposed to 19-day old CYA filter extracts. Without the activating system, viability dropped 20% from 122% (CYA blank) to 97% (inoculated filter on CYA). With the activating system the viability of the MP48 cells dropped 39% from 102% to 62%, an increase in toxicity almost twice as great as without the activating system.

A



B

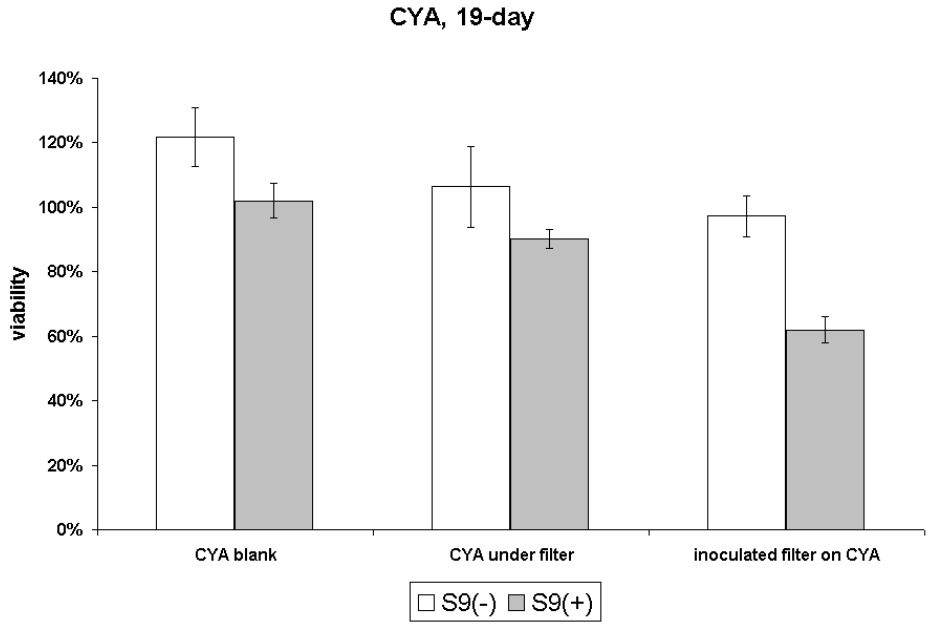


Figure 2-4. Toxicity of extracts of 19-day old cultures of *A. versicolor* to MP48 fetal rat lung cells. A – MEA extracts tested with and without S9 and cofactors. B – CYA extracts tested with and without S9 and cofactors. Columns represent arithmetic means and standard deviations of n=3 replicates of a single sample. Results from 'cells in serum-free media + DMSO' were used as the media control for viability calculations.

2.4 Discussion

Although the cytotoxicity analyses were conducted in triplicate, these results were three analyses of a single sample. HPLC analyses for sterigmatocystin were run on the same sample, but only a single analysis was completed without any repeats. This limitation reduces the certainty of conclusions from this data. However, there are several interesting observations that can be made.

First, the sterigmatocystin analyses, HPLC traces and toxicity data all indicate that sterigmatocystin and other secondary metabolites are not secreted into the substrate at detectable levels and are likely intracellular compounds, supporting reports by Filtenborg et. al. (Filtenborg, Frisvad, Svendsen 1983). Neither the age of the culture (5 vs. 19 days), nor the type of growth media (MEA vs. CYA), affects this result. Although studies on the ciliostatic activity of fungal extracts indicated *A. versicolor* metabolites could be extracellular (Piecková and Kunová 2002), the method used for separating intracellular and extracellular toxins may have affected these results. Fungal cultures in that study were grown in liquid media Erlenmyer flask and filtered through Whatman No.3 filter paper, which has a pore size of 6 µm. The filtered fungal mass was extracted for intracellular toxins, and the filtrate was extracted for extracellular toxins. It is possible that small fungal fragments passed through the filter paper. Therefore, what was extracted might not have been dissolved extracellular toxins, but intracellular metabolites associated with the fragments. In this study, subsequent tests were not designed to account for toxin that might be 'lost' to the substrate, as these results show that the toxin stays with the fungal material.

Another observation is that the concentration of sterigmatocystin increases with the age of the culture on both MEA and CYA. However, the concentration of sterigmatocystin in these samples did not directly correlate with the survival of the MP48 cells. Figure 2-5 compares the sterigmatocystin concentrations with cytoxicity results of the inoculated filters. The concentrations of sterigmatocystin in the fungal extracts used to treat the MP48 cells were calculated from the nmols of sterigmatocystin measured by HPLC (shown in Table 2-1). The diamond markers show the expected survival of cells exposed to the MEA and CYA filter extracts, which were derived from the standard curve presented in Figure 2-2. The measured viabilities of the exposed cells are represented by the triangle markers.

The measured survival for the 5-day old MEA and CYA filter extracts were very close to expected viabilites. Concentrations of sterigmatocystin increased with time on both MEA and CYA, but CYA extracts contained twice as much sterigmatocystin as the MEA extracts. Surprisingly, the 19 day extracts were less toxic than expected. Only 28% and 20% of the cells exposed to the MEA and CYA filter extracts, respectively, were expected to survive, based on the standard curve presented in Figure 2-2. Actual survival rates for the 19-day MEA- and CYA-exposed cells were measured at 40% and 62%, respectively. Interestingly, the CYA sample was less toxic than the MEA sample, even though it contained almost twice as much sterigmatocystin.

It is possible that the additional secondary metabolites produced on the substrates at 19 days, as shown in the C2 HPLC traces in Figure 2-1, may account in part for these unexpected results. The additional compounds may have interacted with toxin-activating

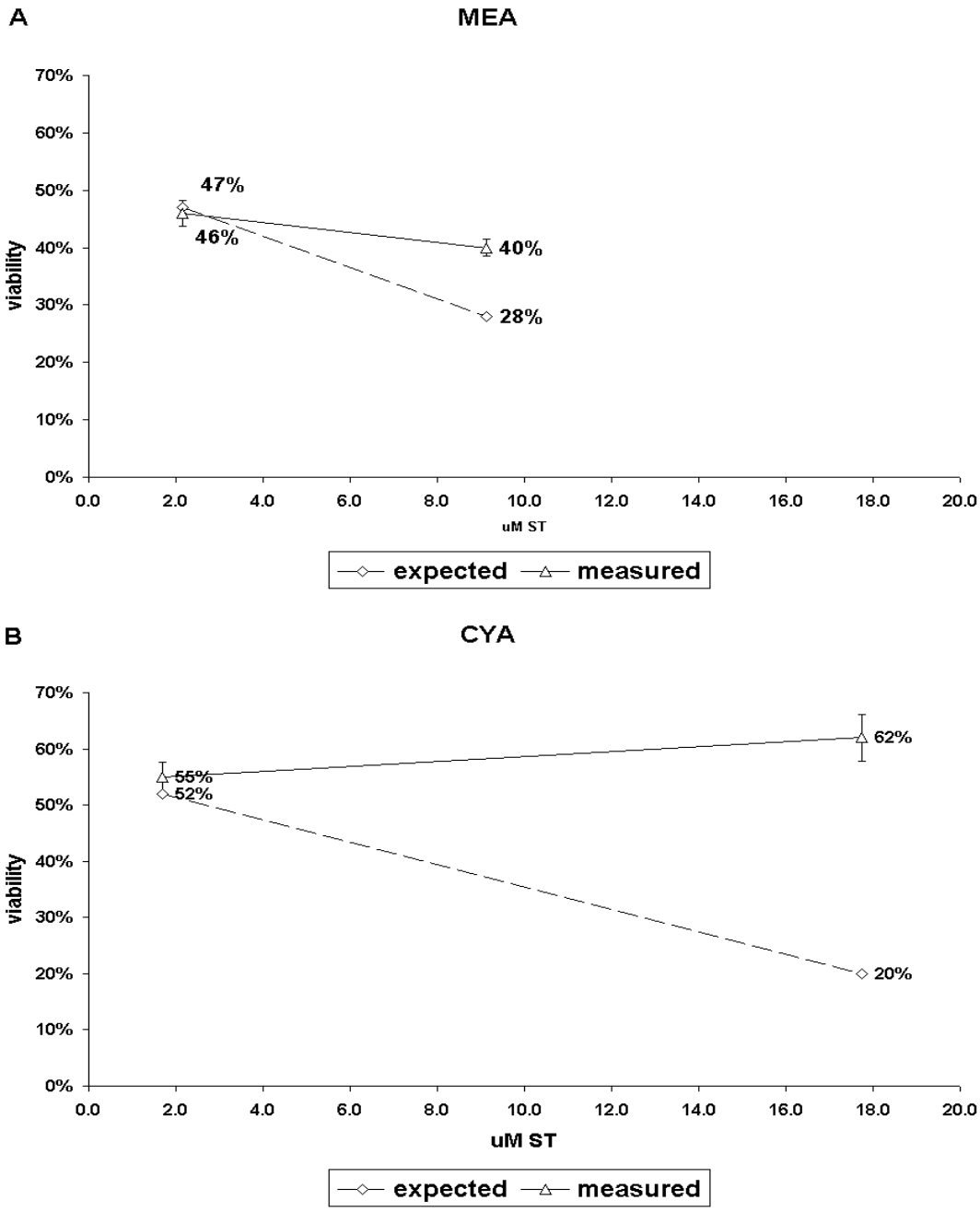


Figure 2-5. Comparison of ST treatment concentrations with expected and measured viabilities of cells exposed to 5- and 19-day extracts of *A. versicolor* on MEA and CYA. A – extracts of inoculated filters on MEA with S9 and cofactors. B – extracts of inoculated filters on CYA with S9 and cofactors. Expected viabilities derived from the standard dose-response curve in Figure 2-2.

enzymes, and reduced their ability to activate sterigmatocystin. Replicate samples would have helped to verify whether this observation was statistically significant.

Chapter 3 – Effects of time and substrate on mycotoxin distribution

3.1 Introduction

As noted in the Chapter 1, the hypothesis of this research is that time and growth substrate affect the amount and distribution of mycotoxins in the fungal particles released from fungal colonies. In an indoor environment, substrates could include materials such as ceiling tile, sheetrock, wallpaper or carpeting. If these materials became wet, they each would supply a different set of nutrients to an inoculating mold. Also, water damage in buildings sometimes occurs over an extended period before being discovered by building occupants. Both substrate differences and time could affect toxin distribution within the mold, and particles released from the colony. The objectives of this set of experiments were to measure the effects of time and growth substrate on the distribution of sterigmatocystin in fixed and removable fungal material produced by *A. versicolor*. Our hypothesis was that the toxin was not evenly distributed between fixed and removable fractions and that toxin distribution in those fractions would be affected by time and growth substrate.

3.2 Materials and Methods

3.2.1 Test Plan

To test this hypothesis, we decided to work with *A. versicolor* over six weeks on two agars, Malt Extract Agar (MEA) and Wallpaper Paste Agar (WPA). MEA is a nutrient-rich media and produces abundant growth. In contrast, WPA is primarily corn starch (Larsen and Frisvad 1994), a carbohydrate-rich but otherwise nutrient-poor media that mimics the paper-backed sheetrock that supports much of the mold growth found in indoor environments. It was chosen as a contrast to MEA, because it produced visibly less growth of *A. versicolor*.

Two inoculated plates (I) and a sterile control plate of each agar were prepared for weekly testing as shown in the set-up scheme outlined in Table 3-1. Each six-week set

Table 3-1 Set-up scheme for plates with MEA and WPA. I = inoculated plates, C = sterile control plates. Set 4 plates used for mass-balance study only.

Age (weeks)	Set 1			Set 2			Set 3			Set 4			
1	I1	I2	C1	I3	I4	C2	I5	I6	C3				
2	I1	I2	C1	I3	I4	C2	I5	I6	C3				
3	I1	I2	C1	I3	I4	C2	I5	I6	C3				
4	I1	I2	C1	I3	I4	C2	I5	I6	C3				
5	I1	I2	C1	I3	I4	C2	I5	I6	C3				
6	I1	I2	C1	I3	I4	C2	I5	I6	C3				
8										I7	I8	I9	C4

was repeated twice for a total of three 6-week sets (n = 6 inoculated plates and 3 controls). At week 0, a set of 12 MEA and 12 WPA plates were inoculated. Two inoculated plates and a sterile control were placed in a plastic bag, sealed with a twist tie

and incubated concurrently at 25°F for a total of six bags of MEA and six bags of WPA plates. Plates were processed at the age in weeks indicated in the first column. Set 4 was used only in a mass-balance study for the toxin, after the other sets were processed.

As outlined in Figure 3-1, five samples were collected from each of the inoculated and control plates. Every sample was analyzed for both sterigmatocystin, a marker of

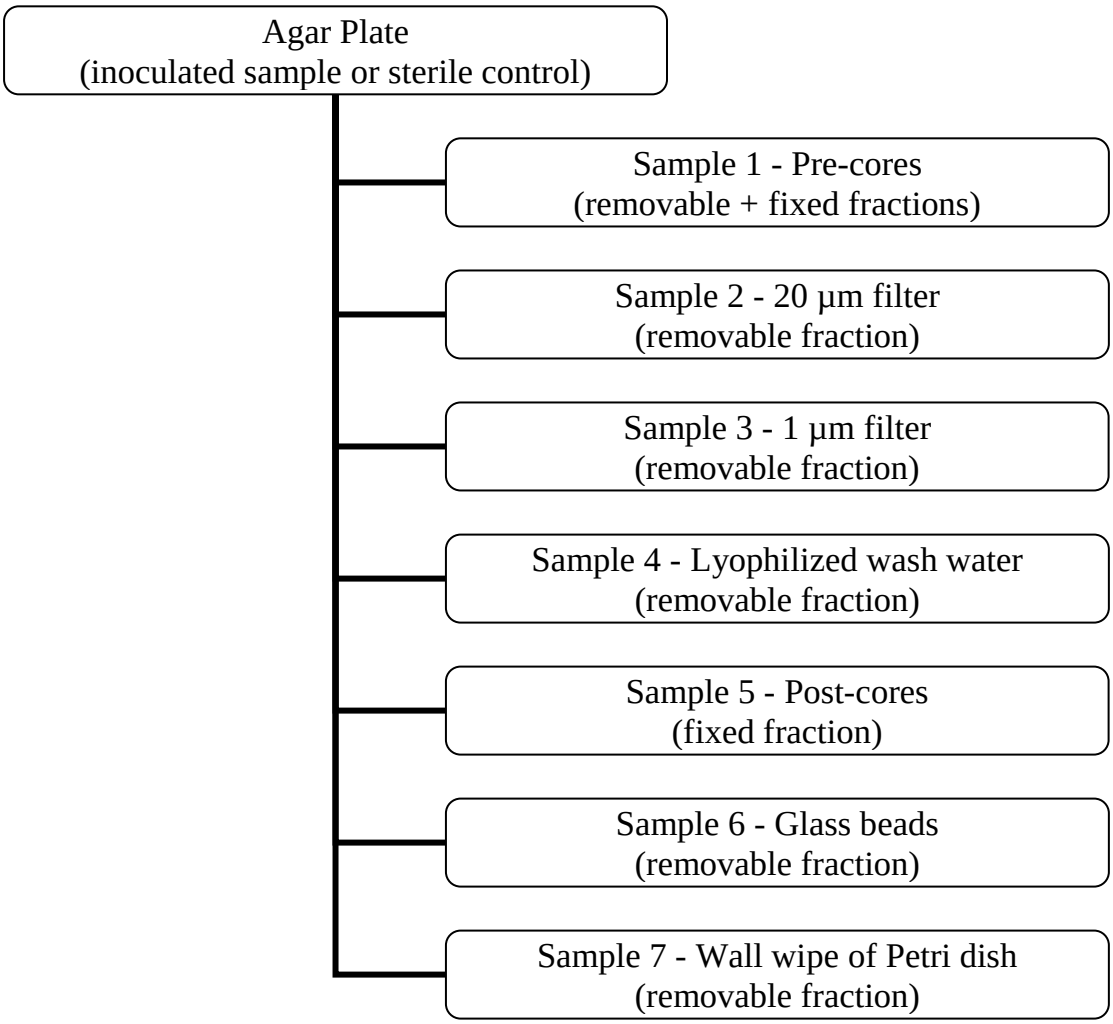


Figure 3-1. Sample processing scheme. Samples 1-5 collected from all plates shown in Table 3-1. Samples 6 and 7 collected only from the plates shown in Set 4.

toxins produced by *A. versicolor*¸ and ergosterol, a marker of fungal growth. Because growth of *A. versicolor* was much greater on MEA than WPA, sterigmatocystin results were standardized two different ways: against the area of the extracted sample, and against ergosterol levels in the same sample. In addition to Samples 1 through 5, Samples 6 and 7 were also collected from a set of plates prepared specially for a mass balance study of the toxin.

3.2.2 Test microorganism

Freeze dried spores (conidia) of *Aspergillus versicolor* (Vuillemin) Tiraboschi, anamorph were purchased from American Type Culture Collection (Manassas, VA). This strain (ATCC#26939) was chosen because it is known to produce sterigmatocystin as well as other secondary metabolites (Hamasaki and others 1967). Because fungal strains have been shown to 'drift' with time, sometimes becoming less vigorous in growth or toxin production (Chang and others 2007), a stock inoculum suspension was prepared and frozen in aliquots for the studies described below.

Briefly, the ATCC *A. versicolor* spores first were cultured on MEA (CM0059) purchased from Remel Inc. (Lenexa KS) in a 150 mm x 15 mm sterile polystyrene Petri dish (BD Biosciences, San Jose, CA). After the plate achieved confluent growth at approximately one week, it was flooded with 20 mL of sterile deionized water with 10% glycerol. The glycerol was added to protect the conidia during freezing. The plate was shaken gently by hand to dislodge the conidia. The resulting suspension was pipetted into a sterile glass bottle. After vortexing to ensure thorough mixing, the concentration of conidia was measured with a hemocytometer and determined to be 3×10^6 conidia per

mL. The suspension was aliquoted into sterile, 1.25 mL microcentrifuge tubes and frozen at -20^{o}C.

3.2.3 Media inoculation

At the beginning of each of the six-week sets, two aliquots of the suspension were removed from the freezer and warmed to room temperature. After vortexing to ensure thorough mixing, one aliquot was used to inoculate twelve sterile 100 X 25 mm polystyrene Petri dishes (product number 351029, BD Biosciences, San Jose, CA). containing MEA and the other to inoculate another twelve plates containing wallpaper paste agar (WPA). Although the plates were designated 100 mm diameter, the covers were larger than the bases, and the diameter of the agar actually measured 80 mm.

The WPA was prepared from cornstarch (Argo, Summit IL), agar (Fisher Scientific, Pittsburg PA) and deionized water (Larsen and Frisvad 1994). Each plate received 0.1 mL of the *A. versicolor* suspension, resulting in approximately 3 x 10^5 conidia deposited on each plate. To spread the inoculum evenly over the surface, 25 mL of sterile, 6-mm glass beads were added to each plate. The plates were shaken 10 times forward and backward, then 10 times side to side to ensure the agar surface was covered with the inoculum. The beads were then removed from the plates, the lids were placed over the plates, and the plates were divided for incubation. Two inoculated plates and an uninoculated control of the same agar were placed in a plastic bag and closed with twist ties for a total of six bags each of MEA and WPA plates: one bag of each media for each week. The plates were incubated in the dark at 25^{o}C until time for processing.

3.2.4 Sample collection

As noted in Section 3.2.1 of this paper, after the designated incubation period, each plate was processed into five different samples to assess toxin levels in each fraction of the fungal material (Figure 3-1). Each week, a set of two inoculated and one sterile control plate with MEA and another set of WPA plates were removed from the incubator. First, three pre-cores were cut from each plate (Sample 1). The pre-cores represent the total amount of fixed and removable fungal material and toxin produced by *A. versicolor* by substrate during the incubation period. Next, the plates were washed and the resulting fungal suspension collected to separate 'removable' fractions of the fungal material (Samples 2, 3 and 4) from the 'fixed' fraction (Sample 5).

The fungal suspension from each sample was filtered to further divide the removable fractions by size. A filter with 20 µm diameter pores (Sample 2) was selected to separate large hyphal strands from the smaller intact conidia, which in the case of *A. versicolor* are about 2-3 µm in diameter (de Hoog and others 2000). Another filter with 1 µm pores (Sample 3) was used to separate intact conidia from smaller fragments, which were expected to pass through into the wash water (Sample 4). The post-cores indicate what remains on the substrate and is not removable by the washing process (Sample 5, fixed fraction). This material consists of hyphae, mycelia and any conidia not removed by the washing process.

For the precore sample, three 9 mm diameter plugs of hyphae, conidia and underlying agar were cut from each plate using an alcohol-rinsed and flame-sterilized metal cork-borer. To reduce the chance for selection bias, the three cores were cut at

approximately 120-degrees from each other on the Petri dish, as shown in Figure 3-2. The three cores from each plate were then combined into a single, clean, glass screw-capped tube for further processing.

After coring, 20 mL of sterile glass beads were added to each plate and the plates were flooded with 10 mL of sterile deionized water. The plates were then placed on a

Figure 3-2. Example of precore sample locations on a set of MEA plates. Uninoculated control plate on the left.

mechanical shaker (Eberbach Corporation, Ann Arbor MI) set at low speed, 160 rpm, for 5 minutes to harvest removable fungal material. The shaker moved the water and beads over the surface of the agar, knocking loose removable conidia, hyphal segments and fragments from the fungal material. The resulting suspension was pipetted into a clean glass 50 mL screw-capped tube, and the plate and beads were shaken twice more with another 7 mL of sterile deionized water each time for a total of 15 minutes of shaking and 24 mL of water. The three wash fractions were consolidated in the 50 mL tube and the suspension was vortexed for 15 seconds on a SP Deluxe Mixer (American Scientific Products, McGaw Park, IL) with the speed control set on 'fast'. The suspension included hyphal fragments, but only conidia were counted with a hemacytomer (Hausser Scientific bright-line hemacytometer, improved Neubauer) to produce the conidial concentration.

The 20 µm filter sample was collected by vacuum filtering the consolidated suspension through a 47 mm diameter, hardened, ashless-grade filter-paper with a pore size of 22 µm (541 Cat No 1541-047, Whatman, Piscataway, NJ) fitted into a stainless steel funnel. The filtered liquid was collected in another clean, glass 50 mL screw-capped tube and the filter (Sample 2) was then removed with flame-sterilized tweezers and placed in a 250 mL clean glass bottle for further analysis. Conidia concentration in the collected filtrate was then counted with the hemacytometer, after vortexing as above. The remaining suspension was pipetted into another stainless steel funnel and vacuum filtered through a 1µm glass fiber filter (Type A/B 47 mm Extra Thick, Pall Corporation, Ann Arbor, MI). As above, the 1 µm filter was removed and placed in a 250 mL bottle for further analysis.

The double-filtered wash water was collected in another clean, glass 50 mL screw-capped tube and, after vortexing, was checked for the presence of fungal particles with the hemacytometer. The wash water was dried on a lyophilizer (Thermovac Industries Corporation, Copiague, N.Y.) for further analysis. However, as the 50 mL tube was too large to fit into the lyophilizing equipment, the liquid was divided between three 20-mL screw-capped glass tubes. Holes were drilled through the plastic caps to accommodate removal of water vapor from the samples on the lyophilizer. The split samples were frozen by rotating the tubes in an ethanol and dry ice bath. The frozen samples were loaded into a lyophilizer jar and placed on the lyophilizer at 0.35 Torr or less until completely dry (usually about 36 hours).

Finally, post-cores were collected from between the pre-cores previously taken from the washed plates (Figure 3-3). Like the pre-cores, the post-cores from each plate

Figure 3-3. Example of pre-and post-core sample locations on a set of Malt Extract Agar Plates. Uninoculated control plate on the left.

were consolidated into a clean, 20-mL glass, screw-capped tube for further processing. The pre-cores holes in the plates contain more water from washing and may appear to be smaller than the post-cores holes. However, both the pre- and post-core holes were cut with the same cork-borer, and are actually the same diameter.

A mass-balance study was also conducted to ensure that all the toxin was accounted for. Stergimatocystin in Sample 1 (precores) was compared to the toxin in the sum of the fixed (Sample 5, postcores) and removable fractions (Samples 2, 3 and 4, filters and wash water) for the existing six sets of samples. In addition to Samples 1-5, two more samples were collected from another set of 8-week old plates, as outlined in Figure 3-1. Sample 6 was the glass beads used to wash the plates. Sample 7 was a gauze wiped used to collect the residual fungal material left around the edge of the Petri dish. Each was placed in a 250 mL clean glass bottle, and then processed and analyzed with the other samples as described in the following section.

3.2.5 Sample preparation

After the samples were collected, 7.5 mL of HPLC-grade methanol was added to the 20 mL tubes with pre- and post-cores (Samples 1 and 5), and to the 250 mL bottles with the 20- and 1-µm filters (Samples 2 and 3), and also to the 250 mL bottles with the glass beads and gauze wipes of the Petri dish walls (Samples 6 and 7, Set 4 only). The wash samples (Sample 4) had been split previously into thirds for lyophilizing. Once dry, 2.5 mL of methanol was added to the residue in each tube. All methanol extracts were subsequently sonicated in a Transsonic TS540 sonicator (Elma, Singen, Germany) for 15 minutes and then vortexed on 'fast' for 15 seconds to extract toxins and ergosterol. The core, filter, bead and wipe samples were then vacuum-filtered into new 20 mL tubes through a clean, stainless steel funnel fitted with a 1.4-µm glass fiber filter without binder (Cat No APFA04700, Millipore, Billerica, MA) to remove particles that could interfere with HPLC analysis. At this point, the triple-split methanol extracts of the lyophilized wash-water samples were consolidated into a single sample as they were vacuum-filtered through the 1.4-µm filter. The volume of filtered methanol in each sample was measured with a pipette. Then, half the sample was left in the 20 mL tube for ergosterol analysis, while the other half was placed into a 4 mL amber glass vial with a Teflon cap for toxin analysis.

3.2.6 Ergosterol sample analysis

The method used for ergosterol analysis was adapted from the methods reported by Pasanen (Pasanen and others 1999) and Jedličková (Jedličková and others 2008). The methanol-extract halves for ergosterol analysis were brought up to 4 mL with appropriate

addition of HPLC-grade methanol. After approximately 480 mg of potassium hydroxide (5 tablets) was added to each tube to saponify the ergosterol in the methanol extract, the tubes were stored in the dark overnight. Usually by the next day the KOH was completely dissolved. If not, the samples were placed alternately in a sonicator and in a Dubnoff Metabolic Shaking Incubator (Precision) set at 65^{o}C and 65 rpm, until all the KOH dissolved.

Because the analytical method then called for extraction of the methanolic-KOH extracts with n-hexane, an internal standard was necessary. To each sample, 18 nmol of 7-dehydrocholesterol was added as the internal standard, along with 1.6 mL of n-hexane for the first extraction. The tubes were then capped tightly and placed in the shaker bath at 65^{o}C and 65 rpm for 30 minutes. After cooling to room temperature, 0.8 mL of deionized water was added to each tube, and the n-hexane layer was removed with a Pasteur pipette and stored in a clean, 4 mL amber glass vial with a Teflon cap. The samples were washed twice more with 1.6 mL of n-hexane and heated and shaken as noted above, but no more water was added. The three hexane fractions were consolidated and taken to dryness on a SpeedVac Plus (Savant). The residue was redissolved in 0.5 mL of HPLC grade methanol for HPLC analysis.

Samples, controls and standards were analyzed with a HPLC (Waters Corporation, Mildford, MA) equipped with a Waters 600E pump, Waters 996 PDA detector, and a Waters 717 autosampler. Samples were separated over a C18 column, 250 x 4.6 mm, Synergi 4 μm Hydro-RP 80A (Phenomenex, Torrance, California, USA) equipped with a guard column. Clean 4 mL amber glass vials with 300 μL glass inserts

were loaded with 200 µL of sample and/or control and placed into the HPLC's autosampler. Ergosterol was analyzed isocratically in HPLC-grade methanol at a flow rate of 1 mL/minute, with a retention time of approximately 17 minutes.

3.2.7 Sterigmatocystin sample analysis

As described in Section 3.2.4, samples and controls were split so they could be analyzed for both ergosterol and sterigmatocystin. The methanol extracts slated for toxin analysis were placed in 4 mL amber glass vials. These vials were taken to dryness on the speed vac. The residue was brought up in 0.5 mL of HPLC grade methanol for HPLC analysis. Clean 4 mL amber glass vials with clear, 300 µL glass inserts were prepared for loading into the HPLC's autosampler, with 200 µL of sample and/or control in each insert.

Samples and controls were separated over the same Synergi column and HPLC system described in Section 3.2.5. A CH-30 column heater (Eppendorf, New York NY) maintained the column at 40^{o}C. Using the method described by Smedsgaard (Smedsgaard 1997) with slight modifications, solvent A was milliQ water with 0.005% (v/v) trifluroracetic acid and solvent B was HPLC-grade acetonitrile with 0.005% (v/v) trifluroracetic acid. Each analysis began with 85% A and 15% B running at 1 mL/min., transitioning to 100% B in 40 minutes, then returning to 85% A and 15% B in 5 minutes and holding for 15 minutes before starting the next analysis. Retention time for sterigmatocystin was approximately 32 minutes.

3.2.8 Statistical analysis

Log transformations of the ergosterol levels per unit area, sterigmatocystin levels per unit area, and ratios of sterigmatocystin divided by ergosterol were computed. The geometric means and standard deviations were calculated for the core samples and the removable fractions for MEA and WPA. The data were analyzed to assess effects of three independent variables (growth substrate, incubation time and fungal fraction) on the level of ergosterol and/or sterigmatocystin. A p-value of 0.05 was used to determine statistical significance.

Three general linear mixed models were fit for each of the outcomes (nmol of ergosterol per cm^2, nmol of sterigmatocystin per cm^2, and nmol of sterigmatocystin per nmol of ergosterol), where associations were modeled using a compound symmetric matrix for agar plates within a set (Table 3-1). The difference in the outcome variables was computed using least-square means for each variable at a given level for the other two variables by including a three-way interaction between media, week, and fraction along with the main effects and all two-way interactions. Observed margins were used to compute the least square means due to missing values in the data. The Tukey-Kramer method for multiple comparisons was used to maintain a familywise error rate of 0.05 for each model. All analyses were performed in SAS version 9.2.

3.3 Results

The results in this section are divided into three categories: ergosterol standardized by area, sterigmatocystin standardized by area, and sterigmatocystin standardized by ergosterol. For the area standardizations, levels were standardized

against the area of the agar the fungal material was removed from. Therefore, ergosterol and sterigmatocystin in the core samples were standardized against 1.91 cm^2 (from three 0.9 cm diameter cores), while 'removable' samples were standardized against 48.3 cm^2 (from the 80 mm diameter agar plate, minus the cores). For standardizations against ergosterol, the nmol of sterigmatocystin were divided by the nmol of ergosterol detected in the same sample.

Because the fungal extracts were split for sterigmatocystin and ergosterol analysis, the results standardized against area show the nmol of compound in half the sample. The total amounts of ergosterol and sterigmatocystin produced by each sample are actually double the amounts shown in Sections 3.3.1 and 3.3.2, respectively. However, because the proportions are the same, conclusions regarding the comparison of compound levels by time, by substrate or by fraction also will not change. Ratios of sterigmatocystin to ergosterol presented in Section 3.3.3 are not affected and remain unchanged either way.

3.3.1 Ergosterol standardized against area

In this section, ergosterol results presented in each figure are compared by substrate, by time and by fungal fraction. Figure 3-4 shows that there were significant differences in overall growth between the two substrates, MEA and WPA. Levels of ergosterol per cm^2 in the pre-cores show that growth of *A. versicolor* on MEA was significantly greater than growth on WPA ($p<0.0001$, Appendix A, Table 3-4-1). Ergosterol levels in the pre-cores from MEA plates ranged from 15-32 nmol/cm^2, whereas levels on WPA were lower, ranging from 0.30 to 0.54 nmol/cm^2.

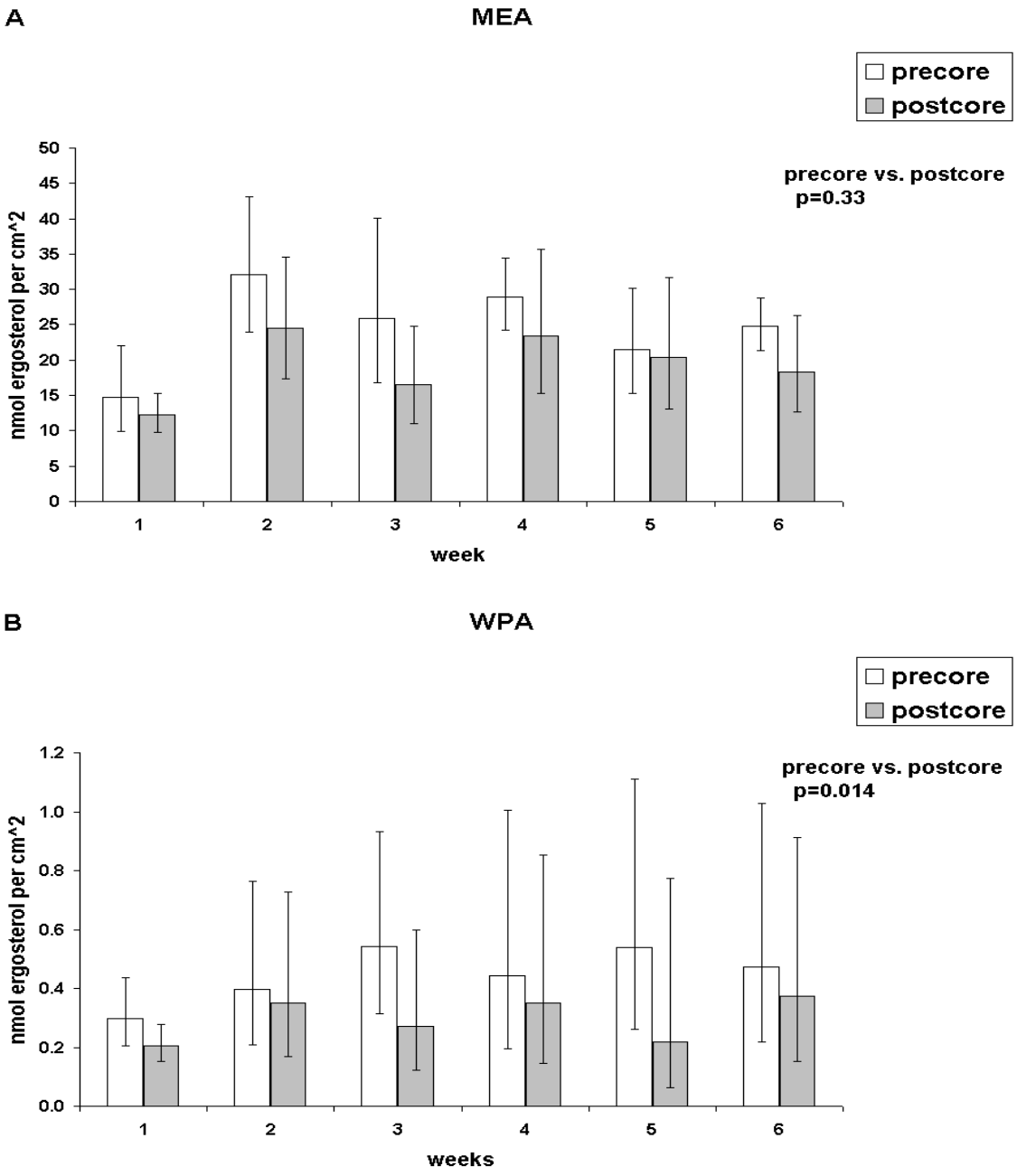


Figure 3-4. Ergosterol per square centimeter in pre- and post-cores. nmol of ergosterol per cm^2 in pre- and post-cores on MEA (A) and WPA (B). Geometric means and standard deviations based on n=6 samples. Neither differences between pre- and post-cores, nor differences over time were statistically significant on either media. Note scale differences: MEA to 50 nmol / cm^2; WPA to 1.2 nmol / cm^2.

Another way of looking at these results is presented in Table 3-2. At 24 nmol/cm^2, average ergosterol levels in the pre-cores on MEA over six weeks were 56 times greater than the 0.44 nmol/cm^2 average ergosterol levels in the pre-cores on WPA. Average ergosterol levels in the post-cores on MEA contained more that 65 times more ergosterol than the post-cores on WPA, indicating that after washing, a slightly greater proportion fungal material remained on the MEA plates than the WPA plates.

Table 3-2. Average ergosterol comparisons by fixed and removable fractions. nmol of ergosterol per cm^2. Geometric means and standard deviations calculated over six-weeks, based on n=36 samples.

	MEA (nmol/cm^2)		**WPA (nmol/cm^2)**		**MEA/WPA Ratio of**
	avg.	**-/+ sd**	**avg.**	**-/+ sd**	**avg's**
Pre-cores	24	16-34	0.44	0.23-0.84	56
Post-cores	19	12-28	0.29	0.13-0.66	65.5
Avg.pre-cores – avg.post-cores	5		0.15		33
Sums of 'removable' fractions	3.0	2.0-4.6	0.25	0.22-0.35	12
Estimated 'removable' percentage	12.5%-21%		34%-57%		

Growth appeared to increase somewhat with time on both substrates (Figure 3-4). On MEA, levels of ergosterol in the precores doubled from 15 nmol/cm^2 at week 1, to 32 nmol/cm^2 at week 2 and then declined slightly in the following weeks. On WPA levels of ergosterol in the precores increased from 0.30 nmol/cm^2 at week 1, to 0.54 nmol/cm^2 at week 3 before leveling off. However, these differences were not statistically significant at a p-value of 0.05 (Appendix A, Tables 3-4-2 and 3-4-3). Similarly, time

did not have a significant effect on the post-cores at a p-value of 0.05 on either MEA or WPA (Appendix A, Tables 3-4-4 and 3-4-5).

Individual weekly differences between the pre- and post-cores (Figure 3-4) were not statistically significant on either substrate (Appendix A, Table 3-4-6). On average over the six weeks, MEA post-cores contained 21% less ergosterol than that measured in MEA pre-cores (Table 3-2), and still this difference was not statistically significant ($p=0.33$, Appendix A, Tables 3-4-6). However, the average 34% difference between the WPA post-cores and pre-cores over the study period was statistically significant ($p=0.014$).

As noted above, MEA post-cores contained an average of 79% of the original ergosterol measured in MEA pre-cores. This indicates that on average, 21% of the total ergosterol, 5 nmol/cm^2, was removed from MEA plates (Table 3-2). WPA post-cores contained an average of 66% of the ergosterol measured in the WPA pre-cores. This means that 34% of the ergosterol, 0.15 nmol/cm^2, was removed from the WPA plates. Although the proportion of removed ergosterol was higher from WPA plates, the ratio of ergosterol removed (MEA/WPA) shows that 33 times more total fungal material was removed from the MEA than the WPA plates.

Data presented in Figure 3-5 show that substrate had a significant effect on the level of ergosterol in the removable fractions (Appendix A, Table 3-5-1). Like the cores, levels of ergosterol per cm^2 in the 20 µm and wash-water fractions from MEA plates were significantly greater than those removed from WPA plates ($p<0.001$). Ergosterol in the 20 µm fraction ranged as high as 4.3 nmol per cm^2 on MEA compared to a high of

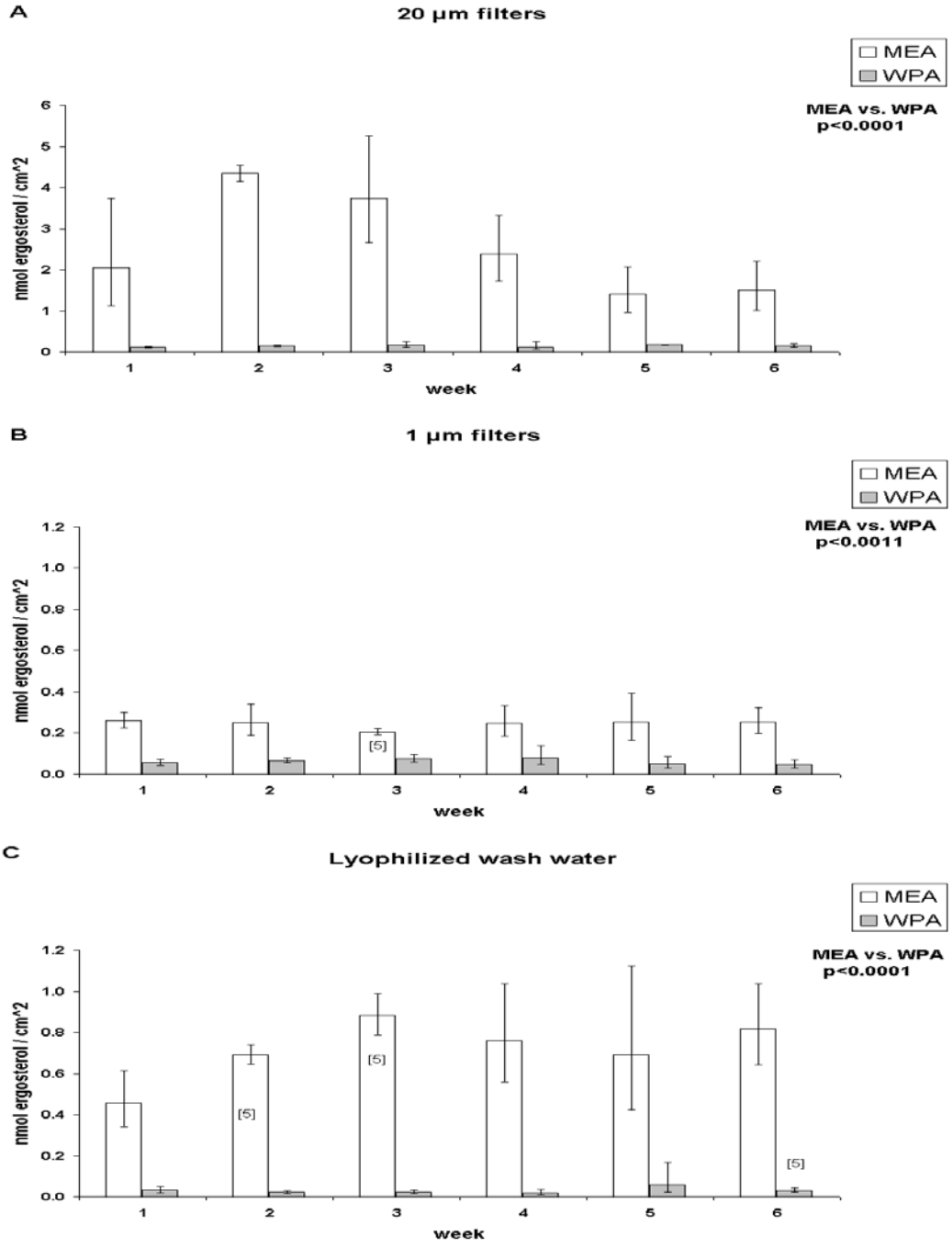


Figure 3-5. Ergosterol in removable fractions. nmol of ergosterol per cm^2 in 20 µm filters (A) 1 µm filters (B) and lyophilized washwater (C) on MEA and WPA. Geometric means and standard deviations based on n=6 samples, except as noted [n]. Note scale differences: (A) to 6 nmol / cm^2; (B) and (C) to 1.2 nmol / cm^2.

0.175 nmol per cm^2 on WPA. Similarly, in the lyophilized wash water fraction, ergosterol peaked at 0.882 nmol per cm^2 on MEA compared to a high of 0.061 nmol per cm^2 on WPA. Ergosterol levels in the 1 µm fractions were more comparable, but still significantly different ($p = 0.0011$). Ergosterol in the 1 µm fraction ranged up to 0.26 nmol per cm^2 on MEA compared to 0.079 nmol per cm^2 on WPA.

Another method of estimating 'removability' is to add up all three 'removable' fractions and compare that number to the pre-core. These sums indicate that washing removed an average of 3 nmol of ergosterol/cm^2 from the MEA plates, but on WPA, only 0.25 nmol/cm^2 was removed, approximately 12 times less (Table 3-2). By this method, 57% of the fungal material on WPA plates was 'removable', whereas only 12.5% of the fungal material was removable from MEA plates. Using both methods (pre- and post-core differences, and sums of removable fractions) gives a range of removability between 12.5-21% for fungal material from MEA plates, and 34-57% for material from WPA plates (Table 3-2).

Over the six week study period, time did not significantly affect the amount of fungal material removed from MEA and WPA plates (Figure 3-5). Although ergosterol levels in the 20 µm fraction at week 2 on MEA were significantly higher than levels at week 5 or week 6 ($p = 0.01$ and 0.03, respectively), overall differences from week 1 to week 6 were not significant at a p-value of 0.05 (Appendix A, Tables 3-5-2, 3-5-3 and 3-5-4). On WPA, there were no significant differences from week 1 to week 6 in any of the removable fractions (Appendix A, Tables 3-5-5, 3-5-6 and 3-5-7).

On both substrates, most of the removed fungal material was captured on the 20 µm filter (Table 3-3). Microscopic examination of the wash water before and after the 20 µm filter showed that the material on this filter consisted of larger hyphal sections, intact conidia and presumably some fragments which were not visible with the standard microscope. On MEA, 69% of the removable ergosterol was detected on the 20 µm filter, 8% on the 1 µm filter and 23% in the wash water. On WPA, 62% of the removable ergosterol was detected on the 20 µm filter, 26% on the 1 µm filter and 12% in the wash water. Microscopic examination of the wash water from both substrates before and after the 1 µm filter confirmed that material on this filter consisted of intact conidia. Although fragments were not visible with this microscope, it is likely that the 1 µm filter also trapped a portion of the fungal fragments. As no microscopically-visible particles were present in the double-filtered wash water, fungal fragments could be the source of the ergosterol. Another possibility is that some amount of ergosterol dissolved in the wash water.

Table 3-3. Average size distribution of ergosterol removed from inoculated plates. nmol of ergosterol per cm^2. Geometric means and standard deviations calculated over six-weeks, based on n=36 samples.

	MEA (nm/cm^2)			**WPA (nm/cm^2)**		
	avg.	**-/+ sd**	**%**	**avg.**	**-/+ sd**	**%**
20 µm filter	2.1	1.3-3.5	69%	0.145	0.10-0.21	62%
1 µm filter	0.26	0.18-0.36	8%	0.061	0.040-0.093	26%
Lyophilized wash water	0.72	0.50-1.0	23%	0.029	0.015-0.056	12%

However, conidia concentrations in the wash water before and after the filters, shown in Figure 3-6, indicate that the distribution of fungal material between the three

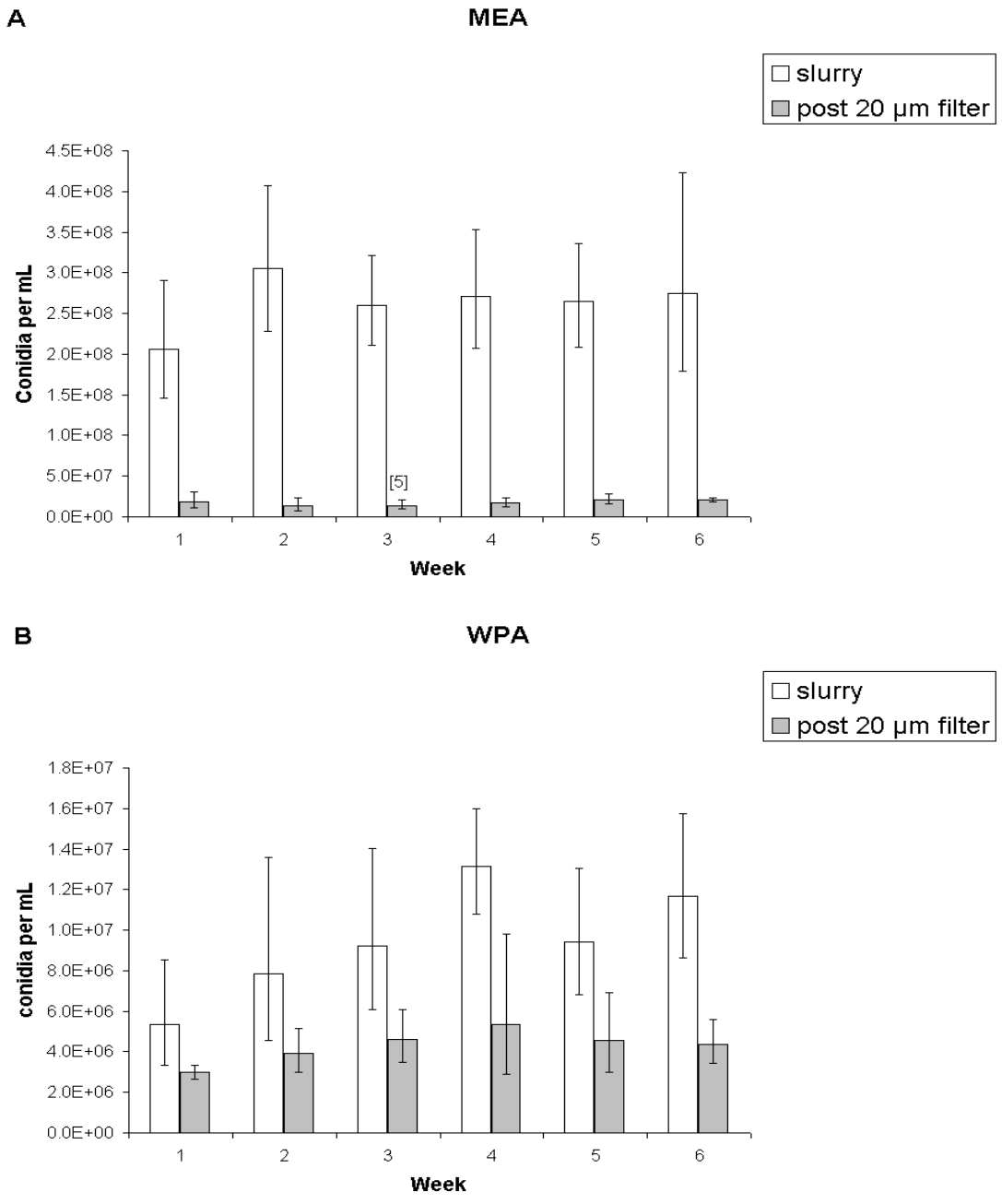


Figure 3-6. Conidia per mL in unfiltered slurry and 20 µm filtered wash water from MEA (A) and WPA (B) plates. Geometric means and standard deviations based on n=6 samples, except as noted [n]. Conidia counts in the 1 µm filtered suspension were zero. Note scale differences: MEA to 4.5E+08 conidia / mL; WPA to1.8E+07 conidia / mL.

removable fractions may be skewed toward the 20 µm filter. The conidia concentrations were determined by microscopic examination of the suspension with a hemocytometer. Although larger sections of hyphae were present in the unfiltered slurry, none were visible after the 1 µm filter and only conidia were counted. Concentrations from the twice-filtered suspension (after the 1 µm filter) are not included because the counts were always below the limits of detectability of the hemocytometer (5×10^4, data not shown).

The data show that less than 10% of the conidia washed from the MEA plates passed through the 20 µm filter. Even though the intact conidia are only 2-3 µm in diameter, the 20 µm filter caught more than 90% of them. For conidia washed from the WPA plates, Figure 3-6 shows that just under 50% of the intact conidia passed through the 20 µm filter. This may be the reason that even though *A. versicolor* produced 12 times more material in the summed 'removable' fractions from MEA than from WPA (Table 3-2), the amount of average ergosterol captured in the 1 µm fraction was only four times greater on MEA than WPA (Table 3-3).

Also, a mass-balance analysis (Figure 3-7) was conducted to determine whether the sum of fungal material in the postcores and removable fractions accounted for material measured in the precores. Figure 3-7 shows that most of the ergosterol was accounted for on MEA, with sums of the postcore, 20 µm filter, 1 µm filter and the lyophilized wash water accounting for 83%-106% of the amount detected in the precore, when adjusted for area. Recovery rates on WPA ranged from 106%-155%. T-tests on the precores and the sums showed that the none of the weekly differences were statistically significant ($p>0.05$).

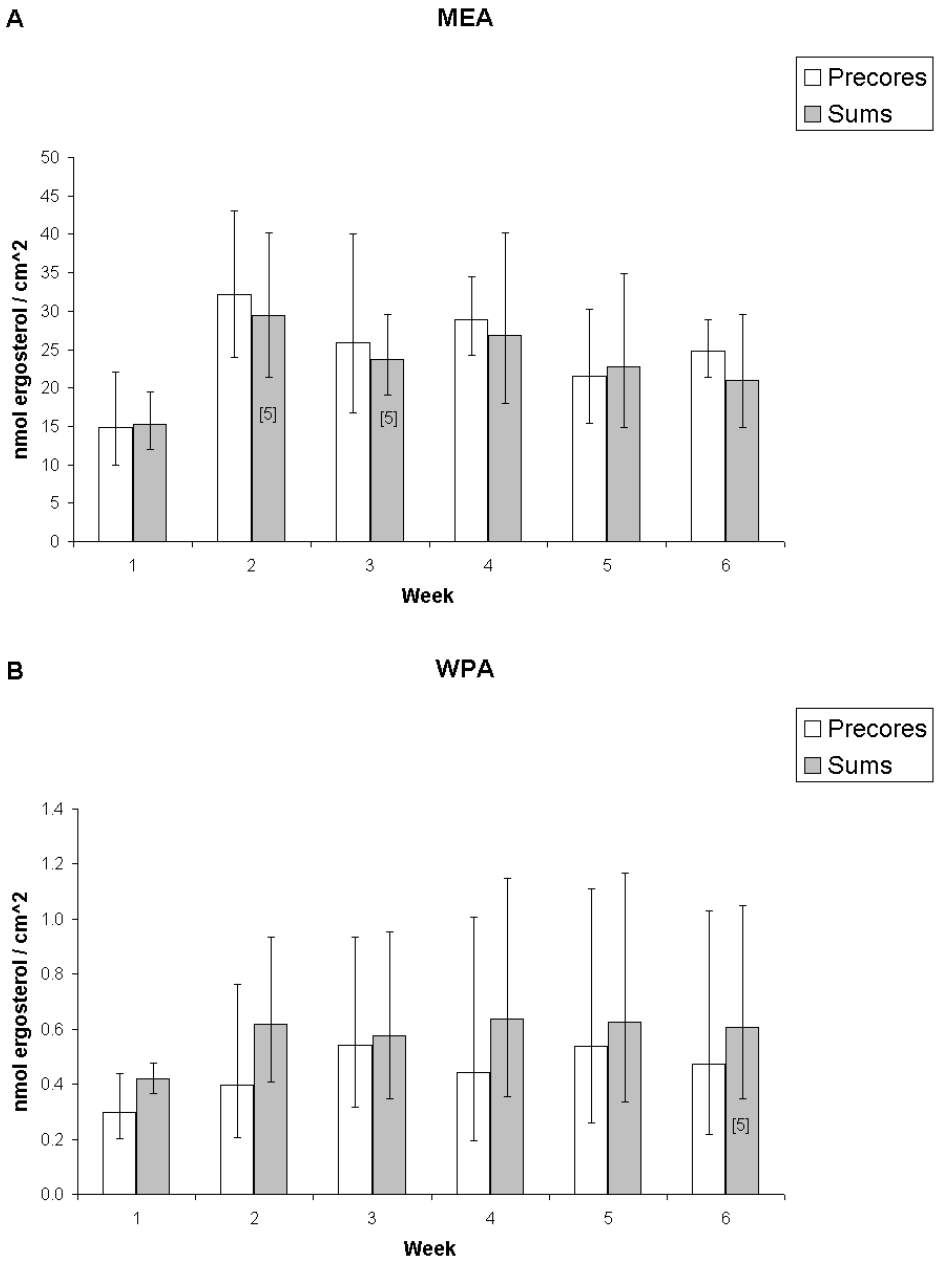


Figure 3-7. Mass Balance Comparison of Ergosterol. Bars represent nmol of ergosterol per cm^2 in precores, versus the sum of nmol of ergosterol per cm^2 in postcores, 20 µm filters, 1 µm filters and lyophilized washwater on MEA (A) and WPA (B). Geometric means and standard deviations based on n=6 samples, except as noted [n]. Note scale differences: MEA and WPA to 50 and 1.4 nmol ergosterol / cm^2, respectively.

3.3.2 Sterigmatocystin (ST) standardized against area

Like the previous section section, sterigmatocystin results presented in each figure are compared by substrate, by time and by fungal fraction. When toxin levels in the pre-cores were standardized against area, it was apparent that substrate had significant effects on production of sterigmatocystin by *A. versicolor* (Figure 3-8). Sterigmatocystin levels in the pre- and post-cores were higher on MEA than on WPA. On MEA, ST levels in the pre-cores ranged from 53 to 81 nmol per cm^2, whereas levels on WPA ranged from 0.13 to 2.0 nmol per cm^2. Substrate differences in the pre-cores ($p<0.0001$) and the post-cores ($p<0.0001$) were statistically significant (Appendix A, Table 3-8-1).

Time had a significant effect on toxin levels in WPA pre-cores, but not MEA pre-cores (Figure 3-8). On MEA, although ST levels in the cores appear to increase through week 4, with pre-cores peaking at 81 nmol per cm^2 before dropping back to 63 nmol per cm^2 at week six (Figure 3-8), the differences were not statistically significant (Appendix A Table 3-8-2). On WPA, ST levels in the week-1 pre-cores were significantly different from those in the pre-cores for week 2 ($p=0.0002$), and weeks 3, 4, 5 and 6 (each $p<0.0001$, Appendix A Table 3-8-3). ST levels in the week 2 precores were significantly different from those for week 4 ($p=0.0085$), week 5 ($p=0.0053$) and week 6 ($p=0.0001$).

Washing removed significant amounts of toxin from WPA, but not MEA plates. On MEA, nmol ST per cm^2 in the pre-cores were not significantly different from those in the post-cores for any of the weeks (Figure 3-8). Over the six weeks, the average difference between pre-cores and post-cores was only about 10%. In contrast, by week 6

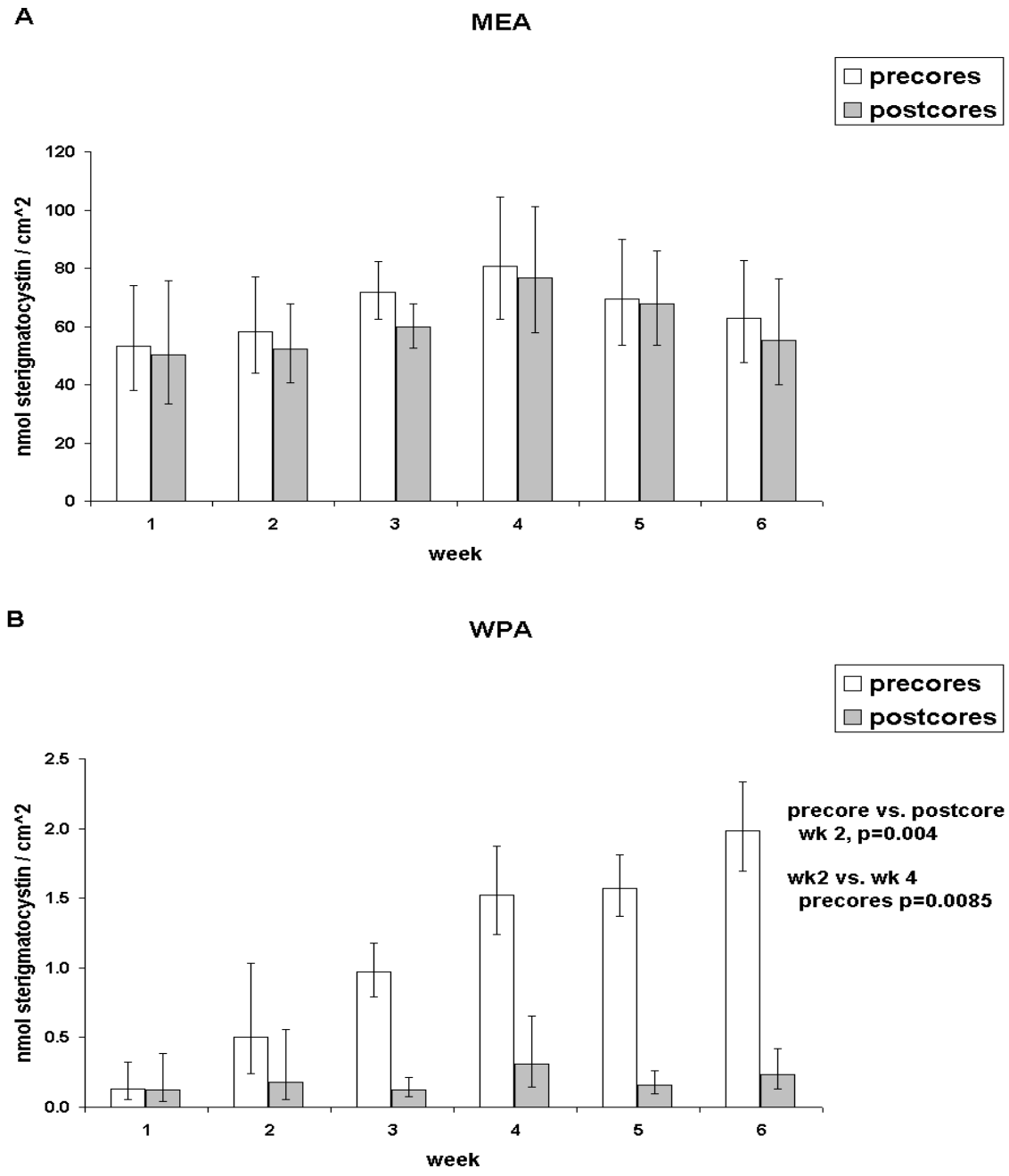


Figure 3-8. Sterigmatocystin per square centimeter in pre- and post-cores. nmol of sterigmatocystin per cm^2 in pre- and post-cores on MEA (A) and WPA (B). Geometric means and standard deviations based on n=6 samples. Note scale differences: MEA to 120 nmol / cm^2; WPA to 2.5 nmol / cm^2.

on WPA, there was approximately a 90% drop in ST concentrations in the post-cores compared to the pre-cores ($p<0.0001$).

Another way of looking at these results is presented in Table 3-4. Average sterigmatocystin levels in the MEA pre-cores (65 nmol/cm^2) were 79 times greater than average sterigmatocystin levels WPA pre-cores (0.82 nmol/cm^2). Average

Table 3-4. Average sterigmatocystin comparisons by fixed and removable fractions. nmol of sterigmatocystin per cm^2. Geometric means and standard deviations calculated over six-weeks, based on n=36 samples.

	MEA (nmol/cm^2)		**WPA (nmol/cm^2)**		**MEA/WPA Ratio of**
	avg.	**+/- sd**	**avg.**	**+/- sd**	**avg's**
Pre-cores	65	49-87	0.82	0.28-2.3	79
Post-cores	60	44-81	0.18	0.08-0.41	333
Pre-cores – Post-cores	5		0.64		7.8
Sums of 'removable' fractions	4.3	3.3-5.8	0.41	0.11-1.5	10.5
Estimated 'removable' percentage	6.6-7.7%		50-78%		

sterigmatocystin levels in the MEA post-cores (60 nmol/cm^2) were 333 times greater than average sterigmatocystin levels WPA pre-cores (0.18 nmol/cm^2). Over the six weeks, MEA post-cores contained an average of 92.3% of the sterigmatocystin measured in MEA pre-cores. This indicates that 7.7% of the total sterigmatocystin (5 nmol/cm^2) was removed from MEA plates (Table 3-4). WPA post-cores contained an average of 22% of the sterigmatocystin measured in the WPA pre-cores. This means that 78% of the sterigmatocystin (0.64 nmol/cm^2) was removed from the WPA plates. Taking the ratio of

toxin removed (MEA/WPA) shows that 7.8 times more toxin was removed from the MEA than the WPA plates.

Substrate had a significant effect on the level of sterigmatocystin in the removable fractions (Figure 3-9). Like the cores, toxin per cm^2 in each of the removable fractions from MEA plates was significantly greater ($p<0.001$) than that removed from WPA plates (Appendix A, Table 3-9-1). However, sterigmatocystin differences between the media were greatest in the 20 µm fraction, ranging up to 4.4 nmol per cm^2 on MEA compared to a high of 0.79 nmol per cm^2 on WPA. In the lyophilized wash water fraction, differences between MEA and WPA were not statistically different at weeks 3, 5 and 6. However, sterigmatocystin peaked at 0.61 nmol per cm^2 on MEA compared to a high of 0.25 nmol per cm^2 on WPA. By week 6 in the 1 µm fraction, sterigmatocystin reached 0.36 nmol per cm^2 on WPA, equivalent to sterigmatocystin levels in the same fraction on MEA. Only the differences between the substrates at week 1 and week 2 were significant in the 1 µm fraction.

Over six weeks, toxin levels in all three removable fractions increased steadily on WPA, but not on MEA (Figure 3-9). Toxin levels on WPA increased each week and were significantly changed over the six week study period (Appendix A, Tables 3-9-5, 3-9-6 and 3-9-7). On MEA, however, although toxin levels increased through week 2 in the 20 µm and lyophilized wash water fractions, they decreased steadily in the following weeks. In no case were any week-to-week differences on MEA statistically significant over the six week study period (Appendix A, Tables 3-9-2, 3-9-3 and 3-9-4).

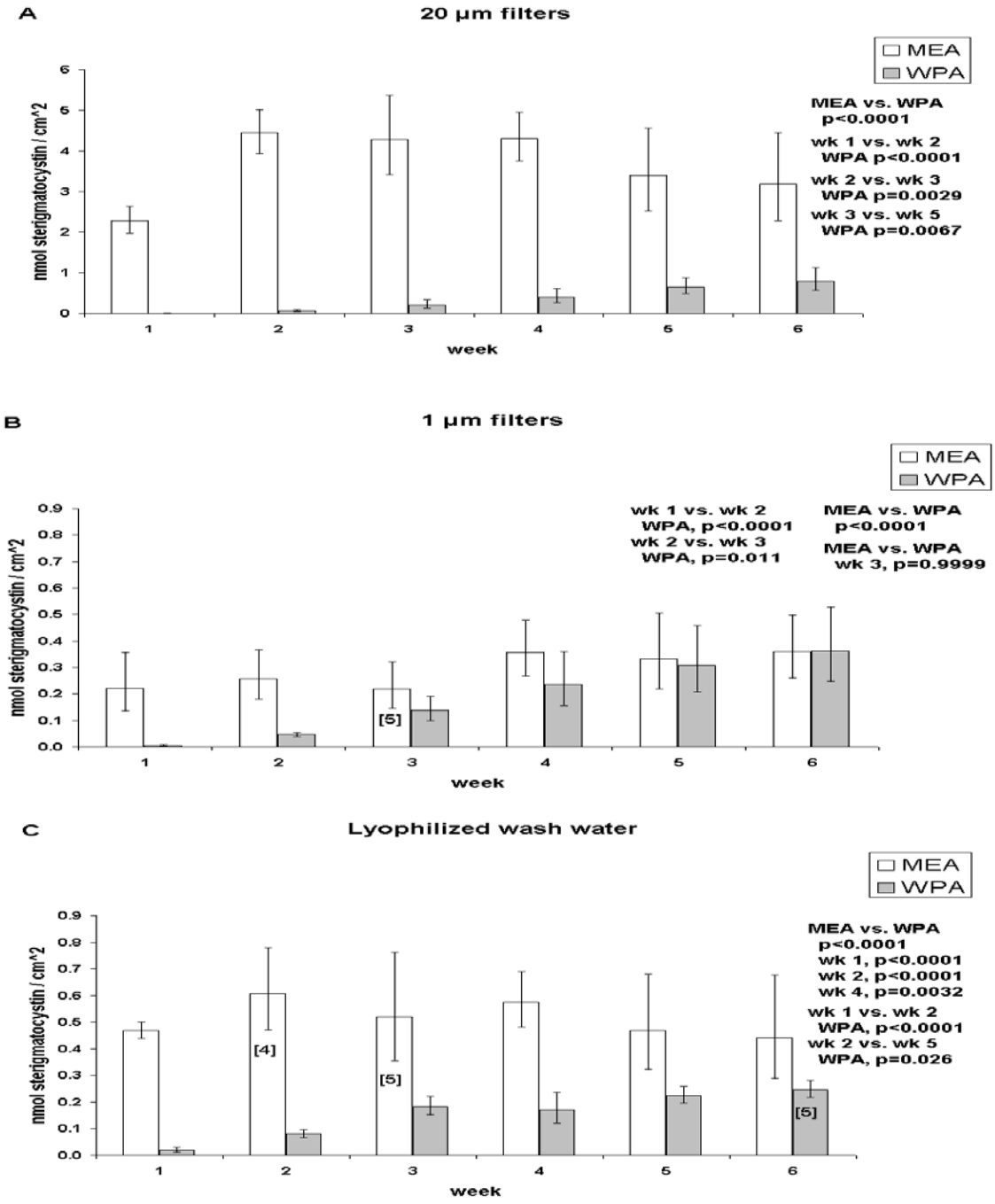


Figure 3-9. Sterigmatocystin in removable fractions. nmol of sterigmatocytin per cm^2 in 20 µm filters (A), 1 µm filters (B) and lyophilized washwater (C) on MEA and WPA. Geometric means & standard deviations based on n=6 samples, except as noted [n]. Note scale differences: MEA and WPA to 6 and 0.9 nmol / cm^2, respectively.

Another method of estimating toxin 'removability' is to add up toxin in all three 'removable' fractions and compare that number to toxin levels in the pre-cores. These sums indicate that washing removed an average of 4.3 nmol of sterigmatocytin/cm^2 from the MEA plates (Table 3-4). On WPA, only 0.41 nmol/cm^2 was removed, approximately 10.5 times less. By this method, 50% of the toxin on WPA plates was 'removable', whereas only 6.7% of the toxin was removable from MEA plates. Using both methods (pre- and post-core differences, and sums of removable fractions) gives a range of toxin removability between 6.6-7.7% for toxin from MEA plates, and 50-78% for toxin from WPA plates.

Table 3-5 shows the distribution of sterigmatocystin between the removable fractions. On MEA, 82% of the removable sterigmatocystin was detected on the 20 µm filter, 7% on the 1 µm filter and 12% in the wash water. In contrast, on WPA, only 44% of the removable sterigmatocystin was detected on the 20 µm filter. The 1 µm filter and lyophilized wash water contained 26% and 30%, respectively, of the removable sterigmatocystin.

Table 3-5. Average distribution of sterigmatocystin removed from inoculated plates. nm / cm^2 derived from geometric means and standard deviations calculated over six-weeks, based on n=36 samples.

	MEA (nm/cm^2)			**WPA (nm/cm^2)**		
	avg.	**+/- sd**	**%**	**avg.**	**+/- sd**	**%**
20 µm filter	3.6	2.6-4.9	82%	0.17	0.031-0.90	44%
1 µm filter	0.29	0.19-0.43	7%	0.10	0.022-0.45	26%
Lyophilized wash water	0.50	0.37-0.69	12%	0.12	0.046-0.29	30%

As noted in Section 3.3.1, however, data presented in Figure 3-9 and Table 3-5 may be skewed toward the larger particles, because more than 90% of the conidia washed from the MEA plates remained on the 20 µm filter, and just over 50% of the intact conidia from the WPA plates were captured on the 20 µm filter.

A mass-balance analysis was conducted to determine whether the sum of sterigmatocystin in the postcores and removable fractions accounted for toxin measured in the precores. Figure 3-10 shows that most of the sterigmatocystin was accounted for on MEA, with sums of the postcore, 20 µm filter, 1 µm filter and the lyophilized wash water accounting for 90-104% of the amount detected in the precore, when adjusted for area. Only the sums for week 3 were statistically different from the precores on MEA. However, recovery rates for weeks 1-6 on WPA (B) were generally lower, with sums accounting for 70-132%. Sums for weeks 3-6 were statistically different from the precores on WPA.

In an effort to determine where sterigmatocystin might have been missed, a set of three 8-week old WPA plates were analyzed as described in Section 3.2.4. Samples 1-7 were collected from these plates, as outlined in Figure 3-1. In addition to Samples 1-5, the pre-cores, post-cores, 20 µm filters, 1 µm filter and lyophilized wash water, two additional samples were analyzed for toxin. Sample 6 comprised the glass beads used to wash the plates. Sample 7 was a piece of gauze used to wipe up the residual fungal material left around the edge of the Petri dish.

The 8-week columns in Figure 3-10 compared the precores, 2.06 nmol, with the sum of the post-cores, 20 µm filters, 1 µm filter and lyophilized wash water, 2.01 nmol.

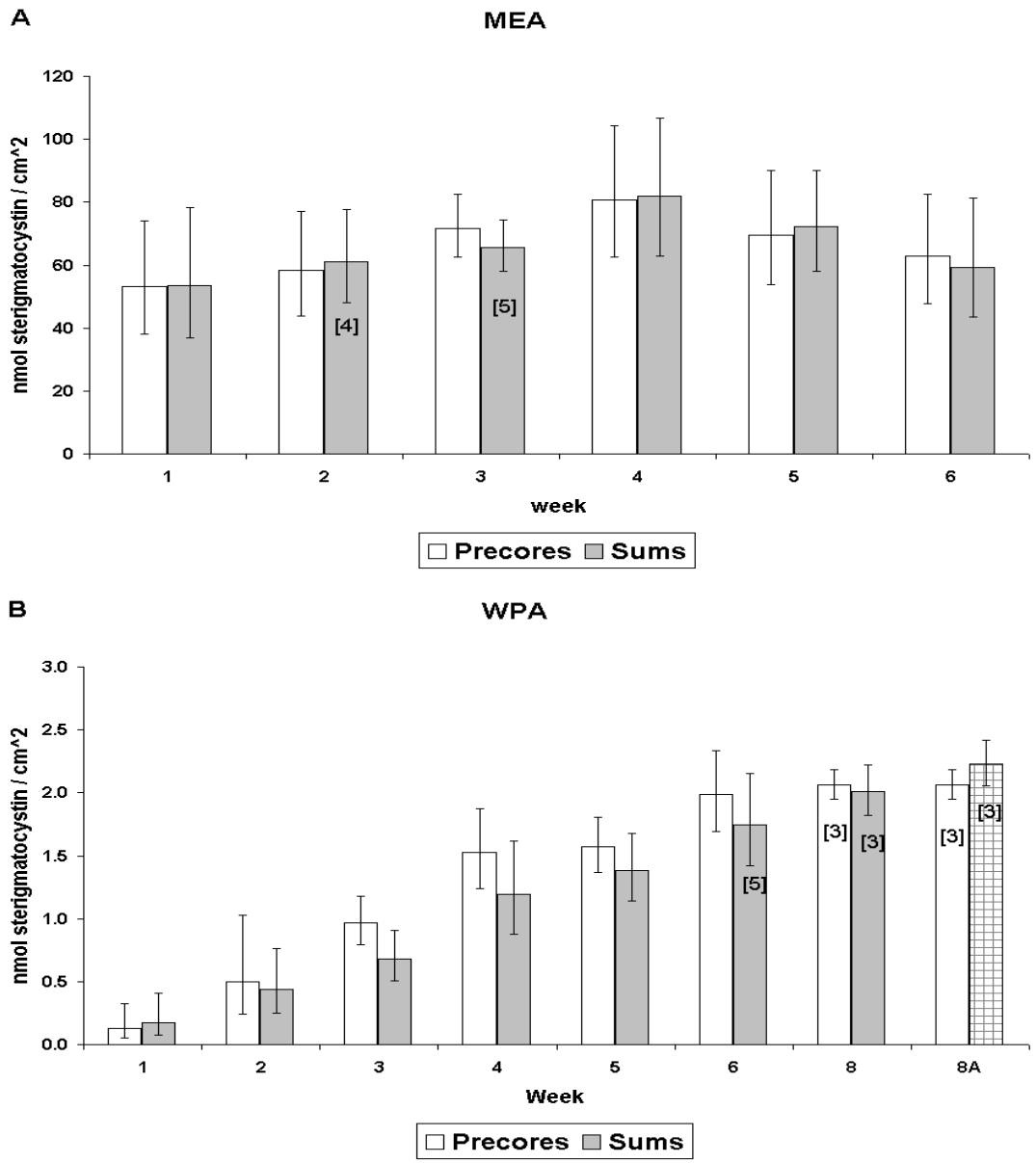


Figure 3-10. Mass balance comparison of sterigmatocystin. Bars represent nmol of sterigmatocystin per cm^2 in precores, versus the sum of nmol of sterigmatocystin per cm^2 in postcores, 20 µm filters, 1 µm filters and lyophilized washwater on MEA (A) and WPA (B). Geometric means and standard deviations based on n=6 samples, except as noted [n]. Week 8A sums included sterigmatocystin recovered from glass beads and the walls of the agar plate. Note scale differences: MEA and WPA to 120 and 3.0 nmol / cm^2, respectively.

The 8A columns compare the precores with the sum of the post-cores, 20 µm filters, 1 µm filter and lyophilized wash water, plus the beads and the wipe sample, 2.23 nmol. The beads and gauze wipe account for an additional 0.22 nmol of sterigmatocystin, or almost an 11% increase. Neither the 8-week nor the 8A sums were statistically different from the 8-week precore average (96.7% and 107%, respectively).

Overall, these results show that although growth of *A. versicolor* on WPA was 56 times less than MEA (Figure 3-4), by week 6, toxin production in removable fractions on WPA was only approximately 4 times less (0.25 – 0.79 nmol) than toxin production levels in removable fractions on MEA (0.36 – 3.2 nmol) (Figure 3-9).

3.3.3 Sterigmatocystin (ST) standardized against ergosterol

Like the previous section sections, sterigmatocystin to ergosterol ratios presented in each figure are compared by substrate, by time and by fungal fraction. In the previous section, toxin levels were standardized against the area of the sample. In this section, because growth levels on MEA and WPA were so different, sterigmatocystin levels were standardized against levels of ergosterol in the same sample. The purpose of this standardization was to investigate, given the same amount of fungal material, whether sterigmatocystin levels would vary between the two substrates, between the fungal fractions, or by time.

Although levels of ergosterol per cm^2 and stergimatocystin per cm^2 were significantly higher on MEA than WPA, Figure 3-11 shows that after week 1, the ratios of nmol of ST per nmol of ergosterol in the pre-cores on MEA and WPA were

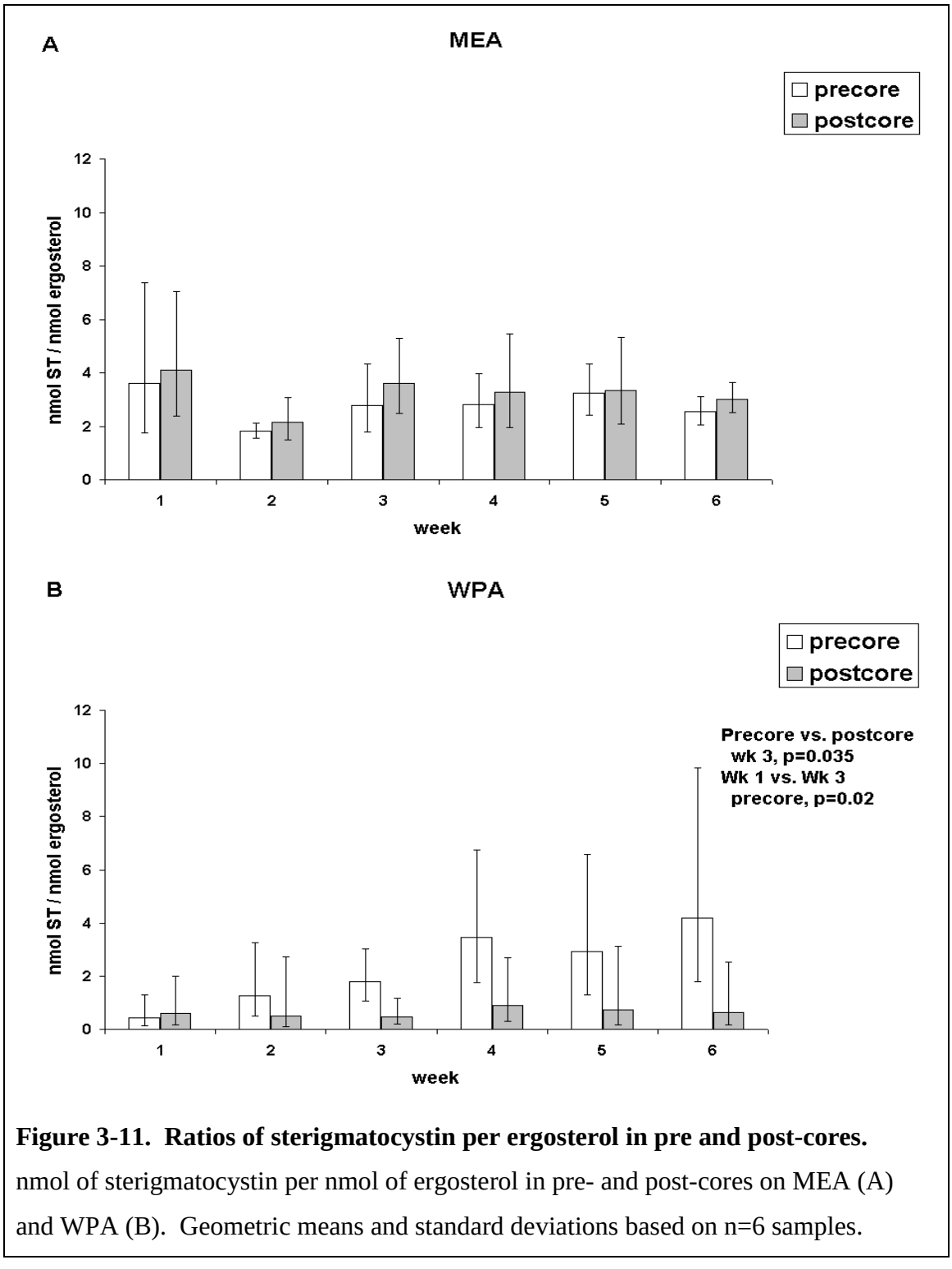


Figure 3-11. Ratios of sterigmatocystin per ergosterol in pre and post-cores. nmol of sterigmatocystin per nmol of ergosterol in pre- and post-cores on MEA (A) and WPA (B). Geometric means and standard deviations based on n=6 samples.

comparable. Statistically, the differences between the precore ratios on MEA and WPA were not significant for any of the weeks, except week 1 ($p=0.0007$) (Appendix A, Table 3-11-1). Ratios in the precores ranged from 1.8 to 3.6 on MEA and from 0.44 to 4.2 on WPA. Over the six weeks, however, the differences between the post-cores on MEA and WPA were statistically significant ($p=0.0002$).

Like ergosterol per cm^2 and stergimatocystin per cm^2on MEA, Figure 3-11 shows that ratios of ST per ergosterol in MEA precores did not change significantly with time (Appendix A, Table 3-11-2). However, ratios in the precores on WPA did increase with time (Appendix A, Table 3-11-3). Differences in the precore ratios between week 1 and weeks 3-6 on WPA were statistically significant. Differences over time in the postcore ratios were not significant on either media (Appendix A, Tables 3-11-4 and 3-11-5).

Precores were significantly different from postcores on WPA, but not MEA. After week 2, ratios in the post-cores on WPA were significantly lower than ratios for the pre-cores from the same week (Appendix A, Table 3-11-6). Post-core ratios on WPA ranged between 0.47 and 0.89, well below the pre-core ratios between 0.44 at week 1, up to 4.2 at week 6. On MEA, however, there was no statistically significant difference between pre-core and post-core ratios. Pre-core ratios ranged between 1.8 and 3.6, and post-core ratios ranged between 2.1 and 4.1.

An alternative analysis of these results is presented in Table 3-6. When toxin levels were standardized against fungal growth, the ratios in the MEA pre-cores were higher than the WPA pre-cores, but the differences were not statistically significant (Appendix A Table 3-11-1) at a p-value of 0.05. On MEA, post-core ratios were slightly

higher than MEA pre-core ratios. On WPA however, post-core ratios were much lower than WPA pre-core ratios. Post-core ratios from MEA plates were 5 times higher than post-core ratios from WPA plates (p=0.0002).

Table 3-6. Comparison of ratios: nmol sterigmatocystin (ST) / nmol ergosterol (erg). Ratios derived from geometric means and standard deviations calculated over six-weeks, based on n=36 samples. * p = 0.0002

	MEA (ST/erg)		WPA (ST/erg)		MEA/WPA ratio of
	avg.	**-/+ sd**	**avg.**	**-/+ sd**	**ST/erg**
pre-cores	2.79	1.77-4.22	1.86	0.63-5.56	1.60
post-cores	3.19	2.05-4.96	0.62	0.18-2.14	5.16*
20 µm filters	1.52	0.98-2.35	1.15	0.24-5.62	1.38
1 µm filters	1.16	0.82-1.66	1.65	0.36-7.49	0.69
Lyophilized wash water	0.72	0.49-1.06	3.85	1.35-11.03	0.18*

A closer examination of the removable fractions from the MEA plates (Figure 3-12) shows that the ratios of ST/ergosterol were lower than in the MEA cores (Figure 3-11). Ratios for the 20 µm filters ranged from 1.0 to 2.4, compared to the post-cores that ranged from 2.1 to 4.1 (p=0.0007, Appendix A Table 3-12-9). Ratios in the 1µm filters and wash samples were lower still, ranging from 0.8 to 1.4 on the filters (p=0.0002) and from 0.54 to 1.0 in the wash samples (p<0.0001).

In contrast, the ratios of sterigmatocystin to ergosterol in the removable fractions from the WPA plates (Figure 3-12) were significantly higher than in the post-cores (Figure 3-11). After week 1, ratios for the 20 µm filters ranged from 0.54 to 5.4, compared to the post-cores that ranged from 0.46 to 0.89 on WPA (p=0.0018, Appendix

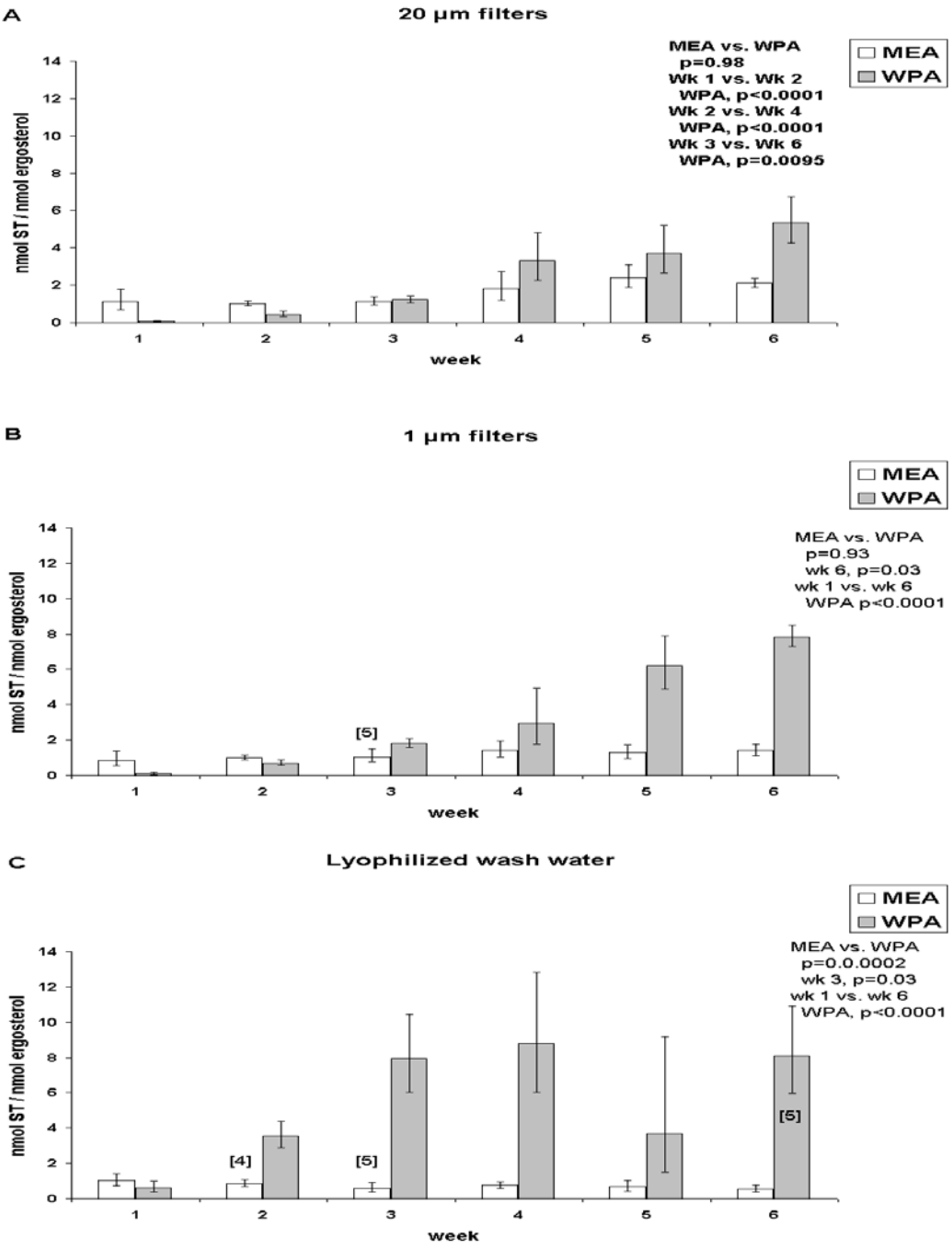


Figure 3-12. Ratios of sterigmatocystin per ergosterol in removable fractions. Bars represent nmol of sterigmatocystin per nmol ergosterol in 20 µm filters, 1 µm filters and lyophilized washwater on MEA (A) and WPA (B). Geometric means and standard deviations based on n=6 samples, except as noted [n].

A Table 3-12-12). The ratios on the 1 µm filters and wash samples were even higher. After week 1, ratios in the 1 µm filters from WPA plates ranged from 0.71 to 7.8 ($p<0.0001$, Appendix A Table 3-12-12), and ratios for the WPA wash water samples ranged between 0.6 and 8.8 ($p<0.0001$, Appendix A Table 3-12-12).

When standardized against ergosterol, Figure 3-12 shows that growth substrate produced significant differences in ST/ergosterol ratios in the wash water, but not in the 1 µm or the 20 µm filters (Appendix A, Table 3-12-1). Over the six week study period, WPA ratios in the wash water ranged from 0.6 to 8.8, but MEA ratios stayed at 1.0 or less ($p=0.0002$). At the end of the study period, WPA ratios were also higher than MEA ratios for the 1 µm and 20 µm fractions, but over the six weeks these differences were not statistically significant at a p-value of 0.05. Data in Table 3-6 also illustrate that particles on the 20 µm filters from the MEA plates were 1.38 times more toxin-rich than the particles on the 20 µm filters from the WPA plates. In contrast, particles on the 1 µm filters from MEA plates were less toxin-rich than the particles on the 1 µm filters from WPA plates. Neither of these comparisons, however, was statistically significant. The ST/ergosterol ratios in the WPA wash water were more than five-times higher than the ratios in the MEA wash water ($p=0.0002$, Appendix A Table 3-12-1).

Time had a significant effect on the ST/ergosterol ratios of the fractions removed from WPA plates, but not MEA plates (Figure 3-12). Over the six weeks, ratios did not increase in any of the fractions removed from MEA plates. Ratios increased steadily, however, for the 1 µm and 20 µm fractions removed from WPA plates. Although ratios in the WPA wash samples leveled off after week 4, the difference over six weeks for all

three removable fractions was statistically significant ($p<0.0001$, Appendix A Tables 3-12-5, 3-12-6 and 3-12-7).

With decreasing particle size, the ratio of sterigmatocystin to ergosterol increased on WPA plates, but decreased on MEA plates (Table 3-6). Although the differences were not statistically significant, particles on the 1 µm filters from WPA plates were 1.4 times (1.65/1.15) more toxin-rich than those on the 20 µm filters. The WPA wash water was 2.6 times more toxin-rich than particles on the 1 µm filters ($p<0.0001$, Appendix A Table 3-12-13). On MEA, particles on the 20 µm filters were slightly but not statistically significantly more toxin-rich than those on the 1 µm filters, and 2.1 times (1.52/0.72) more toxin rich than the MEA wash water ($p=0.0012$, Appendix A Table 3-12-10).

3.4 Discussion

The objectives of this set of experiments were to measure the effects of time and growth substrate on the distribution of sterigmatocystin in fixed and removable fungal material produced by *A. versicolor*. The hypothesis was that the toxin was not evenly distributed between fixed and removable fractions and that toxin distribution in those fractions would be affected by time and growth substrate.

3.4.1 Analytical methods

Filters were used to separate larger fungal particles from intact conidia (2-3 µm) and fragments (<1 µm). While we expected to find a certain amount of conidia and fragments on the 20 µm filter, and some fragments on the 1 µm filter, conidial counts before and after the 20 µm filter indicated that more than 50% of the WPA conidia and

more than 90% of the MEA conidia were trapped on that filter. The lower percentage of conidia from WPA plates on the 20 µm filter may be due to the lower average concentration of conidia in the WPA than the MEA slurry (9.4E+06 vs. 2.6E+08 conidia per mL, respectively). With almost 30 times more conidia, the MEA suspension may have plugged the filter, causing it to filter more efficiently and trap more of the smaller particles than the filter used for the less concentrated WPA suspension. Previous research indicates fungal colonies may release a high proportion of particles smaller than 1 µm in diameter (Górny and others 2001),and many of these particles may have been similarly caught on the 1 µm filter. Because particles of this size were not visible with our microscope, we could not use the hemocytometer to verify this hypothesis with counts before and after the 1 µm filter. Potential effects of this finding are discussed in the following sections.

In order to ensure our analytical methods were accounting for distribution of ergosterol and sterigmatocystin in the cores and removable fractions, levels of these compounds per unit area in the postcores and removable fractions were summed and compared with levels measured in the precores. Ergosterol sums from MEA plates were comparable to levels in the MEA precores. Sums ranged between 83% and 106% of the precores. On WPA, sums ranged between 106% and 155%, appearing to show that there was more ergosterol in the sums than in the pre-cores. However, none of the differences were statistically significant.

Part of the reason for this difference between the two substrates may have been the greater ‘removability’ of fungal material from WPA plates. Because a smaller

percentage (12.5-21%) of material washed off MEA plates than washed off WPA plates (34-57%), post-cores accounted for a greater share of the sum on MEA plates. On WPA, removable fractions often accounted for better than half the sum. This means that any variability in the analysis of those three removable fractions would contribute a proportionately larger amount of variability to the sum, compared to the removable fractions from MEA. In this case, the variability worked to increase the sums over the precores.

Substrate differences were also apparent in the mass balance analysis of sterigmatocysin. Again, sums from the MEA plates compared well with the MEA precores, with stergimatocystin sums ranging between 90% and 104% of the precores, and none of the differences being statistically significant. However, the sums from the WPA plates were slightly lower, between 70% and 132% of the precores, and the levels in the sums for weeks 3-6 were statistically lower than those in the precores. Some of the difference (11%) could be explained by residual toxin left on the glass beads used for washing the plates and the walls of the petri dish.

Like ergosterol, however, stergimatocystin differences between the sums and precores may be due primarily to the greater 'removability' of toxin from WPA as compared to MEA plates. In fact, on WPA, sterigmatocytin was even more 'removable' than ergosterol, with between 6.6% and 7.7% being removed from MEA plates, but between 50% and 78% being removed from WPA plates. Any losses due to sampling or analytical variation would tend to be amplified in the WPA results compared to the MEA results. It is unclear why the stergimatocystin sums were systematically less than the

precores, while the ergosterol sums were greater than the precores, but the difference may have to do with the efficiency of the analytical method.

3.4.2 Growth and removal of fungal material

Growth of *A. versicolor* was 56 times greater on MEA than WPA (Table 3-2). This finding supports the results of other researchers (Moularat and Robine 2008), who have demonstrated varying amounts of fungal growth on building materials such as glass fabric (fiberglass wallpaper), compressed fiberglass filters, paper wallpaper and vinyl wallpaper. Measured weekly, fungal growth was not significantly affected by time on either MEA or WPA (Figure 3-4). Changes between the weeks were not statistically significant, and indicated that fungal growth peaks relatively quickly, within 1-2 weeks, and then stabilizes. Time only slightly affected the amount of fungal material removed from MEA plates (20 µm increased then decreased), but not WPA plates (Figure 3-5). These results indicate that although a substrate in a building may be contaminated for a considerable length of time, fungal particles released from that substrate are unlikely to increase over time, assuming other environmental factors remain constant.

Previous research has shown the potential for substrate to influence the amount of fungal material removed from a substrate. Moving air caused higher numbers of fragments and spores to be released from ceiling tiles inoculated with *A. versicolor* compared to inoculated agar plates (Górny and others 2001). In our experiments also, substrate had a substantial effect on the amount of fungal material that was 'removable' from the different substrates. Although the total amount of material removed from the inoculated MEA plates was 15 to 33 times greater than that removed from WPA plates

(Table 3-2), this range is lower than the factor of 56 between the MEA and WPA precores. In fact, the percentage of material removed from the MEA plates, between 12.5% and 21%, was lower than the percentage of fungal material removed from the WPA plates, between 34% and 57%. In a building, therefore, even though one substrate might produce several times more growth than another, the amounts of fungal material removed from those substrates could be less disparate than the growth levels would indicate. More specifically, mold-resistant sheetrock might produce less growth than standard sheetrock, but may not necessarily release fewer fungal particles. The substrate which is less supportive of growth may lead to development of a colony which is structurally weaker and more likely to be removed by environmental changes.

It is not clear, however, that substrate affects the size of the removable fungal particles. More of the particles from MEA than WPA plates (69% vs. 62%) were trapped on the 20 μm filter (Table 3-3). However, many of the particles on the 20 μm filter, particularly from the MEA plates, may be smaller (2-3 μm), intact conidia. We cannot then be sure that a greater percentage of the particles removed from MEA plates are large and likely to deposit quickly on surfaces inside a building. Nor, conversely, can we be sure that a greater percentage of particles removed from WPA plates were respirable compared to those removed from MEA plates (38% and 31%, respectively). However, we can conclude that at least 38% of the particles removed from WPA plates fall into the 'respirable' size range as do at least 31% of the particles removed from MEA plates.

From an exposure assessment standpoint, respirable particles are of greater concern. Respirable particles are likely to stay airborne longer and, therefore, pose a

greater exposure risk. If inhaled, they are more likely to travel into the lower airways of the lungs. If they deposit in the lower airways and alveoli, they will be more difficult to clear than those depositing in the upper airways. They will have more contact time with surrounding tissue and may be able to exert more significant biological effects.

3.4.3 Toxin production and distribution

Toxin concentrations in the precores indicated that average production of stergimatocystin was 79 times greater on MEA than on WPA (Table 3-4). This finding supports the results of other researchers (Moularat and Robine 2008), who have demonstrated varying amounts of toxin production on building materials such as glass fabric (fiberglass wallpaper), compressed fiberglass filters, paper wallpaper and vinyl wallpaper. With time, stergimatocystin concentrations in the pre-cores remained steady on MEA, but increased significantly on WPA (Figure 3-8). These results indicate that in a building, although fungal contamination on water-damaged building surfaces may not increase with time, the toxin content of that fungal material may continue to increase.

Like ergosterol, 'removable' stergimatocystin was also affected by substrate and time. Previous research had indicated species differences in toxin-removability from inoculated rice (Palmgren and Lee 1986). Toxin from *Aspergillus parasiticus* and *Penicillium oxalicum* remained mostly in the rice-hyphae matrix, whereas the conidia removed with air contained most of the toxin from *Aspergillus niger* and *Aspergillus fumigatus*. Our experiments showed the influence of substrate on removability within the same fungal species.

Although between 15 and 33 times more fungal material was removable from MEA than from WPA plates (Table 3-2), the difference in removable stergimatocystin was much closer. Only between 7.8 and 10.5 times more stergimatocystin was removable from MEA plates than from WPA plates (Table 3-4). This means than on a percentage basis, substrate affects removability of toxin even more than fungal material. In a building, therefore, one water-damaged substrate might produce several times more growth than another. However, the amounts of toxin removed from those substrates by environmental forces such as air or vibration could be much more comparable.

With time, stergimatocystin concentrations in the removable fractions from the WPA plates increased, but weekly differences in removable fraction from the MEA plates were not statistically significant (Figure 3-9). Overall, average stergimatocystin levels were approximately 8 to 10 times greater in removable particles from MEA plates than WPA plates. However, due to uncertainty about the distribution of intact conidia and fragments between the 'removable' fractions, it is more difficult to draw conclusions about the effects on substrate on toxin levels in conidia and fragments.

The data in Figure 3-9 show that by week 6, sterigmatocystin in the 20 µm fraction on MEA was almost four times greater than that on WPA (3.2 nmol / 0.8 nmol), but differences between the substrates in the wash water (0.44 nmol / 0.25 nmol), and 1 µm fractions (0.36 nmol / 0.36 nmol) were not statistically significant. More than 90% of the MEA conidia and more than 50% of the WPA conidia, however, were trapped in the 20 µm fractions. Therefore, toxin associated with conidia and fragments, especially from MEA, may be greater than levels indicated by the 1 µm and wash water fractions. Based

on data presented in Table 3-5, however, we can conclude that at least 19% and 56% of toxin removed from MEA and WPA plates, respectively, is associated with respirable-sized particles.

3.4.4 Ratios of sterigmatocystin / ergosterol

Another way of analyzing this data is by considering the ratio of the nmols of sterigmatocystin divided by the nmols of ergosterol detected in the same sample. The ratios in the precores show that on average, substrate has little effect on the amount of toxin produced by a given amount of fungal material. Precore ratios for MEA and WPA were 2.79 and 1.86, respectively (Table 3-6).

However, the ratios also show that substrate has a significant effect on where the toxin distributes within the fungus. On MEA, the average sterigmatocystin / ergosterol ratio was highest in the postcores (3.25). Ratios dropped progressively from the 20 µm filters (1.61), to the 1 µm filters (1.18), and were lowest in the lyophilized wash water samples (0.74). These results indicate that on MEA, sterigmatocystin concentrates in the fixed fraction of the fungus that is not removed from the plate. The toxin is progressively less concentrated in the largest removable particles, and is least concentrated in the wash water particles. We know there are many conidia and perhaps fragments on the 20 µm filters, and it is likely there are fragments on the 1 µm filters. If these particles were more efficiently size-separated, it is likely that in this case, toxin to ergosterol ratios for the 20 µm and the 1 µm filters would increase.

On WPA, the order of toxin distribution is reversed. The average ratio of sterigmatocystin / ergosterol is highest in the wash water fraction (3.85), and decreases

progressively through the 1 µm filters (1.65) and the 20 µm filters (1.15), until reaching the lowest point in the post-cores (0.62). Again, if particles were more efficiently size-separated, it is likely that ratios for the 1 µm and 20 µm filters would decrease. Overall however, these results indicate that on WPA the smallest particles are the most toxin-rich, and on average they contain more than 5 times more sterigmatocystin than the same size particles from MEA (3.85/0.72). When considering potential exposures to such particles, it is unclear which is more hazardous: exposure to more numerous, but less toxin-rich particles such as those from MEA, or exposure to fewer, but more toxin-laden particles such as those from WPA.

Finally, time had a significant effect on the sterigmatocystin / ergosterol ratios from WPA, but not MEA plates (Figures 3-10 and 3-11). Ratios for the cores and removable fractions on MEA did not change significantly with time, but ratios of the precores and removable fractions on WPA all increased from week 1 to week 6. In an indoor environment with water damage, fungal particle counts may not increase with time, but the toxin content of those particles could increase with time. Importantly, the smallest of the respirable particles could be the most toxin-rich and due to their size, the most likely to deposit in the lowest reaches of the respiratory system.

Chapter 4 – Sterigmatocystin in wash water – particulate-associated or dissolved?

4.1 Introduction

Results from the previous section showed the presence of both sterigmatocystin and ergosterol in the wash water. Because most sources list these compounds as 'insoluble' in water, and no published solubilities were located, it could be assumed that the ergosterol is from small fragments that passed through the 1 µm filter and that the sterigmatocystin is associated with these small fragments. However, another possibility is that these compounds dissolved in the wash water and were not associated with particulates at all. We used dynamic light scattering and scanning electron microscopy to look for particles in the wash water itself, and in the lyophilized material from the wash water. We also experimented to derive the solubility of stergimatocystin in water.

4.2 Materials and methods

4.2.1 Electron microscopy

Initial examination of the wash water was carried out in the College of Science and Engineering's Characterization Facility at the University of Minnesota, which receives partial support from National Science Foundation through the Materials Research Science and Engineering Center program. A drop or two of the wash water was

placed on a mount, dried and stained with uranyl acetate and then examined by transmission electron microscopy (TEM).

Subsequently, lyophilized material from the wash water samples was analyzed by scanning electron microscopy (SEM) at the College of Biological Science's Imaging Center. Working inside a biological safety cabinet to ensure protection from the solid material, small amounts of the lyophilized material from WPA and MEA samples and controls were mounted on aluminum stubs using double-sided carbon adhesive tabs. The material was sputter coated with gold-palladium, and observed under a Hitachi S3500N scanning electron microscope with an accelerating voltage of 5 kV.

4.2.2 Dynamic light scattering (DLS)

Approximately 1 mL of the double-filtered wash water solutions were passed through 0.45 μm syringe filters (Millipore) into 0.5-in.-diameter optical glass tubes that were scrupulously dusted. Researchers in Dr. Timothy P. Lodge's lab in the Department of Chemistry at the University Of Minnesota made DLS measurements of these solutions. The measurements were carried out at 25^{o}C using a custom-built photometer equipped with an electrically heated silicon oil bath, a Lexel 75 Ar+ laser operating at 488 nm, a Brookhaven BI-DS photomultiplier, and a Brookhaven BI-9000 correlator (Liu, Hillmyer, Lodge 2009). The intensity autocorrelation functions, g2(t), were recorded at three angles ranging from 60 to 120 degrees with respect to the laser beam direction, and accepted when the baseline differences (calculated vs measured) were less than 0.1%. A double exponential expression (eq 1) was used to fit the autocorrelation functions to extract the decay rates, Γ_1 and Γ_2.

$$g_2(t) - 1 = [A_1 \exp(-\Gamma_1 t) + A_2 \exp(-\Gamma_2 t)]^2 \qquad \text{(eq. 1)}$$

After obtaining Γ_1 and Γ_2 at different scattering angles, two mutual diffusion coefficients D_m were determined by linearly fitting Γ_1 and Γ_2 versus q^2, where $q = (4\pi n/\lambda)\sin(\theta/2)$ and n, λ, and θ are the solvent refractive index, laser wavelength, and scattering angle, respectively. The hydrodynamic radius (R_h) was obtained from the Stokes-Einstein equation (eq 2), where k_B, T, and η_s are the Boltzmann constant, absolute

$$R_h = k_B T/6\pi\eta_s D_m \qquad \text{(eq. 2)}$$

temperature, and solvent viscosity, respectively. Inverse Laplace transforms were also performed using constrained regularization program REPES in order to obtain the decay rate distributions, which reflect the size distribution of the particles.

4.2.3 Sterigmatocystin Solubility

On WPA, sterigmatocystin levels in the pre-cores increased through week 6 (Figure 3-8). Maximums measured in the pre-cores at week 6 were 2 nmol per cm^2. Because the sample was split for ergosterol analysis, the plate actually contained 2 x 2 nmol/cm^2 x 48.33 cm^2 = 193 nmol. We used 24 mL of sterile deionized water to wash the plates, meaning that if all the sterigmatocystin dissolved, there would have been a maximum of 193 nmol / 24 mL = 8.05 nmol / mL. On MEA, sterigmatocystin levels in the pre-cores over the six weeks increased and then decreased (Figure 3-8). Average levels were measured at 105 nmol / cm^2. If all this toxin had dissolved, concentrations in the wash water would have been 2 x 105 nmol/cm2 x 48.33 cm2 / 24 mL = 423 nmol / mL.

For the solubility test, an intermediate level of 23 nmol of sterigmatocystin / mL of wash water (140 nmol / 6 mL) was used. Working with the stock solution of sterigmatocytin in ethanol, 140 nmol (210 µL of 0.67 mM sterigmatocystin) were placed in each of two centrifuge tubes. The ethanol volume was reduced under a stream of nitrogen, and then 6 mL of MilliQ deionized water was added to each tube. At this point there was no visible precipitate. The tubes were then shaken for 15 minutes on a shaker a low speed.

The tubes were spun for 10 minutes at 7000 rpm (12,000 g) and afterwards precipitate was visible in the bottom of the tubes. The top 3 mL of water from each tube was transferred to a clean glass tube for further processing, taking care not to disturb the solid at the bottom. The 3 mL of water were then frozen and lyophilized. When dry, 3.25 mL of methanol was added to each tube and the tubes were then sonicated for 15 minutes, vortexed for 15 seconds, filtered through a 1.4 µm Millipore filter into a new glass tube, and finally transferred into a 4 mL amber glass vial. The vials were taken to dryness on a speedvac and the residues brought up in 0.5 mL of HPLC-grade methanol. Two injections of each sample, ranging from 10 to 100 µL, were analyzed by HPLC according to the methods detailed in section 3.2.6.

4.3 Results

4.3.1 Electron microscopy

Examination of the wash water with TEM showed no detectable particles in either MEA or WPA samples. The lyophilized material examined by SEM is pictured in Figure

4-1. The WPA sample was a sticky yellow material, and was not amenable to analysis by SEM. The microscopist noted that the filamentous structures visible in the three samples

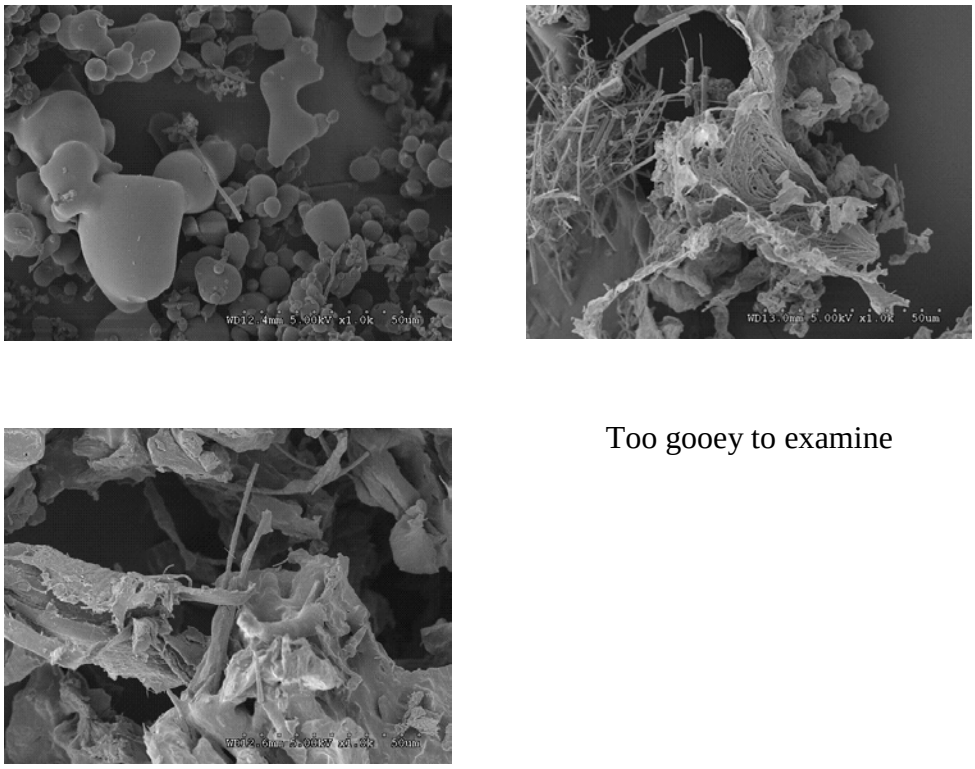


Figure 4-1. SEM photographs of lyophilized material from wash water samples. Magnification for all pictures 1,000x.

looked too regular and too long to be hyphal fragments that made it through the 1-micron filter, and speculated that the fiberglass filter may have been the source.

4.3.2 Dynamic light scattering (DLS)

The DLS results in Table 4-1 appear to indicate that there were 1.36 times more particles in the MEA wash water sample than in the control, and 28.4 times more

particles in the WPA wash water sample than in the control. The diameter of the particles in the WPA sample (cumulant R_h) appears larger than the diameter of particles in the WPA control (225 nm and 143 nm, respectively). Particles in the MEA sample, however, appear to have smaller diameters than particles in the MEA control (137 nm and 195 nm, respectively).

Table 4-1. DLS results. The cumulant R_h indicates the average size (diameter in nm) of the particles in the liquid. The double R_h (nm) indicates particle length and width. The average intensity indicates the number of particles in the liquid.

Sample ID	Average Intensity (kcp)	Cumulant R_h (nm)	Double R_h (nm)
WPA Control	15.4	143	37/262
WPA	437.3	225	67/425
MEA Control	35.0	195	N/A
MEA	47.7	137	48/205

4.3.3 Sterigmatocystin solubility

Results from the four injections produced an average sterigmatocystin solubility of 0.95 nmol/mL. Although 24 mL of water were used to wash the inoculated plates, after filtration through the 20 and 1 µm filters, about 20 mL were usually collected for lyophilization. Because the samples were ultimately split for toxin and ergosterol analysis, dissolved sterigmatocystin could account for 0.95 nmol/mL x 10 mL = 9.5 nmol of sterigmatocystin in each wash water sample. When standardized against the area of the plate, this would account for 0.20 nmol/cm^2.

4.4 Discussion

The TEM analysis of the filtered wash water samples and the SEM analysis of the lyophilized wash water samples did not help to clarify whether there were particulates in the wash water samples. The wash water may have been too dilute to see the expected fragments. Particles were visualized with the SEM analysis, but appeared to be other than fungal and possibly fiberglass fibers from the 1µm glass fiber filter (Pall Type A/B 47 mm Extra Thick) used to separate conidia from the wash water.

Results from the dynamic light scattering analysis of the wash water also were not conclusive. The analysis was run only on a single sample, and the differences were not great enough to make definitive determinations regarding particle size or number. Again, concentrating the sample may increase chances of detecting differences between the samples and controls.

By weeks 5 and 6, sterigmatocystin was detected in the wash water from the WPA plates at 0.23 and 0.24 nmol/cm^2, respectively (Figure 3-9). Average levels in wash water from the MEA plates were higher at approximately 0.84 nmol/cm^2. The solubility calculated in section 4.3.3 would account for almost all the toxin in the WPA wash water (0.20 nmol/cm^2 vs. 0.23 nmol/cm^2 measured), and for approximately 25% of the toxin in the MEA wash water. It is possible, however, that centrifuging did not remove all the undissolved sterigmatocystin. Some stergimatocystin particles, too small to be visible, may have been transferred with the 3 mL of supernatant from the centrifuged sterigmatocystin suspension, and may have accounted for at least part of the detected toxin. If the experiment were repeated, filtering through a 1 µm filter would

help ensure that any small particles are removed. At this point, it is not possible to determine whether the toxin in the wash water is dissolved or particulate-bound.

Chapter 5 – Conclusions.

The hypothesis of this research is that time and growth substrate affect the amount and distribution of mycotoxins in the fungal particles released from fungal colonies. This topic is important, because exposure to mycotoxins associated with small fungal fragments may cause adverse health effects. Understanding how time and substrate affect the distribution of toxins within fungal structures, and their release into the environment can help us better estimate potential risks of exposure to these toxin-carrying particles. The following paragraphs provide a brief review of the knowledge on this topic before and after this research.

5.1 What was known previously

Fungal particles are part of the outdoor and indoor environment. In a building with water damage, molds may colonize wet organic materials. Environmental factors such as air currents or vibration may cause fungal colonies to release particles to the indoor environment. A large proportion of these particles are likely to be sub-micron fragments (Górny and others 2002) which can outnumber intact particles by two orders of magnitude. Such fragments are not detectable with current viable- or total particulate-samplers. Particles released from moldy sheetrock or ventilation ductwork could directly expose building occupants to fine fungal fragments. In addition, laboratory studies have shown that air pressure differences can cause fine particles to migrate through slits between compartments (Mosley and others). This indicates that exposure could also occur due to fragments moving from inaccessible spaces such as damp wall cavities or

crawl spaces through small openings around lights, utility lines or electrical outlets into occupied spaces.

Occupants of moldy buildings often report symptoms such as eye, nose and throat irritation and headaches, although these symptoms have not been causally linked to mold exposures. However, biological effects from exposure to fungal particles and their associated compounds have been documented in cell culture and animals. Extracts of agars inoculated with *Aspergillus spp.* and *Penicillium* were toxic to various cells (Bünger and others 2004). Primary and secondary fungal metabolites can act as irritants, allergens, toxins or even carcinogens (Douwes and others 1996)(Jussila and others 2002), (Seo and others 2008) (WHO-IARC 1976)(Purchase and Van Der Watt, J. J. 1970). The primary metabolites include chitins, glucans and ergosterol. The secondary metabolites such as mycotoxins are species-specific. In some cases, toxins may be detoxified by formation of glucuro-conjugates or glutathione adducts (Slominski and others 2005).

The location of secondary metabolites in fungal structures is variable by species and substrate. Some mycotoxins may locate primarily in the hyphae, others in the conidia, and still others may be secreted outside the fungal structure into the growth substrate. There was conflicting information about whether stergimatocystin was primarily intracellular (Filtenborg, Frisvad, Svendsen 1983), or whether it could be extracellular (Piecková and Kunová 2002). Various building materials inoculated with *A. versicolor* were assessed for mycotoxin content (Moularat and Robine 2008). Some substrates produced fungal growth but no stergimatocystin, while others produced less fungal growth, but abundant stergimatocystin. The toxin was also detected in aerosols

collected from the various substrates, and in all size fractions (inhalable, thoracic and alveolar) from a single substrate).

When building occupants inhale airborne fungal fragments, where those particles deposit in the lungs depends on several factors including the size and shape of the particle, and breathing pattern and air flow rate in the lungs. In general, as particles decrease in size, they are likely to move deeper into the lungs. Very small particles are considered 'respirable', meaning they can reach the lowest branches of the lungs. Particles with diameters smaller than 0.5 μm may eventually deposit by diffusion in the alveoli. Subsets of the population may be more at risk of adverse health effects from exposure to these particles. Respiratory deposition models predict that, due breathing pattern differences, deposition rates in the lower airways of children may be 4-5 time greater than deposition rates in adults (Cho and others 2005).

The body has protective mechanisms to clear material such as fungal particles. Some may be removed entirely from the body by coughing or nose blowing. Sometimes, clearance mechanisms move the particles to the stomach and gastrointestinal tract, the lymphatics and lymph nodes, or the pulmonary vasculature. For example, cilia under the mucous in the tracheobronchial region move in concert to bring particles up to where they can be swallowed. In the alveolar region, where there are no cilia, macrophages may phagocytize the particles. The macrophages can then be cleared by either lymphatic drainage or movement on the alveolar fluid layer to the mucociliary escalator.

The body may also detoxify fungal metabolites associated with the particles. For example, incubation of stergimatocystin with porcine tracheal epithelial cells produced a

glucuronide-conjugate (Slominski and others 2005). Such conjugates make the compounds more water soluble which helps to eliminate them from the body more quickly. Similarly, ergosterol can be metabolized to the more water-soluble 17α,24-dihydroxyergosterol by P450 enzymes (Slominski and others 2005). There are also indications this metabolite has antiproliferative and anticarcinogenic properties. However, in an *in vitro* chick trachea model, sterigmatocystin has been shown to inhibit the movement of cilia (Jesenska and Bernat 1994). Such inhibition could reduce the clearance of toxin-laden particles by the mucociliary escalator, allow more contact time with surrounding tissue and increase the potential for adverse health effects.

5.2 What is known now due to this research

Research presented in Chapter 2 supports previous research indicating that fungal extracts are toxic to cells *in vitro*. Extracts of *Aspergillus spp.* and *Penicillium* cultured on YES were toxic to various cells (Bünger and others 2004), and not all the toxicity could be accounted for by the presence of known toxins such as sterigmatocystin. Similarly, data presented in Chapter 2 indicated that extracts of *A. versicolor* grown on MEA and CYA are toxic to MP48s, an immortalized pre-type II alveolar epithelial cell line. These experiments also indicated, however, that the substrate could affect toxicity, as extracts from MEA were more toxic to the MP48s that CYA extracts. After 5 days, the stergimatocystin concentrations of the extracts could account for the toxicity to the cells, but in contrast to the previous research, at 19 days, even with increased stergimatocystin concentrations, the toxicity of the fungal extracts were less than expected. It is possible that other metabolites produced by the fungal culture

overwhelmed the ability of the P450 enzymes to activate the sterigmatocystin to its more toxic form.

The research described in Chapter 3 supports previous research (Brasel and others 2005a) that showed mycotoxins could be found on fungal fragments. Our experiments go beyond previous research to show that the substrate affects the removability of these compounds. Although more total stergimatocystin and ergosterol were 'removable' from inoculated MEA than WPA plates, greater percentages of the fungal material and toxin were removed from WPA plates than from MEA plates.

The percentage of sterigmatocystin removed from WPA plates was greater than the percentage of ergosterol, producing ST/ergosterol ratios in the removable fractions greater than 1. Previous research also has shown varying amounts of toxin and ergosterol produced in mold growing on building materials (Moularat and Robine 2008). Findings detailed in Chapter 3 show that, in addition, the toxin to ergosterol ratios in removable fractions vary by particle size, by substrate and by time. Ratios for removable fractions from MEA plates were less than 1 for the wash water fraction, close to 1 for the 1 µm filter fraction, and approximately 1.5 for the 20 µm filter fraction. In contrast, ratios for removable fractions from WPA plates were all greater than 1, and unlike the MEA ratios, they increased with time. After six weeks, ratios peaked above 5 in the 20 µm fraction, close to 8 in the 1 µm fraction and slightly above 8 in the wash water fraction.

5.3 Implications

Indoor building materials can be limited in nutrients and overall poor growth substrates for fungal colonization. As shown in the previous section, fungal growth that does occur on such substrates could be high in toxin content and a large percentage of that toxin-containing material may be removable from the substrate. The particles may span a ranges of sizes, but more numerous, smaller fragments are likely to contain higher concentrations of toxins. Building occupants inhaling these toxin-rich, fungal particles could be exposed to the associated primary and secondary metabolites and experience a variety of health effects.

Inhaled fungal particles could deposit throughout the respiratory tract. Larger particles deposited in the upper respiratory system may be removed by coughing, sneezing or ingestion of material moved by the mucociliary escalator. Previous research, however, indicates that sterigmatocystin inhibits the movement of cilia in an *in vitro* chick trachea model (Jesenska and Bernat 1994). Such effects may slow clearance of fungal particles and extend the duration of exposure to fungal metabolites.

Smaller particles could deposit throughout the respiratory system, but unlike large particles, they are small enough to reach the deepest branches and alveoli of the lungs. Although porcine tracheal epithelial cells were able to detoxify sterigmatocystin by producing a sulfo- or glucuronide-conjugates (Slominski and others 2005), cells in the alveoli may not. In fact, the MP48 cells tested in Chapter 2 are pre-type II alveolar epithelial cells which showed significant toxicity to sterigmatocystin when incubated with S9. Because fine particles deposited in the nasopharyngeal region may translocate

to the central nervous system via the olfactory nerve (Oberdörster and others 2004), translocation of fine fungal particles carrying mycotoxins could potentially affect the nervous system also.

5.4 Future research

To help advance the research described in the previous paragraphs, future research should focus on the areas described below.

5.4.1 Solubility

Solubility studies for sterigmatocystin should be repeated, and studies for ergosterol solubility should be added. The studies should have more replicates and supernatant removed from the centrifuged sample should be filtered through a 1 µm filter or smaller to remove any fine, undissolved particles of toxin before analysis. Deriving more accurate numbers for both sterigmatocystin and ergosterol solubility will help to better determine whether the detection of these compounds in wash water is due to the presence of fungal fragments, or dissolved compounds.

5.4.2 Particles in wash water

The nature of the sterigmatocystin and ergosterol in the wash water needs to be defined. Electron microscopy was not initially successful, but if the samples were first concentrated to 1 mL or less, chances to see fungal particles would be increased by at least a factor of 20. The TEM analysis could be repeated by first concentrating the wash sample, then possibly using cryo TEM (freeze and slice sections of wash water for analysis). SEM analysis has more inherent exposure risks for the researchers and

analysts, since the toxin-containing lyophilized material has to be manipulated and placed on the mounting stage. The SEM pictures also showed structures in the controls that in the samples, appeared to get coated with a substance: potentially toxin. TEM analysis of the wash water may be a safer way to look for fungal particles.

5.4.3 Toxicity of extracts of separated fractions

In Chapter 2, the fungal extracts tested for toxicity on the MP48 cells were from inoculated filters. Like a pre-core sample, the fungal material on the inoculated filter included all fungal structures. However, the experiments described in Chapter 3 separated the fungal material into fixed (post-cores) and size-separated, removable fractions (20 µm filter, 1 µm filter and lyophilized wash water). Results showed differing levels of toxins in each of these fractions with ratios of sterigmatocystin/ergosterol highest in the post-cores on MEA, but highest in the lyophilized wash water on WPA. Each of these compounds are markers for other primary and secondary metabolites produced by the fungus, and other metabolites could certainly be present in the extracts. Testing those extracts on the MP48 cells would give a more complete picture of potential hazards of exposure to these fractions.

5.4.4 Factors affecting particle release

Health risk is a combination of exposure potential and toxicity of the compound of concern. The previous sections detail research to better characterize the nature of the fungal toxins and their potential toxicity. We would also like to better define the potential for exposure to these fungal particles by investigating what triggers release from

the substrate. Previous research indicates that fungal particles are released in bursts to the indoor atmosphere, that many of the particles are too small to be detected with current sampling instruments and that these particles are associated with "sick building syndrome". However, probably due to poor exposure assessment methods, researchers have been hard-pressed to tie exposures to documented health effects.

To fill this gap, researchers could inoculate plates of wall paper agar and building materials such as sheet rock and incubate them under controlled temperature, humidity, light and air movement conditions that simulate indoor environments (damp wall cavities or rooms in homes or offices). The controlled laboratory environment would be continuously monitored for release of particles, and collected particles could be size-separated, counted, and evaluated for ergosterol and toxin content. With this set up, particle size, release rate and toxin content could be measured, and the effects of environmental parameters such as temperature, humidity, light and age of culture on these parameters could be evaluated. This experiment would be designed to capture fungal fragments that may be released only intermittently, and could therefore help elucidate why previous exposure sampling has not been well correlated with reported health effects. Eventually, contaminated indoor environments could also be monitored for particles and toxins in a similar fashion.

5.4.5 Biomarkers

Finding good biomarkers for fungal exposure would improve our ability to document exposures and detect health effects associated with exposure. If we focused on toxins such as sterigmatocystin, DNA adducts with the N^7 atom of guanine are a

possibility (Essigmann and others 1979). However, other markers such as monohydroxy-sterigmatocystin, dihydroxy-sterigmatocystin, glutathione adducts, or glucuronide- or sulfo-conjugates are also possible (Slominski and others 2005). Secondary effects, such as increases in mRNA levels of P450s could also be monitored. However, if we focus on ergosterol, biomarkers such as 17α,24-dihydroxyergosterol are possible (Slominski and others 2005).

5.4.6 Summary

Completion of studies in these areas will better characterize the toxicity, hazard potential and exposure potential for the fungal particles. Other common species of indoor mold could also be tested in this system. More data in these areas will enable researchers to better characterize the health risks associated with exposure to fungal particles.

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Appendix A

Table 3-4-1. Ergosterol /cm^2 in pre- and post-cores – media comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	**MEA**	1-6		precore	**WPA**	1-6	**<.0001**
precore	**MEA**	1		precore	**WPA**	1	**<.0001**
precore	**MEA**	2		precore	**WPA**	2	**<.0001**
precore	**MEA**	3		precore	**WPA**	3	**<.0001**
precore	**MEA**	4		precore	**WPA**	4	**<.0001**
precore	**MEA**	5		precore	**WPA**	5	**<.0001**
precore	**MEA**	6		precore	**WPA**	6	**<.0001**
post core	**MEA**	1-6		post core	**WPA**	1-6	**<.0001**
post core	**MEA**	1		post core	**WPA**	1	**<.0001**
post core	**MEA**	2		post core	**WPA**	2	**<.0001**
post core	**MEA**	3		post core	**WPA**	3	**<.0001**
post core	**MEA**	4		post core	**WPA**	4	**<.0001**
post core	**MEA**	5		post core	**WPA**	5	**<.0001**
post core	**MEA**	6		post core	**WPA**	6	**<.0001**

Table 3-4-2. Ergosterol /cm^2 in pre-cores – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	MEA	**1**		precore	MEA	**2**	0.5439
precore	MEA	**1**		precore	MEA	**3**	0.9856
precore	MEA	**1**		precore	MEA	**4**	0.8529
precore	MEA	**1**		precore	MEA	**5**	1
precore	MEA	**1**		precore	MEA	**6**	0.9967
precore	MEA	**2**		precore	MEA	**3**	1
precore	MEA	**2**		precore	MEA	**4**	1
precore	MEA	**2**		precore	MEA	**5**	1
precore	MEA	**2**		precore	MEA	**6**	1
precore	MEA	**3**		precore	MEA	**4**	1
precore	MEA	**3**		precore	MEA	**5**	1
precore	MEA	**3**		precore	MEA	**6**	1
precore	MEA	**4**		precore	MEA	**5**	1
precore	MEA	**4**		precore	MEA	**6**	1
precore	MEA	**5**		precore	MEA	**6**	1

Table 3-4-3. Ergosterol /cm^2 in pre-cores – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	WPA	**1**		precore	WPA	**2**	1
precore	WPA	**1**		precore	WPA	**3**	0.9625
precore	WPA	**1**		precore	WPA	**4**	1
precore	WPA	**1**		precore	WPA	**5**	0.9692
precore	WPA	**1**		precore	WPA	**6**	0.9997
precore	WPA	**2**		precore	WPA	**3**	1
precore	WPA	**2**		precore	WPA	**4**	1
precore	WPA	**2**		precore	WPA	**5**	1
precore	WPA	**2**		precore	WPA	**6**	1
precore	WPA	**3**		precore	WPA	**4**	1
precore	WPA	**3**		precore	WPA	**5**	1
precore	WPA	**3**		precore	WPA	**6**	1
precore	WPA	**4**		precore	WPA	**5**	1
precore	WPA	**4**		precore	WPA	**6**	1
precore	WPA	**5**		precore	WPA	**6**	1

Table 3-4-4. Ergosterol /cm^2 in post-cores – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
post core	MEA	**1**		post core	MEA	**2**	0.8012
post core	MEA	**1**		post core	MEA	**3**	1
post core	MEA	**1**		post core	MEA	**4**	0.9006
post core	MEA	**1**		post core	MEA	**5**	0.9979
post core	MEA	**1**		post core	MEA	**6**	1
post core	MEA	**2**		post core	MEA	**3**	1
post core	MEA	**2**		post core	MEA	**4**	1
post core	MEA	**2**		post core	MEA	**5**	1
post core	MEA	**2**		post core	MEA	**6**	1
post core	MEA	**3**		post core	MEA	**4**	1
post core	MEA	**3**		post core	MEA	**5**	1
post core	MEA	**3**		post core	MEA	**6**	1
post core	MEA	**4**		post core	MEA	**5**	1
post core	MEA	**4**		post core	MEA	**6**	1
post core	MEA	**5**		post core	MEA	**6**	1

Table 3-4-5. Ergosterol /cm^2 in post-cores – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
post core	WPA	**1**		post core	WPA	**2**	0.9935
post core	WPA	**1**		post core	WPA	**3**	1
post core	WPA	**1**		post core	WPA	**4**	0.9936
post core	WPA	**1**		post core	WPA	**5**	1
post core	WPA	**1**		post core	WPA	**6**	0.9622
post core	WPA	**2**		post core	WPA	**3**	1
post core	WPA	**2**		post core	WPA	**4**	1
post core	WPA	**2**		post core	WPA	**5**	0.9996
post core	WPA	**2**		post core	WPA	**6**	1
post core	WPA	**3**		post core	WPA	**4**	1
post core	WPA	**3**		post core	WPA	**5**	1
post core	WPA	**3**		post core	WPA	**6**	1
post core	WPA	**4**		post core	WPA	**5**	0.9996
post core	WPA	**4**		post core	WPA	**6**	1
post core	WPA	**5**		post core	WPA	**6**	0.9949

Table 3-4-6. Ergosterol /cm^2 in pre- and post-cores – fraction comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
post core	MEA	1-6		**precore**	MEA	1-6	0.3323
post core	MEA	1		**precore**	MEA	1	1
post core	MEA	2		**precore**	MEA	2	1
post core	MEA	3		**precore**	MEA	3	0.9999
post core	MEA	4		**precore**	MEA	4	1
post core	MEA	5		**precore**	MEA	5	1
post core	MEA	6		**precore**	MEA	6	1
post core	WPA	1-6		**precore**	WPA	1-6	**0.0136**
post core	WPA	1		**precore**	WPA	1	1
post core	WPA	2		**precore**	WPA	2	1
post core	WPA	3		**precore**	WPA	3	0.7821
post core	WPA	4		**precore**	WPA	4	1
post core	WPA	5		**precore**	WPA	5	0.2146
post core	WPA	6		**precore**	WPA	6	1

Table 3-5-1 Ergosterol /cm^2 in removable fractions – media comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	**MEA**	1-6		20 u	**WPA**	1-6	**<.0001**
20 u	**MEA**	1		20 u	**WPA**	1	**<.0001**
20 u	**MEA**	2		20 u	**WPA**	2	**<.0001**
20 u	**MEA**	3		20 u	**WPA**	3	**<.0001**
20 u	**MEA**	4		20 u	**WPA**	4	**<.0001**
20 u	**MEA**	5		20 u	**WPA**	5	**<.0001**
20 u	**MEA**	6		20 u	**WPA**	6	**<.0001**
1 u	**MEA**	1-6		1 u	**WPA**	1-6	**0.0011**
1 u	**MEA**	1		1 u	**WPA**	1	**0.0118**
1 u	**MEA**	2		1 u	**WPA**	2	0.0817
1 u	**MEA**	3		1 u	**WPA**	3	0.7901
1 u	**MEA**	4		1 u	**WPA**	4	0.3635
1 u	**MEA**	5		1 u	**WPA**	5	**0.0046**
1 u	**MEA**	6		1 u	**WPA**	6	**0.0023**
wash	**MEA**	1-6		wash	**WPA**	1-6	**<.0001**
wash	**MEA**	1		wash	**WPA**	1	**<.0001**
wash	**MEA**	2		wash	**WPA**	2	**<.0001**
wash	**MEA**	3		wash	**WPA**	3	**<.0001**
wash	**MEA**	4		wash	**WPA**	4	**<.0001**
wash	**MEA**	5		wash	**WPA**	5	**<.0001**
wash	**MEA**	6		wash	**WPA**	6	**<.0001**

Table 3-5-2 Ergosterol /cm^2 in removable fractions – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	MEA	**1**		20 u	MEA	**2**	0.621
20 u	MEA	**1**		20 u	MEA	**3**	0.9581
20 u	MEA	**1**		20 u	MEA	**4**	1
20 u	MEA	**1**		20 u	MEA	**5**	1
20 u	MEA	**1**		20 u	MEA	**6**	1
20 u	MEA	**2**		20 u	MEA	**3**	1
20 u	MEA	**2**		20 u	MEA	**4**	0.9618
20 u	MEA	**2**		20 u	MEA	**5**	**0.0128**
20 u	MEA	**2**		20 u	MEA	**6**	**0.0313**
20 u	MEA	**3**		20 u	MEA	**4**	0.9999
20 u	MEA	**3**		20 u	MEA	**5**	0.0875
20 u	MEA	**3**		20 u	MEA	**6**	0.1772
20 u	MEA	**4**		20 u	MEA	**5**	0.9945
20 u	MEA	**4**		20 u	MEA	**6**	0.9997
20 u	MEA	**5**		20 u	MEA	**6**	1

Table 3-5-3 Ergosterol /cm^2 in removable fractions – time comparisons – MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
1 u	MEA	**1**		1 u	MEA	**2**	1
1 u	MEA	**1**		1 u	MEA	**3**	1
1 u	MEA	**1**		1 u	MEA	**4**	1
1 u	MEA	**1**		1 u	MEA	**5**	1
1 u	MEA	**1**		1 u	MEA	**6**	1
1 u	MEA	**2**		1 u	MEA	**3**	1
1 u	MEA	**2**		1 u	MEA	**4**	1
1 u	MEA	**2**		1 u	MEA	**5**	1
1 u	MEA	**2**		1 u	MEA	**6**	1
1 u	MEA	**3**		1 u	MEA	**4**	1
1 u	MEA	**3**		1 u	MEA	**5**	1
1 u	MEA	**3**		1 u	MEA	**6**	1
1 u	MEA	**4**		1 u	MEA	**5**	1
1 u	MEA	**4**		1 u	MEA	**6**	1
1 u	MEA	**5**		1 u	MEA	**6**	1

Table 3-5-4 Ergosterol /cm^2 in removable fractions – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
wash	MEA	**1**		wash	MEA	**2**	0.9995
wash	MEA	**1**		wash	MEA	**3**	0.9471
wash	MEA	**1**		wash	MEA	**4**	0.9977
wash	MEA	**1**		wash	MEA	**5**	1
wash	MEA	**1**		wash	MEA	**6**	0.9771
wash	MEA	**2**		wash	MEA	**3**	1
wash	MEA	**2**		wash	MEA	**4**	1
wash	MEA	**2**		wash	MEA	**5**	1
wash	MEA	**2**		wash	MEA	**6**	1
wash	MEA	**3**		wash	MEA	**4**	1
wash	MEA	**3**		wash	MEA	**5**	1
wash	MEA	**3**		wash	MEA	**6**	1
wash	MEA	**4**		wash	MEA	**5**	1
wash	MEA	**4**		wash	MEA	**6**	1
wash	MEA	**5**		wash	MEA	**6**	1

Table 3-5-5 Ergosterol /cm^2 in removable fractions – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	WPA	**1**		20 u	WPA	**2**	1
20 u	WPA	**1**		20 u	WPA	**3**	1
20 u	WPA	**1**		20 u	WPA	**4**	1
20 u	WPA	**1**		20 u	WPA	**5**	1
20 u	WPA	**1**		20 u	WPA	**6**	1
20 u	WPA	**2**		20 u	WPA	**3**	1
20 u	WPA	**2**		20 u	WPA	**4**	1
20 u	WPA	**2**		20 u	WPA	**5**	1
20 u	WPA	**2**		20 u	WPA	**6**	1
20 u	WPA	**3**		20 u	WPA	**4**	1
20 u	WPA	**3**		20 u	WPA	**5**	1
20 u	WPA	**3**		20 u	WPA	**6**	1
20 u	WPA	**4**		20 u	WPA	**5**	1
20 u	WPA	**4**		20 u	WPA	**6**	1
20 u	WPA	**5**		20 u	WPA	**6**	1

Table 3-5-6 Ergosterol /cm^2 in removable fractions – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
1 u	WPA	**1**		1 u	WPA	**2**	1
1 u	WPA	**1**		1 u	WPA	**3**	1
1 u	WPA	**1**		1 u	WPA	**4**	1
1 u	WPA	**1**		1 u	WPA	**5**	1
1 u	WPA	**1**		1 u	WPA	**6**	1
1 u	WPA	**2**		1 u	WPA	**3**	1
1 u	WPA	**2**		1 u	WPA	**4**	1
1 u	WPA	**2**		1 u	WPA	**5**	1
1 u	WPA	**2**		1 u	WPA	**6**	1
1 u	WPA	**3**		1 u	WPA	**4**	1
1 u	WPA	**3**		1 u	WPA	**5**	0.9999
1 u	WPA	**3**		1 u	WPA	**6**	0.9976
1 u	WPA	**4**		1 u	WPA	**5**	0.9995
1 u	WPA	**4**		1 u	WPA	**6**	0.9919
1 u	WPA	**5**		1 u	WPA	**6**	1

Table 3-5-7 Ergosterol /cm^2 in removable fractions – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
wash	WPA	**1**		wash	WPA	**2**	1
wash	WPA	**1**		wash	WPA	**3**	1
wash	WPA	**1**		wash	WPA	**4**	0.9986
wash	WPA	**1**		wash	WPA	**5**	0.8926
wash	WPA	**1**		wash	WPA	**6**	1
wash	WPA	**2**		wash	WPA	**3**	1
wash	WPA	**2**		wash	WPA	**4**	1
wash	WPA	**2**		wash	WPA	**5**	0.0751
wash	WPA	**2**		wash	WPA	**6**	1
wash	WPA	**3**		wash	WPA	**4**	1
wash	WPA	**3**		wash	WPA	**5**	0.1021
wash	WPA	**3**		wash	WPA	**6**	1
wash	WPA	**4**		wash	WPA	**5**	**0.0103**
wash	WPA	**4**		wash	WPA	**6**	1
wash	WPA	**5**		wash	WPA	**6**	0.7344

Table 3-5-8 Ergosterol /cm^2 in removable fractions – fraction comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	MEA	1-6		**20 u**	MEA	1-6	**<.0001**
precore	MEA	1		**20 u**	MEA	1	**<.0001**
precore	MEA	2		**20 u**	MEA	2	**<.0001**
precore	MEA	3		**20 u**	MEA	3	**<.0001**
precore	MEA	4		**20 u**	MEA	4	**<.0001**
precore	MEA	5		**20 u**	MEA	5	**<.0001**
precore	MEA	6		**20 u**	MEA	6	**<.0001**
precore	MEA	1-6		**1 u**	MEA	1-6	**<.0001**
precore	MEA	1		**1 u**	MEA	1	**<.0001**
precore	MEA	2		**1 u**	MEA	2	**<.0001**
precore	MEA	3		**1 u**	MEA	3	**<.0001**
precore	MEA	4		**1 u**	MEA	4	**<.0001**
precore	MEA	5		**1 u**	MEA	5	**<.0001**
precore	MEA	6		**1 u**	MEA	6	**<.0001**
precore	MEA	1-6		**wash**	MEA	1-6	**<.0001**
precore	MEA	1		**wash**	MEA	1	**<.0001**
precore	MEA	2		**wash**	MEA	2	**<.0001**
precore	MEA	3		**wash**	MEA	3	**<.0001**
precore	MEA	4		**wash**	MEA	4	**<.0001**
precore	MEA	5		**wash**	MEA	5	**<.0001**
precore	MEA	6		**wash**	MEA	6	**<.0001**

Table 3-5-9 Ergosterol /cm^2 in removable fractions – fraction comparisons - MEA.

post core	MEA	1-6	**20 u**	MEA	1-6	**<.0001**
post core	MEA	1	**20 u**	MEA	1	**<.0001**
post core	MEA	2	**20 u**	MEA	2	**<.0001**
post core	MEA	3	**20 u**	MEA	3	**<.0001**
post core	MEA	4	**20 u**	MEA	4	**<.0001**
post core	MEA	5	**20 u**	MEA	5	**<.0001**
post core	MEA	6	**20 u**	MEA	6	**<.0001**
post core	MEA	1-6	**1 u**	MEA	1-6	**<.0001**
post core	MEA	1	**1 u**	MEA	1	**<.0001**
post core	MEA	2	**1 u**	MEA	2	**<.0001**
post core	MEA	3	**1 u**	MEA	3	**<.0001**
post core	MEA	4	**1 u**	MEA	4	**<.0001**
post core	MEA	5	**1 u**	MEA	5	**<.0001**
post core	MEA	6	**1 u**	MEA	6	**<.0001**
post core	MEA	1-6	**wash**	MEA	1-6	**<.0001**
post core	MEA	1	**wash**	MEA	1	**<.0001**
post core	MEA	2	**wash**	MEA	2	**<.0001**
post core	MEA	3	**wash**	MEA	3	**<.0001**
post core	MEA	4	**wash**	MEA	4	**<.0001**
post core	MEA	5	**wash**	MEA	5	**<.0001**
post core	MEA	6	**wash**	MEA	6	**<.0001**

Table 3-5-10 Ergosterol /cm^2 in removable fractions – fraction comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	MEA	1-6		**1 u**	MEA	1-6	**<.0001**
20 u	MEA	1		**1 u**	MEA	1	**<.0001**
20 u	MEA	2		**1 u**	MEA	2	**<.0001**
20 u	MEA	3		**1 u**	MEA	3	**<.0001**
20 u	MEA	4		**1 u**	MEA	4	**<.0001**
20 u	MEA	5		**1 u**	MEA	5	**<.0001**
20 u	MEA	6		**1 u**	MEA	6	**<.0001**
20 u	MEA	1-6		**wash**	MEA	1-6	**<.0001**
20 u	MEA	1		**wash**	MEA	1	**<.0001**
20 u	MEA	2		**wash**	MEA	2	**<.0001**
20 u	MEA	3		**wash**	MEA	3	**0.0002**
20 u	MEA	4		**wash**	MEA	4	**0.0108**
20 u	MEA	5		**wash**	MEA	5	0.7554
20 u	MEA	6		**wash**	MEA	6	0.9542
1 u	MEA	1-6		**wash**	MEA	1-6	**<.0001**
1 u	MEA	1		**wash**	MEA	1	0.9842
1 u	MEA	2		**wash**	MEA	2	**0.0304**
1 u	MEA	3		**wash**	MEA	3	**0.0007**
1 u	MEA	4		**wash**	MEA	4	**0.015**
1 u	MEA	5		**wash**	MEA	5	0.0742
1 u	MEA	6		**wash**	MEA	6	**0.0072**

Table 3-5-11 Ergosterol /cm^2 in removable fractions – fraction comparisons – WPA.

Fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	WPA	1-6		**20 u**	WPA	1-6	**<.0001**
precore	WPA	1		**20 u**	WPA	1	0.1373
precore	WPA	2		**20 u**	WPA	2	0.0708
precore	WPA	3		**20 u**	WPA	3	**0.0085**
precore	WPA	4		**20 u**	WPA	4	**0.0013**
precore	WPA	5		**20 u**	WPA	5	**0.0139**
precore	WPA	6		**20 u**	WPA	6	**0.0085**
precore	WPA	1-6		**1 u**	WPA	1-6	**<.0001**
precore	WPA	1		**1 u**	WPA	1	**<.0001**
precore	WPA	2		**1 u**	WPA	2	**<.0001**
precore	WPA	3		**1 u**	WPA	3	**<.0001**
precore	WPA	4		**1 u**	WPA	4	**<.0001**
precore	WPA	5		**1 u**	WPA	5	**<.0001**
precore	WPA	6		**1 u**	WPA	6	**<.0001**
precore	WPA	1-6		**wash**	WPA	1-6	**<.0001**
precore	WPA	1		**wash**	WPA	1	**<.0001**
precore	WPA	2		**wash**	WPA	2	**<.0001**
precore	WPA	3		**wash**	WPA	3	**<.0001**
precore	WPA	4		**wash**	WPA	4	**<.0001**
precore	WPA	5		**wash**	WPA	5	**<.0001**
precore	WPA	6		**wash**	WPA	6	**<.0001**

Table 3-5-12 Ergosterol /cm^2 in removable fractions – fraction comparisons - WPA.

post core	WPA	1-6		**20 u**	WPA	1-6	**0.0001**
post core	WPA	1		**20 u**	WPA	1	0.9839
post core	WPA	2		**20 u**	WPA	2	0.2656
post core	WPA	3		**20 u**	WPA	3	0.9997
post core	WPA	4		**20 u**	WPA	4	**0.0358**
post core	WPA	5		**20 u**	WPA	5	1
post core	WPA	6		**20 u**	WPA	6	0.165
post core	WPA	1-6		**1 u**	WPA	1-6	**<.0001**
post core	WPA	1		**1 u**	WPA	1	**0.0008**
post core	WPA	2		**1 u**	WPA	2	**<.0001**
post core	WPA	3		**1 u**	WPA	3	**0.002**
post core	WPA	4		**1 u**	WPA	4	**<.0001**
post core	WPA	5		**1 u**	WPA	5	**<.0001**
post core	WPA	6		**1 u**	WPA	6	**<.0001**
post core	WPA	1-6		**wash**	WPA	1-6	**<.0001**
post core	WPA	1		**wash**	WPA	1	**<.0001**
post core	WPA	2		**wash**	WPA	2	**<.0001**
post core	WPA	3		**wash**	WPA	3	**<.0001**
post core	WPA	4		**wash**	WPA	4	**<.0001**
post core	WPA	5		**wash**	WPA	5	**0.0013**
post core	WPA	6		**wash**	WPA	6	**<.0001**

Table 3-5-13 Ergosterol /cm^2 in removable fractions – fraction comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	WPA	1-6		**1 u**	WPA	1-6	**<.0001**
20 u	WPA	1		**1 u**	WPA	1	0.6343
20 u	WPA	2		**1 u**	WPA	2	0.4588
20 u	WPA	3		**1 u**	WPA	3	0.4848
20 u	WPA	4		**1 u**	WPA	4	1
20 u	WPA	5		**1 u**	WPA	5	**0.0019**
20 u	WPA	6		**1 u**	WPA	6	**0.0076**
20 u	WPA	1-6		**wash**	WPA	1-6	**<.0001**
20 u	WPA	1		**wash**	WPA	1	**0.001**
20 u	WPA	2		**wash**	WPA	2	**<.0001**
20 u	WPA	3		**wash**	WPA	3	**<.0001**
20 u	WPA	4		**wash**	WPA	4	**<.0001**
20 u	WPA	5		**wash**	WPA	5	**0.0349**
20 u	WPA	6		**wash**	WPA	6	**<.0001**
1 u	WPA	1-6		**wash**	WPA	1-6	**<.0001**
1 u	WPA	1		**wash**	WPA	1	0.9889
1 u	WPA	2		**wash**	WPA	2	**0.0284**
1 u	WPA	3		**wash**	WPA	3	**0.0045**
1 u	WPA	4		**wash**	WPA	4	**0.0002**
1 u	WPA	5		**wash**	WPA	5	1
1 u	WPA	6		**wash**	WPA	6	0.9998

Table 3-8-1. Sterigmatocystin /cm^2 in pre- and post-cores – media comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	**MEA**	1-6		precore	**WPA**	1-6	**<.0001**
precore	**MEA**	1		precore	**WPA**	1	**<.0001**
precore	**MEA**	2		precore	**WPA**	2	**<.0001**
precore	**MEA**	3		precore	**WPA**	3	**<.0001**
precore	**MEA**	4		precore	**WPA**	4	**<.0001**
precore	**MEA**	5		precore	**WPA**	5	**<.0001**
precore	**MEA**	6		precore	**WPA**	6	**<.0001**
post core	**MEA**	1-6		post core	**WPA**	1-6	**<.0001**
post core	**MEA**	1		post core	**WPA**	1	**<.0001**
post core	**MEA**	2		post core	**WPA**	2	**<.0001**
post core	**MEA**	3		post core	**WPA**	3	**<.0001**
post core	**MEA**	4		post core	**WPA**	4	**<.0001**
post core	**MEA**	5		post core	**WPA**	5	**<.0001**
post core	**MEA**	6		post core	**WPA**	6	**<.0001**

Table 3-8-2. Sterigmatocystin /cm^2 in pre-cores – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	MEA	**1**		precore	MEA	**2**	1
precore	MEA	**1**		precore	MEA	**3**	1
precore	MEA	**1**		precore	MEA	**4**	0.9999
precore	MEA	**1**		precore	MEA	**5**	1
precore	MEA	**1**		precore	MEA	**6**	1
precore	MEA	**2**		precore	MEA	**3**	1
precore	MEA	**2**		precore	MEA	**4**	1
precore	MEA	**2**		precore	MEA	**5**	1
precore	MEA	**2**		precore	MEA	**6**	1
precore	MEA	**3**		precore	MEA	**4**	1
precore	MEA	**3**		precore	MEA	**5**	1
precore	MEA	**3**		precore	MEA	**6**	1
precore	MEA	**4**		precore	MEA	**5**	1
precore	MEA	**4**		precore	MEA	**6**	1
precore	MEA	**5**		precore	MEA	**6**	1

Table 3-8-3. Sterigmatocystin /cm^2 in pre-cores – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	WPA	**1**		precore	WPA	**2**	**0.0002**
precore	WPA	**1**		precore	WPA	**3**	**<.0001**
precore	WPA	**1**		precore	WPA	**4**	**<.0001**
precore	WPA	**1**		precore	WPA	**5**	**<.0001**
precore	WPA	**1**		precore	WPA	**6**	**<.0001**
precore	WPA	**2**		precore	WPA	**3**	0.8166
precore	WPA	**2**		precore	WPA	**4**	**0.0085**
precore	WPA	**2**		precore	WPA	**5**	**0.0053**
precore	WPA	**2**		precore	WPA	**6**	**0.0001**
precore	WPA	**3**		precore	WPA	**4**	0.9995
precore	WPA	**3**		precore	WPA	**5**	0.9979
precore	WPA	**3**		precore	WPA	**6**	0.6288
precore	WPA	**4**		precore	WPA	**5**	1
precore	WPA	**4**		precore	WPA	**6**	1
precore	WPA	**5**		precore	WPA	**6**	1

Table 3-8-4. Sterigmatocystin /cm^2 in post-cores – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
post core	MEA	**1**		post core	MEA	**2**	1
post core	MEA	**1**		post core	MEA	**3**	1
post core	MEA	**1**		post core	MEA	**4**	0.9999
post core	MEA	**1**		post core	MEA	**5**	1
post core	MEA	**1**		post core	MEA	**6**	1
post core	MEA	**2**		post core	MEA	**3**	1
post core	MEA	**2**		post core	MEA	**4**	1
post core	MEA	**2**		post core	MEA	**5**	1
post core	MEA	**2**		post core	MEA	**6**	1
post core	MEA	**3**		post core	MEA	**4**	1
post core	MEA	**3**		post core	MEA	**5**	1
post core	MEA	**3**		post core	MEA	**6**	1
post core	MEA	**4**		post core	MEA	**5**	1
post core	MEA	**4**		post core	MEA	**6**	1
post core	MEA	**5**		post core	MEA	**6**	1

Table 3-8-5. Sterigmatocystin /cm^2 in post-cores – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
post core	WPA	**1**		post core	WPA	**2**	1
post core	WPA	**1**		post core	WPA	**3**	1
post core	WPA	**1**		post core	WPA	**4**	0.0983
post core	WPA	**1**		post core	WPA	**5**	1
post core	WPA	**1**		post core	WPA	**6**	0.8458
post core	WPA	**2**		post core	WPA	**3**	1
post core	WPA	**2**		post core	WPA	**4**	0.2614
post core	WPA	**2**		post core	WPA	**5**	1
post core	WPA	**2**		post core	WPA	**6**	0.9688
post core	WPA	**3**		post core	WPA	**4**	0.1306
post core	WPA	**3**		post core	WPA	**5**	1
post core	WPA	**3**		post core	WPA	**6**	0.898
post core	WPA	**4**		post core	WPA	**5**	0.817
post core	WPA	**4**		post core	WPA	**6**	1
post core	WPA	**5**		post core	WPA	**6**	1

Table 3-8-6. Sterigmatocystin /cm^2 in pre- and post-core – time comparisons.

Fraction	media	week	VS.	_fraction	_media	_week	Adj P
post core	MEA	1-6		**precore**	MEA	1-6	0.9903
post core	MEA	1		**precore**	MEA	1	1
post core	MEA	2		**precore**	MEA	2	1
post core	MEA	3		**precore**	MEA	3	1
post core	MEA	4		**precore**	MEA	4	1
post core	MEA	5		**precore**	MEA	5	1
post core	MEA	6		**precore**	MEA	6	1
post core	WPA	1-6		**precore**	WPA	1-6	**<.0001**
post core	WPA	1		**precore**	WPA	1	1
post core	WPA	2		**precore**	WPA	2	**0.0004**
post core	WPA	3		**precore**	WPA	3	**<.0001**
post core	WPA	4		**precore**	WPA	4	**<.0001**
post core	WPA	5		**precore**	WPA	5	**<.0001**
post core	WPA	6		**precore**	WPA	6	**<.0001**

Table 3-9-1 Sterigmatocystin /cm^2 in removable fractions – media comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	**MEA**	1-6		20 u	**WPA**	1-6	**<.0001**
20 u	**MEA**	1		20 u	**WPA**	1	**<.0001**
20 u	**MEA**	2		20 u	**WPA**	2	**<.0001**
20 u	**MEA**	3		20 u	**WPA**	3	**<.0001**
20 u	**MEA**	4		20 u	**WPA**	4	**<.0001**
20 u	**MEA**	5		20 u	**WPA**	5	**<.0001**
20 u	**MEA**	6		20 u	**WPA**	6	**0.0002**
1 u	**MEA**	1-6		1 u	**WPA**	1-6	**<.0001**
1 u	**MEA**	1		1 u	**WPA**	1	**<.0001**
1 u	**MEA**	2		1 u	**WPA**	2	**<.0001**
1 u	**MEA**	3		1 u	**WPA**	3	0.9999
1 u	**MEA**	4		1 u	**WPA**	4	1
1 u	**MEA**	5		1 u	**WPA**	5	1
1 u	**MEA**	6		1 u	**WPA**	6	1
wash	**MEA**	1-6		wash	**WPA**	1-6	**<.0001**
wash	**MEA**	1		wash	**WPA**	1	**<.0001**
wash	**MEA**	2		wash	**WPA**	2	**<.0001**
wash	**MEA**	3		wash	**WPA**	3	0.0624
wash	**MEA**	4		wash	**WPA**	4	**0.0032**
wash	**MEA**	5		wash	**WPA**	5	0.6845
wash	**MEA**	6		wash	**WPA**	6	0.986

Table 3-9-2 Sterigmatocystin /cm^2 in removable fractions – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	MEA	**1**		20 u	MEA	**2**	0.7936
20 u	MEA	**1**		20 u	MEA	**3**	0.8828
20 u	MEA	**1**		20 u	MEA	**4**	0.8688
20 u	MEA	**1**		20 u	MEA	**5**	1
20 u	MEA	**1**		20 u	MEA	**6**	1
20 u	MEA	**2**		20 u	MEA	**3**	1
20 u	MEA	**2**		20 u	MEA	**4**	1
20 u	MEA	**2**		20 u	MEA	**5**	1
20 u	MEA	**2**		20 u	MEA	**6**	1
20 u	MEA	**3**		20 u	MEA	**4**	1
20 u	MEA	**3**		20 u	MEA	**5**	1
20 u	MEA	**3**		20 u	MEA	**6**	1
20 u	MEA	**4**		20 u	MEA	**5**	1
20 u	MEA	**4**		20 u	MEA	**6**	1
20 u	MEA	**5**		20 u	MEA	**6**	1

Table 3-9-3 Sterigmatocystin /cm^2 in removable fractions – time comparisons – MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
1 u	MEA	**1**		1 u	MEA	**2**	1
1 u	MEA	**1**		1 u	MEA	**3**	1
1 u	MEA	**1**		1 u	MEA	**4**	0.9982
1 u	MEA	**1**		1 u	MEA	**5**	1
1 u	MEA	**1**		1 u	MEA	**6**	0.9976
1 u	MEA	**2**		1 u	MEA	**3**	1
1 u	MEA	**2**		1 u	MEA	**4**	1
1 u	MEA	**2**		1 u	MEA	**5**	1
1 u	MEA	**2**		1 u	MEA	**6**	1
1 u	MEA	**3**		1 u	MEA	**4**	0.9993
1 u	MEA	**3**		1 u	MEA	**5**	1
1 u	MEA	**3**		1 u	MEA	**6**	0.9991
1 u	MEA	**4**		1 u	MEA	**5**	1
1 u	MEA	**4**		1 u	MEA	**6**	1
1 u	MEA	**5**		1 u	MEA	**6**	1

Table 3-9-4 Sterigmatocystin /cm^2 in removable fractions – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
wash	MEA	**1**		wash	MEA	**2**	1
wash	MEA	**1**		wash	MEA	**3**	1
wash	MEA	**1**		wash	MEA	**4**	1
wash	MEA	**1**		wash	MEA	**5**	1
wash	MEA	**1**		wash	MEA	**6**	1
wash	MEA	**2**		wash	MEA	**3**	1
wash	MEA	**2**		wash	MEA	**4**	1
wash	MEA	**2**		wash	MEA	**5**	1
wash	MEA	**2**		wash	MEA	**6**	1
wash	MEA	**3**		wash	MEA	**4**	1
wash	MEA	**3**		wash	MEA	**5**	1
wash	MEA	**3**		wash	MEA	**6**	1
wash	MEA	**4**		wash	MEA	**5**	1
wash	MEA	**4**		wash	MEA	**6**	1
wash	MEA	**5**		wash	MEA	**6**	1

Table 3-9-5 Sterigmatocystin /cm^2 in removable fractions – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	WPA	**1**		20 u	WPA	**2**	**<.0001**
20 u	WPA	**1**		20 u	WPA	**3**	**<.0001**
20 u	WPA	**1**		20 u	WPA	**4**	**<.0001**
20 u	WPA	**1**		20 u	WPA	**5**	**<.0001**
20 u	WPA	**1**		20 u	WPA	**6**	**<.0001**
20 u	WPA	**2**		20 u	WPA	**3**	**0.0029**
20 u	WPA	**2**		20 u	WPA	**4**	**<.0001**
20 u	WPA	**2**		20 u	WPA	**5**	**<.0001**
20 u	WPA	**2**		20 u	WPA	**6**	**<.0001**
20 u	WPA	**3**		20 u	WPA	**4**	0.8287
20 u	WPA	**3**		20 u	WPA	**5**	**0.0067**
20 u	WPA	**3**		20 u	WPA	**6**	**0.0002**
20 u	WPA	**4**		20 u	WPA	**5**	0.9986
20 u	WPA	**4**		20 u	WPA	**6**	0.7601
20 u	WPA	**5**		20 u	WPA	**6**	1

Table 3-9-6 Sterigmatocystin /cm^2 in removable fractions – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
1 u	WPA	**1**		1 u	WPA	**2**	**<.0001**
1 u	WPA	**1**		1 u	WPA	**3**	**<.0001**
1 u	WPA	**1**		1 u	WPA	**4**	**<.0001**
1 u	WPA	**1**		1 u	WPA	**5**	**<.0001**
1 u	WPA	**1**		1 u	WPA	**6**	**<.0001**
1 u	WPA	**2**		1 u	WPA	**3**	**0.0113**
1 u	WPA	**2**		1 u	WPA	**4**	**<.0001**
1 u	WPA	**2**		1 u	WPA	**5**	**<.0001**
1 u	WPA	**2**		1 u	WPA	**6**	**<.0001**
1 u	WPA	**3**		1 u	WPA	**4**	0.9879
1 u	WPA	**3**		1 u	WPA	**5**	0.3693
1 u	WPA	**3**		1 u	WPA	**6**	0.0699
1 u	WPA	**4**		1 u	WPA	**5**	1
1 u	WPA	**4**		1 u	WPA	**6**	0.9999
1 u	WPA	**5**		1 u	WPA	**6**	1

Table 3-9-7 Sterigmatocystin /cm^2 in removable fractions – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
wash	WPA	**1**		wash	WPA	**2**	**<.0001**
wash	WPA	**1**		wash	WPA	**3**	**<.0001**
wash	WPA	**1**		wash	WPA	**4**	**<.0001**
wash	WPA	**1**		wash	WPA	**5**	**<.0001**
wash	WPA	**1**		wash	WPA	**6**	**<.0001**
wash	WPA	**2**		wash	WPA	**3**	0.2869
wash	WPA	**2**		wash	WPA	**4**	0.5279
wash	WPA	**2**		wash	WPA	**5**	**0.026**
wash	WPA	**2**		wash	WPA	**6**	**0.0158**
wash	WPA	**3**		wash	WPA	**4**	1
wash	WPA	**3**		wash	WPA	**5**	1
wash	WPA	**3**		wash	WPA	**6**	1
wash	WPA	**4**		wash	WPA	**5**	1
wash	WPA	**4**		wash	WPA	**6**	1
wash	WPA	**5**		wash	WPA	**6**	1

Table 3-9-8 Sterigmatocystin /cm^2 in removable fractions – fraction comparisons.

Fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	MEA	1-6		**20 u**	MEA	1-6	**<.0001**
precore	MEA	1		**20 u**	MEA	1	**<.0001**
precore	MEA	2		**20 u**	MEA	2	**<.0001**
precore	MEA	3		**20 u**	MEA	3	**<.0001**
precore	MEA	4		**20 u**	MEA	4	**<.0001**
precore	MEA	5		**20 u**	MEA	5	**<.0001**
precore	MEA	6		**20 u**	MEA	6	**<.0001**
precore	MEA	1-6		**1 u**	MEA	1-6	**<.0001**
precore	MEA	1		**1 u**	MEA	1	**<.0001**
precore	MEA	2		**1 u**	MEA	2	**<.0001**
precore	MEA	3		**1 u**	MEA	3	**<.0001**
precore	MEA	4		**1 u**	MEA	4	**<.0001**
precore	MEA	5		**1 u**	MEA	5	**<.0001**
precore	MEA	6		**1 u**	MEA	6	**<.0001**
precore	MEA	1-6		**wash**	MEA	1-6	**<.0001**
precore	MEA	1		**wash**	MEA	1	**<.0001**
precore	MEA	2		**wash**	MEA	2	**<.0001**
precore	MEA	3		**wash**	MEA	3	**<.0001**
precore	MEA	4		**wash**	MEA	4	**<.0001**
precore	MEA	5		**wash**	MEA	5	**<.0001**
precore	MEA	6		**wash**	MEA	6	**<.0001**

Table 3-9-9 Sterigmatocystin /cm^2 in removable fractions – fraction comparisons.

post core	MEA	1-6		**20 u**	MEA	1-6	**<.0001**
post core	MEA	1		**20 u**	MEA	1	**<.0001**
post core	MEA	2		**20 u**	MEA	2	**<.0001**
post core	MEA	3		**20 u**	MEA	3	**<.0001**
post core	MEA	4		**20 u**	MEA	4	**<.0001**
post core	MEA	5		**20 u**	MEA	5	**<.0001**
post core	MEA	6		**20 u**	MEA	6	**<.0001**
post core	MEA	1-6		**1 u**	MEA	1-6	**<.0001**
post core	MEA	1		**1 u**	MEA	1	**<.0001**
post core	MEA	2		**1 u**	MEA	2	**<.0001**
post core	MEA	3		**1 u**	MEA	3	**<.0001**
post core	MEA	4		**1 u**	MEA	4	**<.0001**
post core	MEA	5		**1 u**	MEA	5	**<.0001**
post core	MEA	6		**1 u**	MEA	6	**<.0001**
post core	MEA	1-6		**wash**	MEA	1-6	**<.0001**
post core	MEA	1		**wash**	MEA	1	**<.0001**
post core	MEA	2		**wash**	MEA	2	**<.0001**
post core	MEA	3		**wash**	MEA	3	**<.0001**
post core	MEA	4		**wash**	MEA	4	**<.0001**
post core	MEA	5		**wash**	MEA	5	**<.0001**
post core	MEA	6		**wash**	MEA	6	**<.0001**

Table 3-9-10 Sterigmatocystin /cm^2 in removable fractions – fraction comparisons.

Fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	MEA	1-6		**1 u**	MEA	1-6	**<.0001**
20 u	MEA	1		**1 u**	MEA	1	**<.0001**
20 u	MEA	2		**1 u**	MEA	2	**<.0001**
20 u	MEA	3		**1 u**	MEA	3	**<.0001**
20 u	MEA	4		**1 u**	MEA	4	**<.0001**
20 u	MEA	5		**1 u**	MEA	5	**<.0001**
20 u	MEA	6		**1 u**	MEA	6	**<.0001**
20 u	MEA	1-6		**wash**	MEA	1-6	**<.0001**
20 u	MEA	1		**wash**	MEA	1	**<.0001**
20 u	MEA	2		**wash**	MEA	2	**<.0001**
20 u	MEA	3		**wash**	MEA	3	**<.0001**
20 u	MEA	4		**wash**	MEA	4	**<.0001**
20 u	MEA	5		**wash**	MEA	5	**<.0001**
20 u	MEA	6		**wash**	MEA	6	**<.0001**
1 u	MEA	1-6		**wash**	MEA	1-6	**0.0007**
1 u	MEA	1		**wash**	MEA	1	0.514
1 u	MEA	2		**wash**	MEA	2	0.5127
1 u	MEA	3		**wash**	MEA	3	0.3815
1 u	MEA	4		**wash**	MEA	4	0.9985
1 u	MEA	5		**wash**	MEA	5	1
1 u	MEA	6		**wash**	MEA	6	1

Table 3-9-11 Sterigmatocystin /cm^2 in removable fractions – fraction comparisons.

Fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	WPA	1-6		**20 u**	WPA	1-6	**<.0001**
precore	WPA	1		**20 u**	WPA	1	**<.0001**
precore	WPA	2		**20 u**	WPA	2	**<.0001**
precore	WPA	3		**20 u**	WPA	3	**<.0001**
precore	WPA	4		**20 u**	WPA	4	**0.0002**
precore	WPA	5		**20 u**	WPA	5	0.164
precore	WPA	6		**20 u**	WPA	6	0.1162
precore	WPA	1-6		**1 u**	WPA	1-6	**<.0001**
precore	WPA	1		**1 u**	WPA	1	**<.0001**
precore	WPA	2		**1 u**	WPA	2	**<.0001**
precore	WPA	3		**1 u**	WPA	3	**<.0001**
precore	WPA	4		**1 u**	WPA	4	**<.0001**
precore	WPA	5		**1 u**	WPA	5	**<.0001**
precore	WPA	6		**1 u**	WPA	6	**<.0001**
precore	WPA	1-6		**wash**	WPA	1-6	**<.0001**
precore	WPA	1		**wash**	WPA	1	**<.0001**
precore	WPA	2		**wash**	WPA	2	**<.0001**
precore	WPA	3		**wash**	WPA	3	**<.0001**
precore	WPA	4		**wash**	WPA	4	**<.0001**
precore	WPA	5		**wash**	WPA	5	**<.0001**
precore	WPA	6		**wash**	WPA	6	**<.0001**

Table 3-9-12 Sterigmatocystin /cm^2 in removable fractions – fraction comparisons.

post core	WPA	1-6	**20 u**	WPA	1-6	1
post core	WPA	1	**20 u**	WPA	1	**<.0001**
post core	WPA	2	**20 u**	WPA	2	0.8087
post core	WPA	3	**20 u**	WPA	3	0.9933
post core	WPA	4	**20 u**	WPA	4	1
post core	WPA	5	**20 u**	WPA	5	**<.0001**
post core	WPA	6	**20 u**	WPA	6	**0.0014**
post core	WPA	1-6	**1 u**	WPA	1-6	**0.0016**
post core	WPA	1	**1 u**	WPA	1	**<.0001**
post core	WPA	2	**1 u**	WPA	2	0.0608
post core	WPA	3	**1 u**	WPA	3	1
post core	WPA	4	**1 u**	WPA	4	1
post core	WPA	5	**1 u**	WPA	5	0.8327
post core	WPA	6	**1 u**	WPA	6	0.9998
post core	WPA	1-6	**wash**	WPA	1-6	**0.0453**
post core	WPA	1	**wash**	WPA	1	**<.0001**
post core	WPA	2	**wash**	WPA	2	0.9996
post core	WPA	3	**wash**	WPA	3	1
post core	WPA	4	**wash**	WPA	4	0.9248
post core	WPA	5	**wash**	WPA	5	1
post core	WPA	6	**wash**	WPA	6	1

Table 3-9-13 Sterigmatocystin /cm^2 in removable fractions – fraction comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	WPA	1-6		**1 u**	WPA	1-6	**0.0018**
20 u	WPA	1		**1 u**	WPA	1	1
20 u	WPA	2		**1 u**	WPA	2	1
20 u	WPA	3		**1 u**	WPA	3	1
20 u	WPA	4		**1 u**	WPA	4	0.9877
20 u	WPA	5		**1 u**	WPA	5	0.5456
20 u	WPA	6		**1 u**	WPA	6	0.41
20 u	WPA	1-6		**wash**	WPA	1-6	0.0532
20 u	WPA	1		**wash**	WPA	1	0.0839
20 u	WPA	2		**wash**	WPA	2	1
20 u	WPA	3		**wash**	WPA	3	1
20 u	WPA	4		**wash**	WPA	4	0.1985
20 u	WPA	5		**wash**	WPA	5	**0.0191**
20 u	WPA	6		**wash**	WPA	6	**0.008**
1 u	WPA	1-6		**wash**	WPA	1-6	0.7683
1 u	WPA	1		**wash**	WPA	1	**0.0023**
1 u	WPA	2		**wash**	WPA	2	0.9831
1 u	WPA	3		**wash**	WPA	3	1
1 u	WPA	4		**wash**	WPA	4	1
1 u	WPA	5		**wash**	WPA	5	1
1 u	WPA	6		**wash**	WPA	6	1

Table 3-11-1. Sterigmatocystin /erg in pre- and post-cores – media comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	**MEA**	1-6		precore	**WPA**	1-6	0.8873
precore	**MEA**	1		precore	**WPA**	1	**0.0007**
precore	**MEA**	2		precore	**WPA**	2	1
precore	**MEA**	3		precore	**WPA**	3	1
precore	**MEA**	4		precore	**WPA**	4	1
precore	**MEA**	5		precore	**WPA**	5	1
precore	**MEA**	6		precore	**WPA**	6	1
post core	**MEA**	1-6		post core	**WPA**	1-6	**0.0002**
post core	**MEA**	1		post core	**WPA**	1	**0.0036**
post core	**MEA**	2		post core	**WPA**	2	**0.0435**
post core	**MEA**	3		post core	**WPA**	3	**0.0011**
post core	**MEA**	4		post core	**WPA**	4	0.388
post core	**MEA**	5		post core	**WPA**	5	**0.0485**
post core	**MEA**	6		post core	**WPA**	6	0.075

Table 3-11-2. Sterigmatocystin /erg in pre-cores – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	MEA	**1**		precore	MEA	**2**	0.9931
precore	MEA	**1**		precore	MEA	**3**	1
precore	MEA	**1**		precore	MEA	**4**	1
precore	MEA	**1**		precore	MEA	**5**	1
precore	MEA	**1**		precore	MEA	**6**	1
precore	MEA	**2**		precore	MEA	**3**	1
precore	MEA	**2**		precore	MEA	**4**	1
precore	MEA	**2**		precore	MEA	**5**	0.9998
precore	MEA	**2**		precore	MEA	**6**	1
precore	MEA	**3**		precore	MEA	**4**	1
precore	MEA	**3**		precore	MEA	**5**	1
precore	MEA	**3**		precore	MEA	**6**	1
precore	MEA	**4**		precore	MEA	**5**	1
precore	MEA	**4**		precore	MEA	**6**	1
precore	MEA	**5**		precore	MEA	**6**	1

Table 3-11-3. Sterigmatocystin /erg in pre-cores – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
precore	WPA	**1**		precore	WPA	**2**	0.3905
precore	WPA	**1**		precore	WPA	**3**	**0.02**
precore	WPA	**1**		precore	WPA	**4**	**<.0001**
precore	WPA	**1**		precore	WPA	**5**	**<.0001**
precore	WPA	**1**		precore	WPA	**6**	**<.0001**
precore	WPA	**2**		precore	WPA	**3**	1
precore	WPA	**2**		precore	WPA	**4**	0.4935
precore	WPA	**2**		precore	WPA	**5**	0.8724
precore	WPA	**2**		precore	WPA	**6**	0.1305
precore	WPA	**3**		precore	WPA	**4**	0.9966
precore	WPA	**3**		precore	WPA	**5**	1
precore	WPA	**3**		precore	WPA	**6**	0.8487
precore	WPA	**4**		precore	WPA	**5**	1
precore	WPA	**4**		precore	WPA	**6**	1
precore	WPA	**5**		precore	WPA	**6**	1

Table 3-11-4. Sterigmatocystin /erg in post-cores – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
post core	MEA	**1**		post core	MEA	**2**	0.9974
post core	MEA	**1**		post core	MEA	**3**	1
post core	MEA	**1**		post core	MEA	**4**	1
post core	MEA	**1**		post core	MEA	**5**	1
post core	MEA	**1**		post core	MEA	**6**	1
post core	MEA	**2**		post core	MEA	**3**	1
post core	MEA	**2**		post core	MEA	**4**	1
post core	MEA	**2**		post core	MEA	**5**	1
post core	MEA	**2**		post core	MEA	**6**	1
post core	MEA	**3**		post core	MEA	**4**	1
post core	MEA	**3**		post core	MEA	**5**	1
post core	MEA	**3**		post core	MEA	**6**	1
post core	MEA	**4**		post core	MEA	**5**	1
post core	MEA	**4**		post core	MEA	**6**	1
post core	MEA	**5**		post core	MEA	**6**	1

Table 3-11-5. Sterigmatocystin /erg in post-cores – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
post core	WPA	**1**		post core	WPA	**2**	1
post core	WPA	**1**		post core	WPA	**3**	1
post core	WPA	**1**		post core	WPA	**4**	1
post core	WPA	**1**		post core	WPA	**5**	1
post core	WPA	**1**		post core	WPA	**6**	1
post core	WPA	**2**		post core	WPA	**3**	1
post core	WPA	**2**		post core	WPA	**4**	0.9511
post core	WPA	**2**		post core	WPA	**5**	1
post core	WPA	**2**		post core	WPA	**6**	1
post core	WPA	**3**		post core	WPA	**4**	0.998
post core	WPA	**3**		post core	WPA	**5**	1
post core	WPA	**3**		post core	WPA	**6**	1
post core	WPA	**4**		post core	WPA	**5**	1
post core	WPA	**4**		post core	WPA	**6**	1
post core	WPA	**5**		post core	WPA	**6**	1

Table 3-11-6. Sterigmatocystin /erg in pre- and post-core – fraction comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
post core	MEA	1-6		**precore**	MEA	1-6	0.954
post core	MEA	1		**precore**	MEA	1	1
post core	MEA	2		**precore**	MEA	2	1
post core	MEA	3		**precore**	MEA	3	1
post core	MEA	4		**precore**	MEA	4	1
post core	MEA	5		**precore**	MEA	5	1
post core	MEA	6		**precore**	MEA	6	1
post core	WPA	1-6		**precore**	WPA	1-6	**<.0001**
post core	WPA	1		**precore**	WPA	1	1
post core	WPA	2		**precore**	WPA	2	0.2634
post core	WPA	3		**precore**	WPA	3	**0.0354**
post core	WPA	4		**precore**	WPA	4	**0.0298**
post core	WPA	5		**precore**	WPA	5	**0.0084**
post core	WPA	6		**precore**	WPA	6	**<.0001**

Table 3-12-1 Sterigmatocystin /erg in removable fractions – media comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	**MEA**	1-6		20 u	**WPA**	1-6	0.983
20 u	**MEA**	1		20 u	**WPA**	1	**<.0001**
20 u	**MEA**	2		20 u	**WPA**	2	0.9942
20 u	**MEA**	3		20 u	**WPA**	3	1
20 u	**MEA**	4		20 u	**WPA**	4	1
20 u	**MEA**	5		20 u	**WPA**	5	1
20 u	**MEA**	6		20 u	**WPA**	6	0.9735
1 u	**MEA**	1-6		1 u	**WPA**	1-6	0.9296
1 u	**MEA**	1		1 u	**WPA**	1	**0.001**
1 u	**MEA**	2		1 u	**WPA**	2	1
1 u	**MEA**	3		1 u	**WPA**	3	1
1 u	**MEA**	4		1 u	**WPA**	4	0.9998
1 u	**MEA**	5		1 u	**WPA**	5	0.0789
1 u	**MEA**	6		1 u	**WPA**	6	**0.026**
wash	**MEA**	1-6		wash	**WPA**	1-6	**0.0002**
wash	**MEA**	1		wash	**WPA**	1	1
wash	**MEA**	2		wash	**WPA**	2	0.4351
wash	**MEA**	3		wash	**WPA**	3	**<.0001**
wash	**MEA**	4		wash	**WPA**	4	**<.0001**
wash	**MEA**	5		wash	**WPA**	5	**0.0278**
wash	**MEA**	6		wash	**WPA**	6	**<.0001**

Table 3-12-2 Sterigmatocystin /erg in removable fractions – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	MEA	**1**		20 u	MEA	**2**	1
20 u	MEA	**1**		20 u	MEA	**3**	1
20 u	MEA	**1**		20 u	MEA	**4**	1
20 u	MEA	**1**		20 u	MEA	**5**	0.9518
20 u	MEA	**1**		20 u	MEA	**6**	0.9979
20 u	MEA	**2**		20 u	MEA	**3**	1
20 u	MEA	**2**		20 u	MEA	**4**	0.9999
20 u	MEA	**2**		20 u	MEA	**5**	0.837
20 u	MEA	**2**		20 u	MEA	**6**	0.9798
20 u	MEA	**3**		20 u	MEA	**4**	1
20 u	MEA	**3**		20 u	MEA	**5**	0.97
20 u	MEA	**3**		20 u	MEA	**6**	0.9991
20 u	MEA	**4**		20 u	MEA	**5**	1
20 u	MEA	**4**		20 u	MEA	**6**	1
20 u	MEA	**5**		20 u	MEA	**6**	1

Table 3-12-3 Sterigmatocystin /erg in removable fractions – time comparisons – MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
1 u	MEA	**1**		1 u	MEA	**2**	1
1 u	MEA	**1**		1 u	MEA	**3**	1
1 u	MEA	**1**		1 u	MEA	**4**	1
1 u	MEA	**1**		1 u	MEA	**5**	1
1 u	MEA	**1**		1 u	MEA	**6**	1
1 u	MEA	**2**		1 u	MEA	**3**	1
1 u	MEA	**2**		1 u	MEA	**4**	1
1 u	MEA	**2**		1 u	MEA	**5**	1
1 u	MEA	**2**		1 u	MEA	**6**	1
1 u	MEA	**3**		1 u	MEA	**4**	1
1 u	MEA	**3**		1 u	MEA	**5**	1
1 u	MEA	**3**		1 u	MEA	**6**	1
1 u	MEA	**4**		1 u	MEA	**5**	1
1 u	MEA	**4**		1 u	MEA	**6**	1
1 u	MEA	**5**		1 u	MEA	**6**	1

Table 3-12-4 Sterigmatocystin /erg in removable fractions – time comparisons - MEA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
wash	MEA	**1**		wash	MEA	**2**	1
wash	MEA	**1**		wash	MEA	**3**	1
wash	MEA	**1**		wash	MEA	**4**	1
wash	MEA	**1**		wash	MEA	**5**	1
wash	MEA	**1**		wash	MEA	**6**	0.9984
wash	MEA	**2**		wash	MEA	**3**	1
wash	MEA	**2**		wash	MEA	**4**	1
wash	MEA	**2**		wash	MEA	**5**	1
wash	MEA	**2**		wash	MEA	**6**	1
wash	MEA	**3**		wash	MEA	**4**	1
wash	MEA	**3**		wash	MEA	**5**	1
wash	MEA	**3**		wash	MEA	**6**	1
wash	MEA	**4**		wash	MEA	**5**	1
wash	MEA	**4**		wash	MEA	**6**	1
wash	MEA	**5**		wash	MEA	**6**	1

Table 3-12-5 Sterigmatocystin /erg in removable fractions – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	WPA	**1**		20 u	WPA	**2**	**<.0001**
20 u	WPA	**1**		20 u	WPA	**3**	**<.0001**
20 u	WPA	**1**		20 u	WPA	**4**	**<.0001**
20 u	WPA	**1**		20 u	WPA	**5**	**<.0001**
20 u	WPA	**1**		20 u	WPA	**6**	**<.0001**
20 u	WPA	**2**		20 u	WPA	**3**	0.4342
20 u	WPA	**2**		20 u	WPA	**4**	**<.0001**
20 u	WPA	**2**		20 u	WPA	**5**	**<.0001**
20 u	WPA	**2**		20 u	WPA	**6**	**<.0001**
20 u	WPA	**3**		20 u	WPA	**4**	0.5569
20 u	WPA	**3**		20 u	WPA	**5**	0.2848
20 u	WPA	**3**		20 u	WPA	**6**	**0.0095**
20 u	WPA	**4**		20 u	WPA	**5**	1
20 u	WPA	**4**		20 u	WPA	**6**	1
20 u	WPA	**5**		20 u	WPA	**6**	1

Table 3-12-6 Sterigmatocystin /erg in removable fractions – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
1 u	WPA	**1**		1 u	WPA	**2**	**<.0001**
1 u	WPA	**1**		1 u	WPA	**3**	**<.0001**
1 u	WPA	**1**		1 u	WPA	**4**	**<.0001**
1 u	WPA	**1**		1 u	WPA	**5**	**<.0001**
1 u	WPA	**1**		1 u	WPA	**6**	**<.0001**
1 u	WPA	**2**		1 u	WPA	**3**	0.6772
1 u	WPA	**2**		1 u	WPA	**4**	**0.0135**
1 u	WPA	**2**		1 u	WPA	**5**	**<.0001**
1 u	WPA	**2**		1 u	WPA	**6**	**<.0001**
1 u	WPA	**3**		1 u	WPA	**4**	1
1 u	WPA	**3**		1 u	WPA	**5**	0.0976
1 u	WPA	**3**		1 u	WPA	**6**	**0.0093**
1 u	WPA	**4**		1 u	WPA	**5**	0.9765
1 u	WPA	**4**		1 u	WPA	**6**	0.5937
1 u	WPA	**5**		1 u	WPA	**6**	1

Table 3-12-7 Sterigmatocystin /erg in removable fractions – time comparisons - WPA.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
wash	WPA	**1**		wash	WPA	**2**	**0.0003**
wash	WPA	**1**		wash	WPA	**3**	**<.0001**
wash	WPA	**1**		wash	WPA	**4**	**<.0001**
wash	WPA	**1**		wash	WPA	**5**	**0.0002**
wash	WPA	**1**		wash	WPA	**6**	**<.0001**
wash	WPA	**2**		wash	WPA	**3**	0.9271
wash	WPA	**2**		wash	WPA	**4**	0.7584
wash	WPA	**2**		wash	WPA	**5**	1
wash	WPA	**2**		wash	WPA	**6**	0.8812
wash	WPA	**3**		wash	WPA	**4**	1
wash	WPA	**3**		wash	WPA	**5**	0.9633
wash	WPA	**3**		wash	WPA	**6**	1
wash	WPA	**4**		wash	WPA	**5**	0.8404
wash	WPA	**4**		wash	WPA	**6**	1
wash	WPA	**5**		wash	WPA	**6**	0.9313

Table 3-12-8 Sterigmatocystin /erg in removable fractions – fraction comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
pre core	MEA	1-6		20 u	MEA	1-6	**0.007**
pre core	MEA	1		20 u	MEA	1	0.1698
pre core	MEA	2		20 u	MEA	2	0.9999
pre core	MEA	3		20 u	MEA	3	0.7957
pre core	MEA	4		20 u	MEA	4	1
pre core	MEA	5		20 u	MEA	5	1
pre core	MEA	6		20 u	MEA	6	1
pre core	MEA	1-6		1 u	MEA	1-6	**0.0002**
pre core	MEA	1		1 u	MEA	1	**0.0122**
pre core	MEA	2		1 u	MEA	2	0.9998
pre core	MEA	3		1 u	MEA	3	0.7772
pre core	MEA	4		1 u	MEA	4	0.9961
pre core	MEA	5		1 u	MEA	5	0.7361
pre core	MEA	6		1 u	MEA	6	0.9998
pre core	MEA	1-6		wash	MEA	1-6	**<.0001**
pre core	MEA	1		wash	MEA	1	0.0821
pre core	MEA	2		wash	MEA	2	0.9986
pre core	MEA	3		wash	MEA	3	**0.0119**
pre core	MEA	4		wash	MEA	4	**0.0509**
pre core	MEA	5		wash	MEA	5	**0.0032**
pre core	MEA	6		wash	MEA	6	**0.0041**

Table 3-12-9 Sterigmatocystin /erg in removable fractions – fraction comparisons

fraction	media	week	VS.	_fraction	_media	_week	Adj P
post core	MEA	1-6		20 u	MEA	1-6	**0.0007**
post core	MEA	1		20 u	MEA	1	**0.0527**
post core	MEA	2		20 u	MEA	2	0.976
post core	MEA	3		20 u	MEA	3	0.2007
post core	MEA	4		20 u	MEA	4	0.9996
post core	MEA	5		20 u	MEA	5	1
post core	MEA	6		20 u	MEA	6	1
post core	MEA	1-6		1 u	MEA	1-6	**<.0001**
post core	MEA	1		1 u	MEA	1	**0.0026**
post core	MEA	2		1 u	MEA	2	0.974
post core	MEA	3		1 u	MEA	3	0.2065
post core	MEA	4		1 u	MEA	4	0.8991
post core	MEA	5		1 u	MEA	5	0.6565
post core	MEA	6		1 u	MEA	6	0.9679
post core	MEA	1-6		wash	MEA	1-6	**<.0001**
post core	MEA	1		wash	MEA	1	**0.0222**
post core	MEA	2		wash	MEA	2	0.9472
post core	MEA	3		wash	MEA	3	**0.0006**
post core	MEA	4		wash	MEA	4	**0.0096**
post core	MEA	5		wash	MEA	5	**0.0022**
post core	MEA	6		wash	MEA	6	**0.0005**

Table 3-12-10 Sterigmatocystin /erg in removable fractions – fraction comparisons

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	MEA	1-6		1 u	MEA	1-6	0.5626
20 u	MEA	1		1 u	MEA	1	1
20 u	MEA	2		1 u	MEA	2	1
20 u	MEA	3		1 u	MEA	3	1
20 u	MEA	4		1 u	MEA	4	1
20 u	MEA	5		1 u	MEA	5	0.999
20 u	MEA	6		1 u	MEA	6	1
20 u	MEA	1-6		wash	MEA	1-6	0.0012
20 u	MEA	1		wash	MEA	1	1
20 u	MEA	2		wash	MEA	2	1
20 u	MEA	3		wash	MEA	3	0.9995
20 u	MEA	4		wash	MEA	4	0.8227
20 u	MEA	5		wash	MEA	5	0.0707
20 u	MEA	6		wash	MEA	6	**0.0282**
1 u	MEA	1-6		wash	MEA	1-6	0.0586
1 u	MEA	1		wash	MEA	1	1
1 u	MEA	2		wash	MEA	2	1
1 u	MEA	3		wash	MEA	3	1
1 u	MEA	4		wash	MEA	4	0.9981
1 u	MEA	5		wash	MEA	5	0.9976
1 u	MEA	6		wash	MEA	6	0.6049

Table 3-12-11 Sterigmatocystin /erg in removable fractions – fraction comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
pre core	WPA	1-6		20 u	WPA	1-6	**0.0357**
pre core	WPA	1		20 u	WPA	1	**<.0001**
pre core	WPA	2		20 u	WPA	2	0.3872
pre core	WPA	3		20 u	WPA	3	1
pre core	WPA	4		20 u	WPA	4	1
pre core	WPA	5		20 u	WPA	5	1
pre core	WPA	6		20 u	WPA	6	1
pre core	WPA	1-6		1 u	WPA	1-6	0.9898
pre core	WPA	1		1 u	WPA	1	**0.0188**
pre core	WPA	2		1 u	WPA	2	0.9998
pre core	WPA	3		1 u	WPA	3	1
pre core	WPA	4		1 u	WPA	4	1
pre core	WPA	5		1 u	WPA	5	0.9664
pre core	WPA	6		1 u	WPA	6	0.9989
pre core	WPA	1-6		wash	WPA	1-6	**0.0004**
pre core	WPA	1		wash	WPA	1	1
pre core	WPA	2		wash	WPA	2	0.4164
pre core	WPA	3		wash	WPA	3	**0.007**
pre core	WPA	4		wash	WPA	4	0.6848
pre core	WPA	5		wash	WPA	5	1
pre core	WPA	6		wash	WPA	6	0.9941

Table 3-12-12 Sterigmatocystin /erg in removable fractions – fraction comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
post core	WPA	1-6		20 u	WPA	1-6	**0.0018**
post core	WPA	1		20 u	WPA	1	**<.0001**
post core	WPA	2		20 u	WPA	2	1
post core	WPA	3		20 u	WPA	3	0.5754
post core	WPA	4		20 u	WPA	4	**0.0469**
post core	WPA	5		20 u	WPA	5	**0.0005**
post core	WPA	6		20 u	WPA	6	**<.0001**
post core	WPA	1-6		1 u	WPA	1-6	**<.0001**
post core	WPA	1		1 u	WPA	1	**0.0005**
post core	WPA	2		1 u	WPA	2	0.9999
post core	WPA	3		1 u	WPA	3	**0.0312**
post core	WPA	4		1 u	WPA	4	0.1216
post core	WPA	5		1 u	WPA	5	**<.0001**
post core	WPA	6		1 u	WPA	6	**<.0001**
post core	WPA	1-6		wash	WPA	1-6	**<.0001**
post core	WPA	1		wash	WPA	1	1
post core	WPA	2		wash	WPA	2	**<.0001**
post core	WPA	3		wash	WPA	3	**<.0001**
post core	WPA	4		wash	WPA	4	**<.0001**
post core	WPA	5		wash	WPA	5	**0.0005**
post core	WPA	6		wash	WPA	6	**<.0001**

Table 3-12-13 Sterigmatocystin /erg in removable fractions – fraction comparisons.

fraction	media	week	VS.	_fraction	_media	_week	Adj P
20 u	WPA	1-6		1 u	WPA	1-6	0.2
20 u	WPA	1		1 u	WPA	1	1
20 u	WPA	2		1 u	WPA	2	1
20 u	WPA	3		1 u	WPA	3	1
20 u	WPA	4		1 u	WPA	4	1
20 u	WPA	5		1 u	WPA	5	1
20 u	WPA	6		1 u	WPA	6	1
20 u	WPA	1-6		wash	WPA	1-6	**<.0001**
20 u	WPA	1		wash	WPA	1	**<.0001**
20 u	WPA	2		wash	WPA	2	**<.0001**
20 u	WPA	3		wash	WPA	3	**<.0001**
20 u	WPA	4		wash	WPA	4	0.5718
20 u	WPA	5		wash	WPA	5	1
20 u	WPA	6		wash	WPA	6	1
1 u	WPA	1-6		wash	WPA	1-6	**<.0001**
1 u	WPA	1		wash	WPA	1	**0.0003**
1 u	WPA	2		wash	WPA	2	**0.0016**
1 u	WPA	3		wash	WPA	3	**0.0081**
1 u	WPA	4		wash	WPA	4	0.3243
1 u	WPA	5		wash	WPA	5	1
1 u	WPA	6		wash	WPA	6	1