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An Examination of Keratinophilic Fungal on Eastern Bluebird Eggshells

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by

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Abstract

This study was designed to evaluate the mycobiota of Eastern Bluebird eggshells and assess the abundance of keratinophilic fungi. Samples of fungi taken from Eastern Bluebird eggshells were subcultured for identification using macroscopic and microscopic characteristics. The occurrence of a significant number of keratinophilic fungi on Eastern Bluebird eggshells poses a potential threat to developing chicks.

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Introduction

Bird and Egg Characteristics

The Eastern Bluebird, *Sialia sialis*, is found in the United States and southern Canada east of the Rocky Mountains (Pearson & Fuertes 1936). The natural nesting sites of the Eastern Bluebird include deserted cavity nests of woodpeckers, hollows in decaying trees, or crevices in rocks (Pearson & Fuertes 1936). The nests are generally comprised of grass, weed stalks, and small pieces of bark and are lined with finer blades of grass (Pearson & Fuertes 1936). Like many songbirds, Eastern Bluebirds lay one egg a day until the clutch, normally 4 or 5 eggs, is complete. Eastern Bluebird eggs are most often incubated by the female (Sibley et al. 2001) and the typical time of incubation is between 10 and 17 days. Many birds begin incubation before the entire clutch is completed which can lead to mortality of the chicks hatched later (Clark & Wilson 1981; Stoleson & Beissinger 1995). Mortality in later hatched chicks has also been attributed to starvation from scramble competition with larger siblings or sibling interference, to fratricide, infanticide, or even suicide depending on the conditions in the nest (O'Connor 1978).

The avian egg is comprised of several components: blastoderm, yolk, albumen, membranes, and eggshell (Romanoff & Romanoff 1949). The blastoderm is the part of the egg that becomes the embryo following fertilization. The yolk functions as a support structure for the blastoderm and provides vital nutrients for the developing embryo. The albumen serves to stabilize the embryo and the yolk within the egg should it be disturbed as well as aiding in cushioning the inner components of the egg. Inside of the eggshell are membranes that further protect the developing embryo should the shell become

compromised as a result of an impact. The eggshell itself is a relatively smooth, hard, calcareous coat that is attached to the outer membrane of the egg. In order to help the growing chick survive, the eggshell needs to be porous enough to allow respiration but dense enough to prevent the entrance of microorganisms and/or desiccation of the chick. In order to aid in these functions, the eggshell is covered with a protein coat called the cuticle. The cuticle, though still porous to gasses, prevents the entrance of microorganisms through the pores of the eggshell (Romanoff & Romanoff 1949).

Experiments with the eggs of the Pearl-Eyed Thrasher (*Margarops fuscatus*) and the Domestic Fowl (*Gallus domesticus*) showed a positive correlation between eggshell microbial densities and infection of egg contents (Cook et al. 2005a). The infection of egg contents occurred rapidly during the period required to lay a clutch; generally within 3-5 days. While the microbiota of the eggshells included several types of organisms, only pseudomonads and fungi were found to be important in the infection process (Cook et al. 2005a). This is because pseudomonads and fungi are able to digest the cuticle which, in turn, compromises the eggshell's water resistant properties and results in desiccation of the egg (Board & Halls 1973; Board et al. 1979). The destruction of the cuticle can also allow microorganisms to infect and disrupt the development of the chick (Romanoff & Romanoff 1949). Increased eggshell microbial densities resulted in decreased hatching success rate (Cook et al. 2003, 2005b).

The effects of incubation on the microbiota of eggshells are significant. When exposed to ambient conditions, bacteria on thrasher eggs multiplied rapidly over a three day period and the proportion of eggs with microbial groups known to be invasive or to promote trans-shell infection increased. In contrast, when incubated by female Pearl-

Eyed Thrashers, the volume of bacteria and fungi on eggshells decreased and the microbial community was more benign (Cook et al. 2005a). In a study by Haftorn (1988), the point below which no embryonic development takes place in an egg, the physiological zero temperature is between 25-27°C. Birds incubate eggs at a temperature much higher than the physiological zero temperature in order to facilitate faster development of the egg and chick (Haftorn 1988). According to Huggins (1941), bluebirds incubate their eggs at an average temperature of 34.1°C. This higher incubation temperature could potentially produce an environment unfit for fungal and bacterial growth.

Fungal parasites on bird eggs

Konillowicz-Kowalska et al. (2011) found keratinophilic and geophilic dermatophytes in the nests of nine bird species including species of *Trichophyton*, *Microsporum* and *Chrysosporium* (Konillowicz-Kowalska et al. 2011). Among the fungal species isolated were *Microsporum gypseum, M. cookei*, and *C. keratynophilum*, all of which have been shown to cause dermatomycoses; fungal infections of the skin of animals (Konillowicz-Kowalska et al. 2011). Each of these fungi could be a potential pathogen to newly hatched chicks when they are exposed to the nest or eggshell remains.

Additional fungi that are associated with bird feathers, bones and eggshells include members of the Phyla Zygomycota and Deuteromycota. The Zygomycota includes fungi characterized by having aseptate, coenocytic hyphae, no motile stage during their life history, and a saprobic or parasitic existence. Fungal taxa within the Zygomycota reproduce asexually by producing sporangia and sporangiospores and sexually through the fusion of gametangia to form a zygosporangium. The

Deuteromycota, or Imperfect Fungi, are known only to reproduce asexually though the production of conidia. The Dematiaceae and Moniliaceae are Families within the Deuteromycota. Fungi in the Dematiaceae are characterized by having darkly pigmented (e.g., brown or black) conidia, conidiophores and/or hyphae and commonly cause leaf spots, blight, and root rot diseases. Fungi in the Moniliaceae are characterized as having hyaline or lightly pigmented conidia, conidiophores and/or hyphae and commonly cause gray mold, wilt, root rot, blight rot of stored seed, and canker diseases.

Members of these groups of fungi often produce secondary metabolites called mycotoxins that are capable of causing illness and death (Bennett & Klich 2003). Absorption of mycotoxins can occur by inhalation of conidia or hyphal fragments which produce a poorly-understood complex of symptoms (Smith & McGinnis 2009). Mycotoxins can also be inhaled from the air after being released by fungi growing on damp cellulose products (Smith & McGinnis 2009). Eastern Bluebird chicks could be exposed to mycotoxins after they hatch, whether it is through contact with the eggshell , nesting materials or inhalation. At high enough concentrations, mycotoxins may disrupt chick development and result in newborn chicks becoming ill or dying.

In this study, I sampled the mycobiota of Eastern Bluebird eggshells. I hypothesized that keratinophilic fungi will be the predominant fungi isolated from Eastern Bluebird eggshells.

Methods

For this study, eggs of Eastern Bluebirds were sampled from nest boxes at sites less than 8 km from Charleston, Coles County, Illinois. These sites were the Maurer farm (DM), the Furry Pasture (FP), the Gary Allen property (GA), the Charleston Country

Club (GC), the Neil Cole property (NC), the Phoenix Orient property (PO), and the Telecom property (TC).

Wearing gloves cleaned with 90% isopropyl alcohol, a sterile cotton swab was moistened with a 1.0% sterile saline solution. One half of the shell surface of the first-laid egg ("A") was swabbed early on the day of laying. Early on the fourth day after laying, the other half of the egg was swabbed. Opposite halves of the shell were swabbed on each day to avoid skewing the results from the swabbing of the first half of the shell. At the same time, one half of the second-laid egg ("B"), third-laid egg ("C"), fourth laid egg ("D"), and fifth laid egg ("E") was swabbed with the second half being swabbed four days later in order to see how the mycobiota varied with time and order of laying.

In order to compare the microbiota of incubated A-eggs with manipulated eggs that are only exposed to ambient temperatures, a sample microbiota from one-half of the shell surface of the A-egg was taken early on the day of laying. Using sterile procedures as described above, the A-egg was removed, along with the top half of its nest, and placed in an unused nest box. A wire screen was stapled across the box hole to prevent disturbance or contamination of the egg. After four days, the other half of the eggshell was swabbed and the egg was replaced in the original nest. The possible accumulation of microbes in nest boxes used to hold eggs was controlled for by utilizing unused nest boxes of different ages.

Swabs of eggshells were placed in a 1 mL of 1.0% sterile saline solution and stored temporarily in a cooler in the field. Within 4 hours of sampling, 0.1 mL of the 1.0% saline solution was streaked onto two plates of MacConkey agar. This medium selects for gram-negative, enteric bacteria which are common pathogens of avian

embryos (Cook et al. 2003, 2005a, Shawkey et al. 2009). The plates were incubated aerobically for five days at 35°C. Each of the plates were marked with a replicate number, nest number, and the egg from which the swab was taken. For example, in (2) DM-4c A, (2) is the replicate number, DM-4c is the nest number, and A is the egg the swab was taken from.

A subset of "B," "C," "D," and "E" eggs were sampled on the day of laying and, using the above methods, the samples were cultured on two plates of each of trypticase soy agar (TSA) and MacConkey agar. TSA agar is used as a general purpose plating medium for culturing non-specific bacteria.

After the initial period of incubation, each plate was examined. Any fungal colonies observed were subcultured using sterile technique onto plates of potato dextrose agar (PDA). These plates were sealed and placed in an incubation chamber for several weeks at 24°C on a 12 hour light: dark cycle. Each plate was marked with the replicate number, nest number, date of original culture, and date of subculture.

Fungal cultures were observed with the naked eye for characterization of morphological features (Appendix A). Images of fungal cultures were recorded using a Nikon Coolpix 995 (Appendix B). Semi-permanent slides of fungal colonies that developed on PDA plates were prepared by placing a small sample of the hyphae in a drop of polyvinyl alcohol-lactic acid glycerol (PVLG) and covering the sample with a glass cover slip. The slides were heated at approximately 50°C for a week on a slide warmer and then stored in slide trays. The slides were observed with an Olympus BX51compound microscope in order to assess microscopic features and identify the fungus growing on each egg. Photomicrographs of micromorphological characters were

prepared using Spot Insight (Appendix C). Fungi were identified using *The Genera of Hyphomycetes* (Seifert et al. 2011), *The Genera of Hyphomycetes* from Soil (Barron 1968), and *Illustrated Genera of Imperfect Fungi* (Barnett and Hunter 1998).

Results

Fungal diversity across all localities was compiled (Table 1). *Alternaria* was the only fungus found at all localities with the remaining genera sparsely interspersed among localities. *Alternaria* also proved to be the most prevalent with respect to order of laying (Table 2). The largest numbers of Alternaria cultures were made on the A-egg and may be the result of the habit of Eastern Bluebirds not to incubate their eggs until the end of the laying of the clutch (e.g., D-egg or E-egg). A comparison was made between the number of keratinophilic and non-keratinophilic fungi isolated at each site (Figure 1). The genus of fungi that occurred with the most frequency across all sites was *Alternaria*, a keratinophilic fungus, at a rate of occurrence of 66.39%. The family that occurred most frequently was the Dematiaceae, at a rate of occurrence of 84.03%. The rate of occurrence of keratinophilic fungi was 71.43%, while the rate of occurrence of non-keratinophilic fungi was 28.57%.

Discussion

A larger portion of the fungi cultured from Eastern Bluebird eggshells were keratinophilic. A high percentage of keratinophilic fungi were expected due to loose feathers and other keratin-containing material found in and around the nest. Kornillowicz-Kowalska et al. (2011) found a mean colonization rate of keratinophilic fungi in the nests of nine bird species of approximately 75%. Mean egg colonization by keratinophilic fungi reported by Kornillowicz-Kowalska et al. (2011) is slightly higher

than the 71.43% occurrence of keratinophilic fungi that we observed. Bluebird nests contain feathers from parent birds and other keratin containing organic compounds that the keratinophilic fungi break down and consume. This allows fungi such as *Alternaria* and *Chrysosporium* to thrive in an environment such as an Eastern Bluebird nest box.

Gliotoxins are produced by species of *Penicillium* and *Trichoderma*. Gliotoxins have demonstrated immunosuppressive potential, including the ability to inhibit phagocytosis by macrophages and antigen-mediated activation of lymphocytes (Lewis et al. 2005). Trichothecenes are a group of extremely toxic mycotoxins produced by species of *Fusarium* and *Trichoderma* in airborne conidia. Trichothecenes inhibit protein synthesis at the initiation and termination stages (Smith & McGinnis 2009). Ochratoxin A is produced by some species of *Penicillium*. Ochratoxin A is a nephrotoxin, a liver toxin, an immune suppressant, a potent agent of malformation of embryos, and a carcinogen (Bennett and Klich 2003). Ochratoxin A, gliotoxins, and trichothecenes could potentially be associated with the nests of Eastern Bluebirds due to the presence of *Penicillium*, *Trichoderma*, and *Fusarium* within the nest and on the eggshells. These toxins could be extremely harmful to developing and newly hatched chicks.

Future studies could focus solely on the mycobiota of eggshells rather than selecting for bacteria. In fact, MacConkey Agar and TSA, selective media for bacteria, could reduce the abundance of fungi that are isolated from eggshells. A second are of stud could be to examined differences between the mycobiota of incubated eggs and eggs exposed to ambient temperatures.

Conclusions

Eggshells and biological materials within the nest are composed largely of keratin. As a result, a large number of fungi isolated were keratinophilic. The high occurrence of keratinophilic fungi growing on the eggshells of Eastern Bluebirds poses a threat to the developing chick.

Table 1. Fungal diversity across all localities. Fungi found on Eastern Bluebird eggshells on the Maurer farm (DM), the Furry Pasture (FP), the Gary Allen property (GA), the Charleston Country Club (GC), the Neil Cole property (NC), the Phoenix Orient property (PO), and the Telecom property (TC).

	DM	GA	GC	FP	NC	PO	TC	Total	Average	Standard Deviation
Alternaria	2	11	10	19	21	4	12	79	11.28571429	7.016986193
Aureobasidium	0	1	2	1	0	1	0	5	0.714285714	0.755928946
Chaetophoma	0	0	0	1	0	0	1	2	0.285714286	0.487950036
Chrysosporium	1	0	0	1	0	0	0	2	0.285714286	0.487950036
Cladosporium	0	0	0	0	0	0	1	1	0.142857143	0.377964473
Curvularia	0	0	1	0	0	0	1	2	0.285714286	0.487950036
Fusarium	0	1	0	2	0	0	0	3	0.428571429	0.786795792
Microsporum	0	1	0	0	1	0	2	4	0.571428571	0.786795792
Mucor	0	0	0	0	2	0	0	2	0.285714286	0.755928946
Oidiodendron	0	0	0	1	0	0	0	1	0.142857143	0.377964473
Penicillium	0	0	0	0	0	0	2	2	0.285714286	0.755928946
Pestalotiopsis	0	0	0	1	0	0	0	1	0.142857143	0.377964473
Rhizopus	0	0	3	0	0	0	1	4	0.666666667	1.211060142
Scopulariopsis	0	0	0	0	1	0	0	1	0.142857143	0.377964473
Torula	0	0	0	1	0	0	0	1	0.142857143	0.377964473
Trichoderma	0	0	0	0	0	0	1	1	0.142857143	0.377964473
Ulocladium	0	1	0	1	6	0	0	8	1.142857143	2.193062655
Total	3	15	16	28	31	5	21	119	17	10.63014581

Table 2. Fungal diversity as a function of egg order. A = First-laid egg, E = Last-laid egg.

	A	**B**	**C**	**D**	**E**	**Total**	**Average**	**Standard Deviation**
Alternaria	48	10	0	15	6	79	15.8	18.82020191
Aureobasidium	3	1	0	0	1	5	1	1.224744871
Chaetophoma	2	0	0	0	0	2	0.4	0.894427191
Chrysosporium	2	0	0	0	0	2	0.4	0.894427191
Cladosporium	1	0	0	0	0	1	0.2	0.447213595
Curvularia	1	1	0	0	0	2	0.4	0.547722558
Fusarium	2	1	0	0	0	3	0.6	0.894427191
Microsporum	2	2	0	0	0	4	0.8	1.095445115
Mucor	2	0	0	0	0	2	0.4	0.894427191
Oidiodendron	0	0	0	0	1	1	0.2	0.447213595
Penicillium	2	0	0	0	0	2	0.4	0.894427191
Pestalotiopsis	0	0	0	0	1	1	0.2	0.447213595
Rhizopus	4	0	0	0	0	4	0.8	1.788854382
Scopulariopsis	0	0	0	0	1	1	0.2	0.447213595
Torula	1	0	0	0	0	1	0.2	0.447213595
Trichoderma	1	0	0	0	0	1	0.2	0.447213595
Ulocladium	5	3	0	0	0	8	1.6	2.302172887
Total	76	18	0	15	10	119	23.8	29.96998498

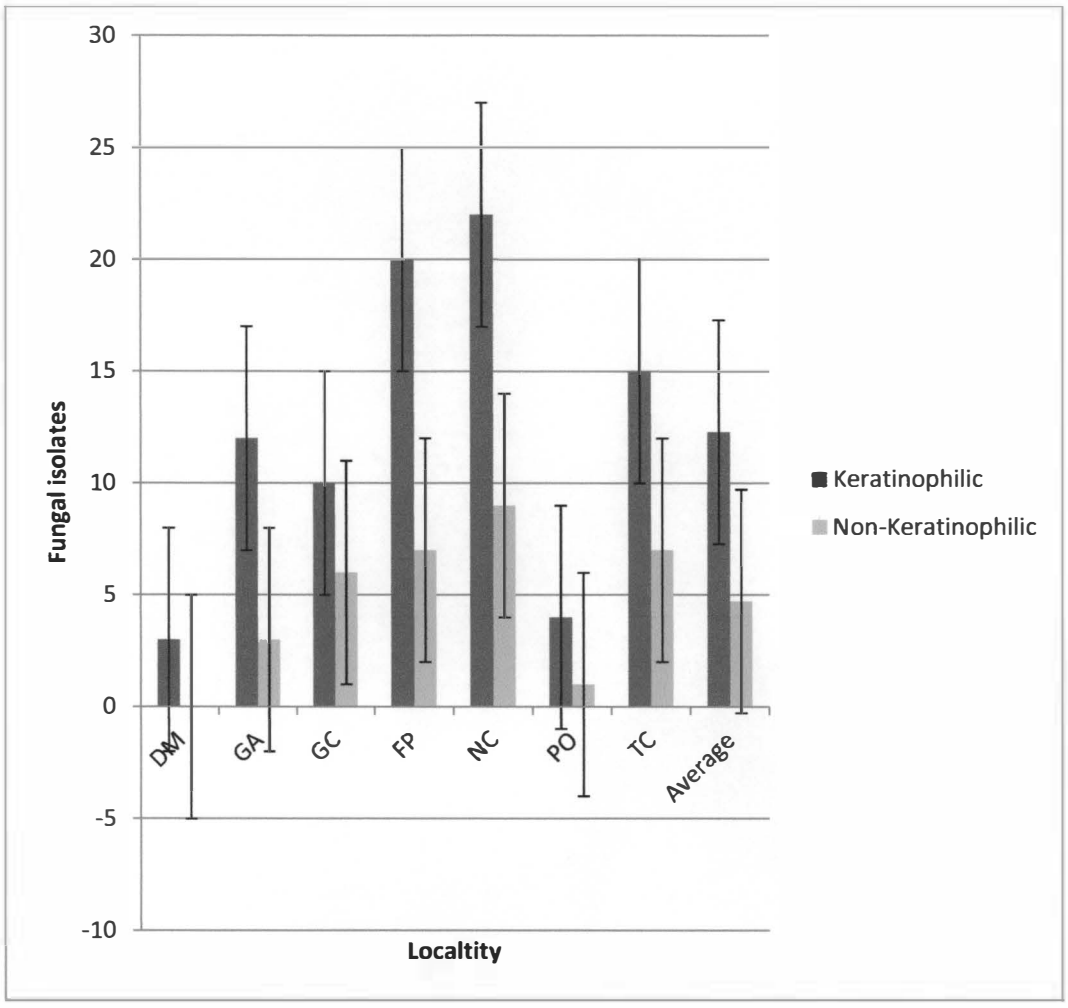


Figure 1. Comparison of Keratinophilic and Non-Keratinophilic Fungi on Eastern Bluebird eggs at the Maurer farm (DM), the Furry Pasture (FP), the Gary Allen property (GA), the Charleston Country Club (GC), the Neil Cole property (NC), the Phoenix Orient property (PO), and the Telecom property (TC).

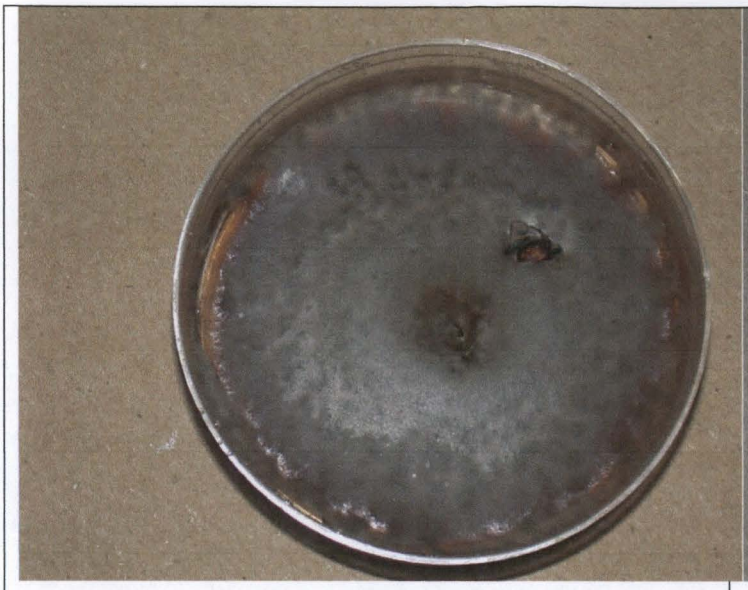
Appendix A. Morphological Characteristics of Fungi Isolated.

	Colony Color	**Reverse Surface**	**Hyphae**	**Reproductive Structures**	**Spores**
Alternaria	Grey to green-black with pale margin	Brown to black	Septate, brown	Simple, brown conidiophores	Transversely and longitudinally septate, solitary or in chains, ovoid to clavate, roughened, brown conidia
Aureobasidium	Mucus-like, white to yellow, then brown to black with grey margin	Pale grey to black	Septate, hyaline to brown	Lacking distinct conidiophores	Oval to cylindrical, unicellular, budding, hyaline conidia
Chaetophoma	Pale to dark green	Brown to black	Septate, hyaline to pale olive	Short, hyaline conidiophores borne in brown pycnidia	Ovoid, unicellular conidia
Chrysosporium	White	Pale yellow	Septate, hyaline	Poorly differentiated conidiophores	Globose to pyriform, intercalary, hyaline, thick-walled conidia
Cladosporium	Olive-green to black, velvety	Black	Septate, brown to black	Branched, brown conidiophores	Ellipsoid to cylindric or irregular, some lemon-shaped, unicellular, olive-brown conidia
Curvularia	Dark green	Dark brown to black	Septate, hyaline to brown	Simple conidiophores with apical hila	Transversely septate, brown conidia with enlarged central cell

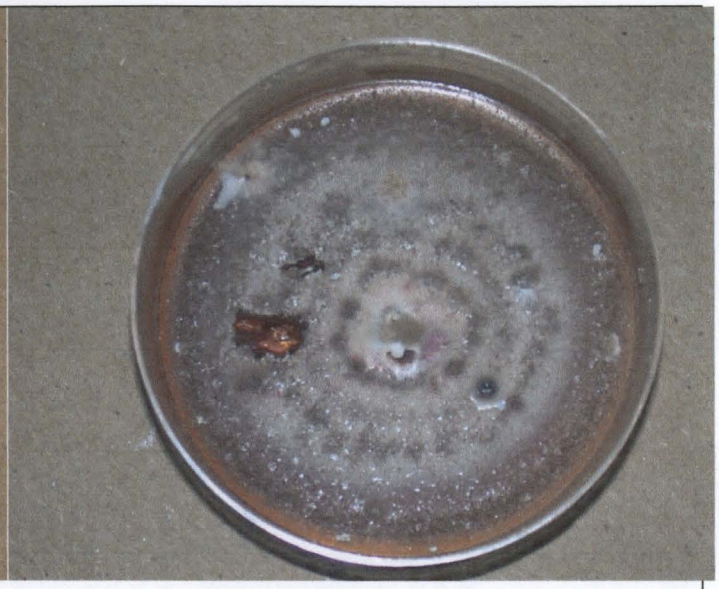
Fusarium	White to pink	Pink center with white margins	Septate, hyaline	Slender, solitary, simple conidiophores	Macroconidia several-celled, canoe-shaped, hyaline; microconidia ovoid to oblong, hyaline
Microsporum	White to grey	Pale yellow	Septate, hyaline	Slender, simple, determinate conidiophores	Solitary, fusoid, several-celled, hyaline macroconidia
Mucor	Grey to brown, fluffy	Pale yellow	Aseptate, hyaline without rhizoids	Hyaline sporangiophore; hyaline, round sporangia with columella	Unicellular, ovoid to ellipsoid, smooth to rough, hyaline to pale brown
Oidiodendron	White	Pale yellow	Hyaline, septate	Sparsely branched, hyaline conidiophores segmenting into conidia	Chains of unicellular, fusoid, hyaline conidia
Penicillium	Blue-green, grey-green, olive-grey	Yellow	Septate, hyaline	Hyaline conidiophores with brush-like phialides	Round, unicellular, hyaline, chains of conidia
Pestalotiopsis	Olive-green	Olive – green to yellow	Septate, hyaline	Short, simple conidiophores; borne in brown pycnidia	Ellipsoid to fusoid, multicellular, brown conidia with hyaline apical appendages
Rhizopus	White to grey, fluffy	Pale yellow	Aseptate, hyaline with rhizoids	Hyaline sporangiophore; hyaline, round sporangia with columella	Unicellular, round to ovoid, smooth or striate, hyaline to pale brown sporangiospore

Scopulariopsis	Light brown	Light brown	Aseptate, hyaline	Hyaline, branched, conidiophores with clustered, annellate conidiogenous cells	Unicellular, globose with truncated base, hyaline conidia produced in basipetal chains
Torula	White to grey or light brown	Light brown	Septate, brown	Short, simple, brown conidiophores	Unicellular to multicellular, chains of brown conidia
Trichoderma	White, then with rings of green	Tan to yellow	Septate, hyaline	Conidiophores with solitary or clustered, flask-shaped phialides	Round to ellipsoid, unicellular, smooth to roughened, green conidia
Ulocladium	Olive-brown to black	Olive-brown to black	Septate, brown	Simple, sympodial, septate, brown conidiophores	Transversely and longitudinally septate, solitary, oval to round, brown conidia

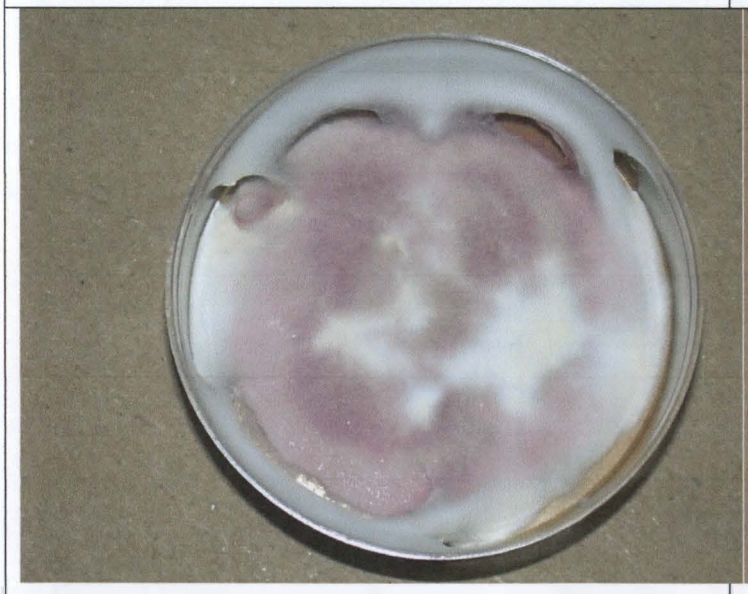
Appendix B. Photographs of Macrophorphology



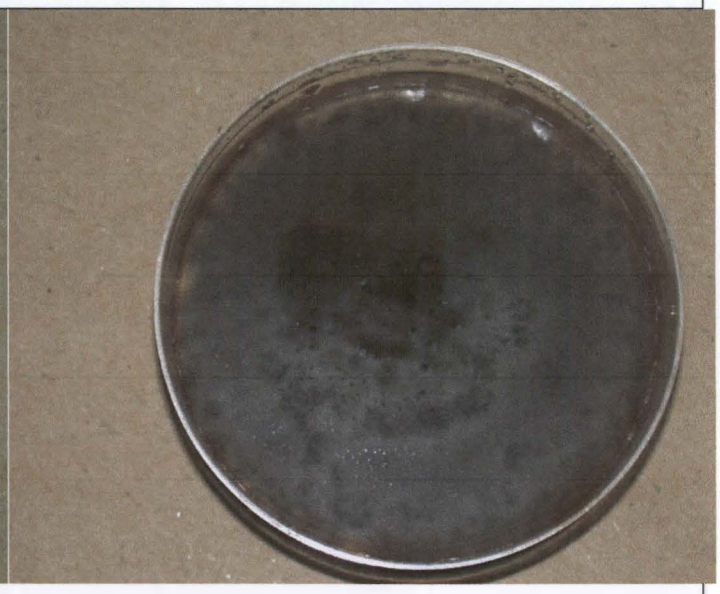
Alternaria



Aureobasidium

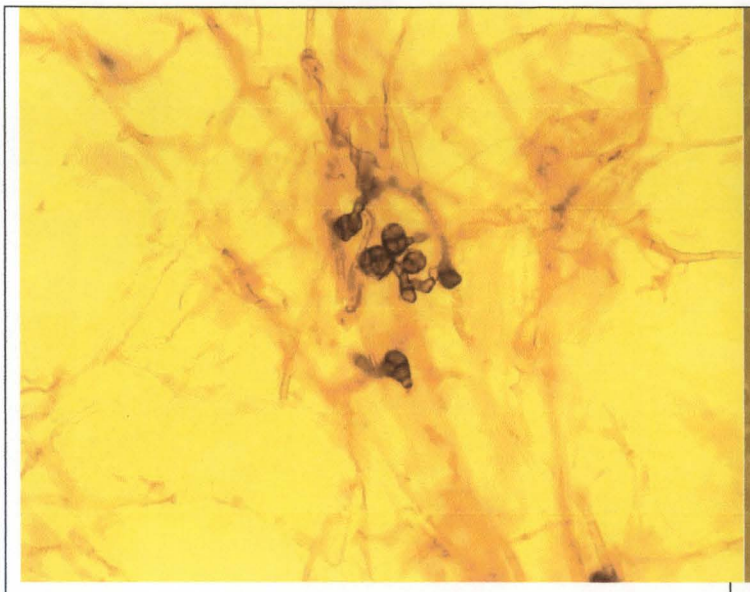


Fusarium

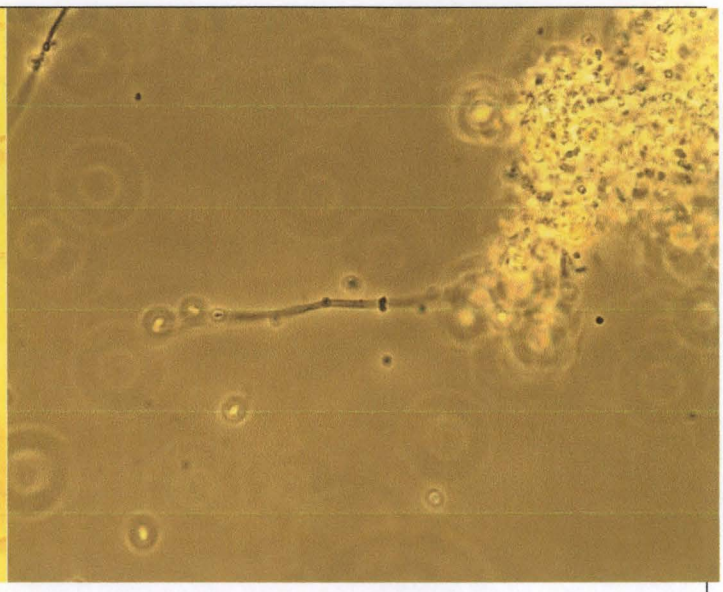


Ulocladium

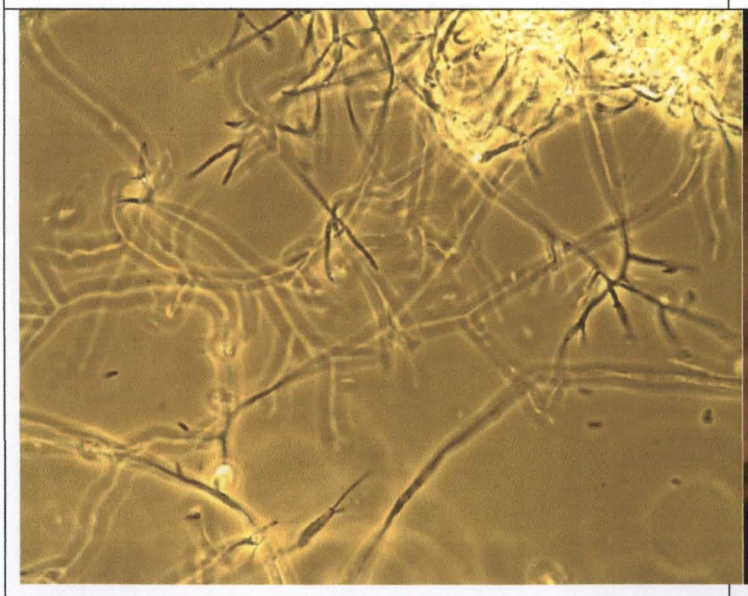
Appendix C. Photomicrographs of Microphorphology



Alternaria (400x)



Aureobasidium (400x)



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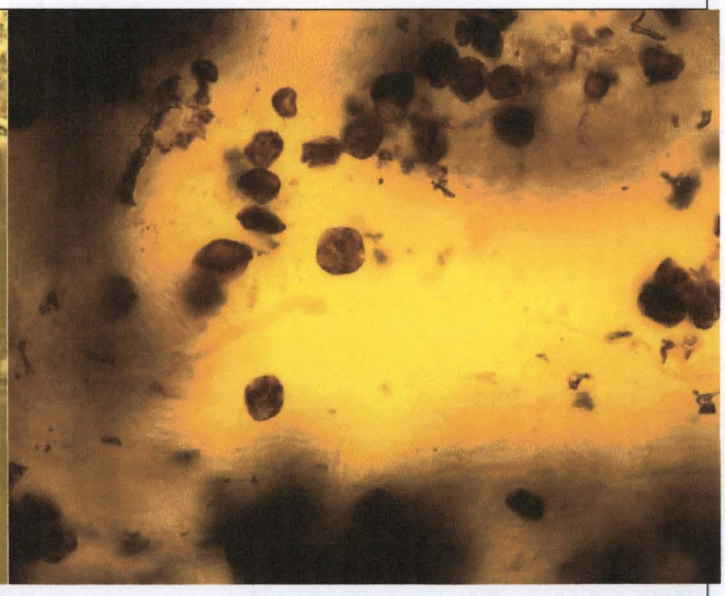

Ulocladium (400x)

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