Original Research Paper

Viable airborne fungi identified in a small town, public building in West Virginia

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Article history
Received: 20 July 2023
Revised: 26 August 2023
Accepted: 31 August 2023

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Abstract: We spend about 90% of our time indoors. The air we breathe contains fungi and other microbes. Airborne fungal communities have been studied in air quality investigations of complaint buildings in response to occupant health issues, water damage, or visible mold growth; however, few studies of the airborne fungal community in non-complaint buildings have been conducted. Moreover, almost all studies of indoor airborne fungal communities are conducted in large cities in urban locations. This study describes a baseline survey of the viable airborne fungi found inside a public building in a small rural town. Fungi were captured passively using an open plate method and isolates were identified to putative taxa through sequence of the nuclear ribosomal internal transcribed spacer (ITS) region. We sampled in two different years (2020 and 2022) and sampled the outside airborne fungal community in 2022. Eighteen fungal taxa were isolated from indoor air; five of these taxa were identified only in 2020, eight taxa were only identified in 2022, and five taxa were identified in both years. *Cladosporium* was the most commonly isolated genus from indoor air. We isolated 17 different fungal taxa in 2022; nine were only captured indoors, four were only captured outside, and four were captured both indoors and outside. This study provides a baseline survey of fungal taxa found inside a public building in a rural location, the variability between years, and the impact of outside air.

Keywords: Indoor air; fungi.

Introduction

There are an estimated 2.2 – 3.8 million species of fungi, though only about 120,000 species have been identified, and even fewer species have been well characterized (Hawksworth & Lücking, 2017). Microbes, including fungi, are generally less studied than the larger, charismatic megafauna and flora. For example, the IUCN (International Union for Conservation of Nature) Red List of Threatened Species has evaluated only 0.5% (640 species) of the estimated 141,541 described species in the Fungi & Protists category, compared to 81% of vertebrates, 2% of invertebrates, and 15% of plants (IUCN, 2022). Despite this paucity of information, fungi are extraordinarily important across ecology, agriculture, industry, and medicine, with both enormous potential benefits and significant concerns. There is growing awareness of the threats fungi pose to human health (Fisher et al., 2020) and the World Health Organization recently released the first WHO fungal priority pathogens list (World Health Organization, 2022).

People are continually exposed to fungal propagules (spores and hyphal fragments) through inhalation of air both indoors and outside. Many species of fungi reproduce through the production of spores, many of these spores are wind-dispersed, and some fungi produce prodigious amounts of spores. A study in the British city of Cardiff found that there are ~85,000 fungal spores per cubic meter of air and it has been suggested that people inhale spores with every breath (Flannigan et al., 2011). In general, the abundance of fungal propagules is reduced inside

compared to outdoors (Shelton et al., 2002). Building heating, ventilation, and air conditioning systems dramatically reduce fungal abundance and species diversity in indoor air (Burge et al., 2000; Horner et al., 2004; Kemp et al., 2003; Kozak et al., 1980; Myers et al., 2021; Solomon et al., 1980; Spiegelman et al., 1963).

Human activity can impact the species of fungi found in indoor air, but the primary source of indoor fungi is the outdoor environment (Adams et al., 2013; Lehtonen et al., 1993; Li & Kendrick, 1995, 1996). A global study of indoor fungi found that geographic location, particularly latitude, is the main predictor of fungal diversity; the type of building does not significantly impact the fungal community (Amend et al., 2010). A nationwide study of fungal diversity in dust samples collected from the outside of houses across the United States identified regionally different fungal communities primarily determined by environmental factors such as precipitation, diversity of vascular plants, and temperature (Barberán, Ladau, et al., 2015). A few genera were cosmopolitan across the United States (*Cladosporium*, *Toxicocladosporium*, and *Alternaria*), but most taxa displayed regionally restricted distribution and many of the taxa isolated had not been previously described (Barberán, Ladau, et al., 2015). Building use factors such as the number of occupants, gender, or the presence of pets impacts the bacterial community composition of indoor air, but has only a limited impact on the fungal community composition of indoor air (Barberán, Dunn, et al., 2015).

Fungal spores for some species (for example, the rust fungus *Puccinia graminis*) can move hundreds to thousands of kilometers by wind (Hirst & Stedman, 1967; Kolmer, 2005). However, dispersal distances are species-dependent and for many species, long-distance dispersal of spores is limited (Golan et al., 2017). A study of ectomycorrhizal fungi found that the diversity of fungal species and the abundance of spores dropped significantly at distances greater than one km from the forest edge (Peay et al., 2012). As such, the fungal species found in airborne propagules is geographically local and dependent upon the local habitat.

Asthma, allergies, and other respiratory symptoms may be associated with indoor air quality. The fungus *Alternaria alternata* is associated with fungal allergies and common in household dust (Salo et al., 2005). Respiratory symptoms are correlated with the abundance of *Penicillium* in indoor air and with the abundance of *Alternaria*, *Penicillium*, and *Cladosporium* in indoor dust (Behbod et al., 2013). However, it is not clear whether resuspended dust is a relevant exposure route for indoor fungi (Nazaroff, 2016).

Water damage alters the fungal community composition of indoor air and increases the indoor fungal spore counts (Chakravarty, 2022; Miller et al., 2000; Niemeier et al., 2006; Rao et al., 2007). In five out of six US cities surveyed, more than 50% of homes exhibited signs of dampness and home dampness was correlated with respiratory symptoms in children (Brunekreef et al., 1989). Damp buildings and visible mold are associated with asthma development (Cho et al., 2006; Gent et al., 2002); however, exposure to house dust with low fungal diversity is also significantly correlated with later development of asthma (Dannemiller et al., 2014).

While many air-quality investigations have focused on buildings with reported occupant health issues, water damage, or visible mold growth, there are few surveys of the normal baseline indoor air fungal community from non-complaint buildings. For example, Shelton *et al.* (Shelton et al., 2002) investigated indoor air fungi from 1717 buildings across the United States; these samples were collected as part of building investigations, generally due to reported issues. A few studies have investigated the airborne fungal community in non-complaint buildings in the United States; most of these studies are restricted to buildings in a single city (Adams et al., 2013; Horner et al., 2004; Lee et al., 2006; Reynolds et al., 2001). In 1994-1998, the US EPA conducted the Building Assessment Survey and Evaluation (BASE) study. Indoor air quality, including airborne fungi, was investigated in 100 non-complaint office buildings across the United States. These office buildings were in 37 cities across 25 continental states; the cities had a population of >100,000 and were primarily in urban (73%) or suburban (23%) locations (Burton et al., 2000). All of these studies were conducted in large metropolitan cities and most of these large cities were in urban or suburban locations. Our study focused on a small town in a rural area.

The objective of this study is to document the viable airborne fungal community in a non-complaint public building located in a small town in rural West Virginia. In this baseline study, we

investigated inter-year variation of the indoor fungal community and examined the contribution of outside airborne fungal propagules to the indoor fungal community.

Materials and Methods

Site Description

The study was conducted in a teaching laboratory in the Byrd Science Center on the Shepherd University campus. Shepherd University is in Shepherdstown, West Virginia, a small town (population ~1500) located in Jefferson County, which is approximately 6% urban and 94% rural, according to the 2020 Census (U.S. Census Bureau, n.d.).

The teaching laboratory is two and a half floors above a parking lot, next to grassy areas, flower gardens, and trees. The lab is temperature-controlled (heating and air-conditioning); however, there are three large windows within the lab that are closed, but not sealed. The laboratory is used to teach the laboratory component of a variety of biology courses including introductory biology, microbiology, genetics, and mycology; approximately 70 different people use the lab during a given semester. In September 2020, social distancing protocols were in place due to the COVID-19 pandemic and only 20-25 different people used the lab in a given week. In September 2022, student traffic was back to normal.

2020 Survey - Indoor

In September 2020, airborne fungi were captured at four sites within the teaching lab and one site in the small, adjacent prep room. These sites were located away from the windows to avoid capturing fungi blown inside on the draft under the window. At each site, two 100 mm petri dishes of Potato Dextrose Yeast Agar (PDYA), two 100 mm petri dishes of Malt Extract Yeast Peptone Agar (MYPA), and two 100 mm petri dishes of Brain-Heart Infusion Agar (BHIA, Carolina Biological cat. no. 781781) were left open to the air for seven hours. Capture plates were closed and incubated at room temperature for three days.

PDYA was made using Potato Dextrose Agar (Carolina Biological cat. no. 786341) with the addition of two grams yeast extract (Fisher Bioreagents cat. no. 7064) per liter of media. MYPA was made using Malt Extract Agar (MP Biomedicals cat. no. 1006817) with the addition of two grams yeast extract (Fisher Bioreagents cat. no. 7064) and one gram peptone (Fisher Bioreagents cat. no. 7063) per liter of media.

2022 Survey – Indoor and Outside

In September 2022, viable airborne fungi were captured inside the teaching laboratory at two of the same sites used in 2020 and a third site on the inside windowsill. Fungi were captured from two sites outside the teaching laboratory: the outside windowsill and a picnic table located on a grassy area immediately outside the Byrd Science Center. At each indoor site, two 100 mm petri dishes of Potato Dextrose Agar (PDA, Becton Dickinson cat. no. 213400) and two 100 mm petri dishes of Malt Extract Agar (MEA, MP Biomedicals cat. no. 1006817) were left open to the air for two hours. For the outdoor sites, three PDA plates and three MEA plates were left open for two hours at each site. Capture plates were incubated at room temperature for five days.

Isolation of captured fungi

From each survey, representative colonies were selected from across the capture plates based on morphological diversity. Individual fungal colonies were transferred from capture plates to fresh culture medium to isolate pure cultures. Isolates were grown on the same culture medium used for capture. Several isolates required additional passages to achieve a pure culture. Pure cultures were grown for DNA extraction and preserved as glycerol stocks.

Identification by DNA Barcode

Mycelium and conidia (if present) were scraped from the surface of approximately one third a culture plate for DNA extraction. DNA was extracted using the Quick DNA Fungal/Bacterial Miniprep Kit (Zymo Research), according to manufacturer's instructions, with the following modification: cell walls were disrupted using two 20-second cycles on a Sonibeast homogenizer (BioSpec). The nuclear ribosomal internal transcribed spacer (ITS) was PCR amplified using 2.5 µL extracted DNA as template, ITS1 and ITS4 primers (Fungal Primer Mix, Carolina Biological), and Illustra PuReTaq Ready-to-Go PCR beads. Amplification protocol consisted of 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C.

Crude PCR product was sequenced in both directions using the M13F and M13R primers

(Genewiz/Azenta Life Science). Each sequence was compared to the Genbank rRNA/ITS fungi database using the blastn program to determine putative genera of fungal isolates. For each isolate, the putative taxon was based on evaluation of the top hits with smallest e-value. All ITS sequences were submitted to Genbank: 31 isolates from September 2020 indoor survey have Genbank (GB) accession numbers OR140017 – OR140047, 21 isolates from September 2022 indoor survey have GB accession numbers OR140048 – OR140070, and 14 isolates from September 2022 outside survey have GB accession numbers OR140071 – OR140084.

Results

Identification of fungi by DNA Barcode

Sixty-six fungal isolates were identified to putative taxa by ITS sequence (Table 1). For 59 of the isolated fungi, comparison of the sequenced ITS region to the Genbank rRNA/ITS fungi database resulted in multiple top blast hits with e-value zero in a single genus. Two isolates did not return any hits with e-value zero. The top match for one isolate captured indoors in 2020 (GB accession number OR140040) was *Stachybotrys limonispora* (GB accession number NR_156604) with e-value 2e-174 and 87% identity; this isolate had other top-scoring matches to different genera in the Hypocreales order, so we assigned this isolate to the Hypocreales order. The top two hits for an isolate captured indoors in 2022 (GB accession number OR140064) were *Pyrenochaetopsis microspora* (GB accession number NR_160059) and *P. indica* (GB accession number NR_160058), both with e-value 3e-172 and approximately 89% identity; we assigned this isolate to the *Pyrenochaetopsis* genus.

Three isolates captured indoors in 2022 (GB accession numbers OR140052, OR140056, and OR140065) returned only one match with e-value zero: *Xenoacrodontium juglandis* (GB accession number NR_175240) with approximately 90% identity. These three isolates were isolated from three separate capture plates on different culture media and different locations within the laboratory. Because the percent identity over the ITS region was only 90%, we assigned these isolates to the genus *Xenoacrodontium.*

Two isolates returned multiple matches with e-value zero, but the matches were to different genera within a family. One isolate (Genbank accession number OR140018) captured indoors in 2020 had more than one hundred matches with e-value zero to different genera in the family Didymellaceae. A second isolate (GB accession number OR140062), captured indoors in 2022, returned matches with e-value zero to a variety of genera in the Cordycipitaceae family.

Table 1. Putative taxa determined by ITS sequence of fungi isolated indoors in 2020, indoors in 2022, and outside in 2022. Numbers indicate the number of separate fungal isolates from each survey identified to each taxon. Most isolates had a clear identification to putative genus by ITS sequence; identification to putative taxa for seven isolates (*) is explained further in the text.

Putative taxa	Indoor 2020	Indoor 2022	Outside 2022	Totals
Alternaria	2			2
Aureobasidium			2	2
Botrytis		1		1
Cercospora	1			1
Cladosporium	10	4	1	15
Cordycipitaceae		1*		1
Didymella	4		1	5
Didymellaceae	1*			1
Entomocorticium		1		1
Epicoccum	3	1	3	7
Fusarium	3	1		4
Mucor			3	3
Myrmecridium		1		1
Nigrospora			1	1
Penicillium	2	4	1	7
Pestalotiopsis		1	2	3
Pseudopithomyces	4	1		5
Pyrenochaetopsis		1*		1
Hypocreales	1*			1
Toxicocladosporium		1		1
Xenoacrodontium		3*		3
Totals	**31**	**21**	**14**	**66**

Indoor viable airborne fungi

The 31 isolates captured in the indoor 2020 survey represent ten different taxa, with the genus *Cladosporium* the most commonly identified (Table

1). The genera *Alternaria*, *Cladosporium*, *Didymella*, *Epicoccum*, *Fusarium*, *Penicillium*, and *Pseudopithomyces* were captured more than once. Twenty-one isolates, representing thirteen taxa were identified in the indoor 2022 survey; *Cladosporium*, *Penicillium*, and *Xenoacrodontium* were captured multiple times (Table 1).

Across both 2020 and 2022, 18 different taxa were identified indoors; however, only five taxa (*Cladosporium*, *Epicoccum*, *Fusarium*, *Penicillium*, and *Pseudopithomyces*) were captured indoors in both years (Table 2). Five taxa were captured indoors in 2020 only and eight taxa were captured indoors in 2022 only (Table 2).

Table 2. Year-to-year variation of fungal taxa identified indoors. Taxa isolated indoors in the 2020 survey, but not the 2022 survey, are listed in the **2020 only** column. Taxa isolated indoors in both 2020 and 2022 surveys are listed in the **Both years** column. Taxa isolated indoors in the 2022 survey, but not the 2020 survey, are listed in the **2022 only** column.

2020 only	Both years	2022 only
Alternaria	*Cladosporium*	*Botrytis*
Cercospora	*Epicoccum*	Cordycipitaceae
Didymella	*Fusarium*	*Entomocorticium*
Didymellaceae	*Penicillium*	*Myrmecridium*
Hypocreales	*Pseudopithomyces*	*Pestalotiopsis*
		Pyrenochaetopsis
		Toxicocladosporium
		Xenoacrodontium

Outside air and indoor fungi

To investigate the contribution of outside air to the indoor fungi, the 2022 survey included the inside windowsill of one of the teaching lab windows and two outside sites, including the outside windowsill of the same window. This window is closed; however, the window is not sealed and there is a visible gap and detectable draft under this window.

Overall, there were visibly more fungal colonies on the outside capture plates (Figure 2) than the indoor capture plates (Figure 1). Indoors, the number of colonies captured decreased at sites further away from the windowsill. More colonies grew on the indoor windowsill capture plates (Figure 1 panels A and B) than on the capture plates from the other two indoor sites (Figure 1, panels C-F).

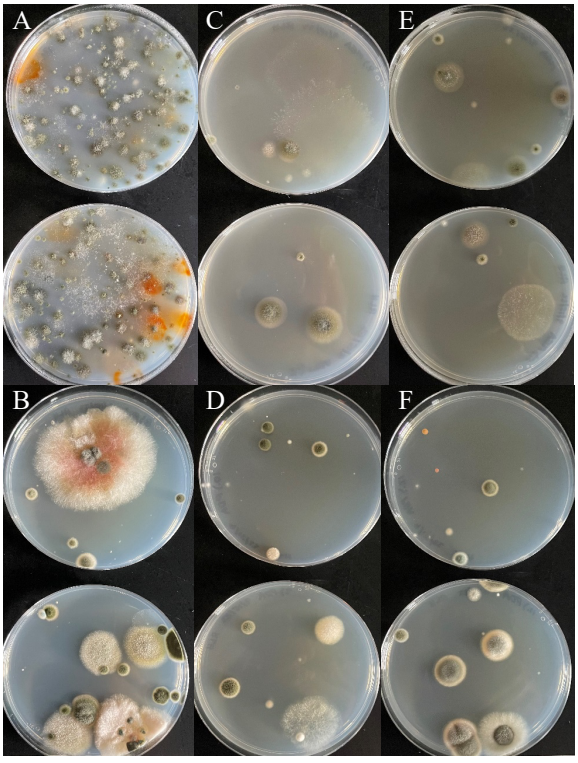

Figure 1. Capture plates from Indoor 2022 survey. Four capture plates from site 1 (panels A, B), site 2 (panels C, D), and site 3 (panels E, F). Site 1 is the indoor windowsill. Panels A, C, and E are fungi captured at each site on Malt Extract Agar. Panels B, D, and F are fungi captured at each site on Potato Dextrose Agar.

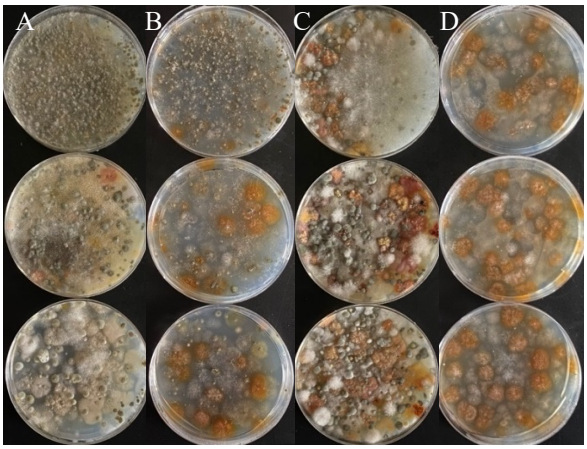

Figure 2. Capture plates from Outside 2022 survey. Six capture plates from outside site 1 (panels A, B) and six capture plates from outside site 2 (panels C, D). Site 1 is the outside windowsill. Panels A and C are fungi captured at each site on Potato Dextrose Agar. Panels B and D are fungi captured at each site on Malt Extract Agar.

Due to the density of colonies on the outdoor capture plates, it was difficult to isolate pure cultures. Only fourteen colonies were isolated and

identified by ITS sequence: 11 from the outdoor windowsill and three from a second outside site near the science building. Eight taxa were identified outside, with the genera *Aureobasidium*, *Epicoccum*, *Mucor*, and *Pestalotiopsis* identified more than once (Table 1).

Of the seventeen taxa captured in 2022, only four taxa (*Cladosporium*, *Epicoccum*, *Penicillium*, and *Pestalotiopsis*) were isolated both indoors and outside (Table 3). Nine taxa from the 2022 survey were captured only indoors and four taxa were captured only outside (Table 3). While *Didymella* was isolated only outside in 2022, it was captured indoors in 2020 (Table 1).

Table 3. Taxa captured in the 2022 survey indoors only, outside only, or both indoors and outside.

Indoor only	Indoor & outside	Outside only
Botrytis	*Cladosporium*	*Aureobasidium*
Cordycipitaceae	*Epicoccum*	*Didymella*
Entomocorticium	*Penicillium*	*Mucor*
Fusarium	*Pestalotiopsis*	*Nigrospora*
Myrmecridium		
Pseudopithomyces		
Pyrenochaetopsis		
Toxicocladosporium		
Xenoacrodontium		

Discussion

The ITS region is the accepted DNA barcode for universal studies across Kingdom Fungi and can generally identify isolates to genus; identification to species usually requires taxon-specific expertise and additional biochemical, morphological, or genetic data (Lücking et al., 2020; Schoch et al., 2012). We assigned 63 of 66 fungal isolates to putative genus by ITS sequence.

While the open-plate method is not a quantifiable method, we saw a much higher abundance of fungal colonies on our outside capture plates than on our inside capture plates (Figures 1 and 2) and we captured more fungi on the windowsill (Figure 1 panels A and B) than elsewhere in the lab (Figure 1 panels C – F). This is consistent with other studies that find higher abundance of fungal propagules outdoors than indoors (Shelton et al., 2002).

Several fungal taxa are commonly identified from indoor air. In the EPA BASE study of non-complaint buildings across the United States, the most commonly identified fungal taxa were *Cladosporium*, *Penicillium*, *Aspergillus*, *Alternaria*, and *Aureobasidium*; *Cladosporium* was cultured from over 85% of the buildings surveyed (Tsai et al., 2007; Womble et al., 1999). In a study of mostly complaint buildings across the United States, the most commonly identified sporulating fungi were *Cladosporium* (found in 86% of buildings), *Penicillium* (found in 80% of buildings), and *Aspergillus* (found in 62% of buildings) (Shelton et al., 2002). The most commonly identified fungal genera in a global study of fungal communities from indoor dust were *Alternaria*, *Cladosporium*, and *Epicoccum* (Amend et al., 2010).

Cladosporium was the most commonly identified taxa in our survey, representing 14 out of the 52 isolates identified indoors. We also identified *Penicillium* in all three of our surveys; however, we did not isolate any *Aspergillus* species. We isolated 18 taxa in the two indoor surveys; only six of these taxa (*Alternaria*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Penicillium*, and *Stachybotrys*) are among the most commonly isolated fungi in buildings of the Northeast, which includes West Virginia (Shelton et al., 2002). Three of our 18 indoor taxa are among the less commonly isolated fungi in buildings of the Northeast: *Botrytis*, *Mucor*, and *Nigrospora* (Shelton et al., 2002). All but *Aureobasidium* and *Mucor* were also identified in the EPA-BASE survey of indoor air of non-complaint buildings (Tsai et al., 2007).

In a study of dust collected outside homes across the United States, 94% of the identified fungal taxa were found in 10% or less of the collected samples (Barberán, Ladau, et al., 2015). While some of the taxa exhibited cosmopolitan distribution, many of the taxa were geographically restricted. Even the supposedly cosmopolitan *Alternaria* and *Cladosporium* displayed geographical patterns with *Alternaria* more common in samples from the Great Plains and *Cladosporium* more common in samples from the eastern United States (Barberán, Ladau, et al., 2015). Similar to other studies, we identified several taxa that appear to be rare: we isolated them in one year, but not the other, and they are not reported in these other large-scale studies.

The EPA-BASE study grouped fungi into categories based on outdoor source (leaf-surface fungi, soil fungi, and water-requiring fungi) and

potentially toxigenic (Macher et al., 2001). Many of fungi we isolated indoors belong to the leaf-surface category and probably arise through ventilation from an outside source: *Alternaria* (2 isolates), *Cladosporium* (14 isolates), and *Epicoccum* (4 isolates). *Penicillium* (6 isolates) are considered soil fungi, with an outdoor source likely. *Botrytis* (1 isolate) and *Fusarium* (4 isolates) are water-requiring fungi that are likely due to an outdoor source, but could grow indoors especially in the presence of excess water. Some *Fusarium* species may also produce mycotoxins.

In general, the fungal taxa isolated in our surveys do not pose a threat to healthy individuals (Flannigan et al., 2011). *Cladosporium* was the most commonly isolated genus in our studies; there are three major species complexes within the *Cladosporium* genus (*C. cladosporioides*, *C. herbarum*, and *C. sphaerospermum*) (Bensch et al., 2018). Based on ITS sequence, 14 of our isolates are *C. cladosporioides* and one is *C. sphaerospermum*. *C. herbarum* and *C. cladosporioides* show greatly reduced growth at 30°C and do not grow at all at 35°C (Briceño & Latorre, 2008). However, *Cladosporium* has been found in studies of healthy human gut microbiomes (Hallen-Adams & Suhr, 2017).

Allergies, asthma, and hypersensitivity diseases can be related to indoor air quality and airborne fungi. The World Health Organization recognizes more than 100 fungal allergens from 29 different species (Borchers et al., 2017). Allergy testing of patients in West Virginia detected sensitivities to a variety of different fungal species that have been identified in indoor air (Beezhold et al., 2008; Shelton et al., 2002). Species tested included several from the same genera identified in our study: 11.8% patients were sensitive to *Alternaria alternata*, 6.9% were sensitive to *Cladosporium sphaerospermum*, 6.9% were sensitive to *Penicillium notatum*, 4.9% were sensitive to *Epicoccum nigrum*, and 4.0% were sensitive to *Penicillium chrysogenum* (Beezhold et al., 2008).

This study involved passive capture of viable airborne fungi on two separate dates and presents a baseline survey of taxa present in indoor air of a public building in a small town in rural, mid-Atlantic United States. This study also resulted in a stock collection of indoor viable fungi from this region. We plan to use this collection to investigate questions with applications to human health, including antifungal resistance in environmental (versus clinical) non-pathogenic fungal isolates and adaptation of environmental isolates to gradual increases in temperature. In the recently released report, the WHO describes two major areas of concern related to fungal disease in humans: first, an increase in antifungal resistance and second global warming (World Health Organization, 2022). Global warming increases the threat from invasive fungal disease both because the habit range for pathogens can change and because new fungal pathogens can emerge through a gradual adaptation to increased temperatures (de Crecy et al., 2009; Mazi et al., 2023).

Acknowledgements

This research was supported by the Shepherd University Biology Department, a student fellowship from NASA-WVSGC (J. Kellison), and a research enhancement award from NASA-WVSGC (L. Robertson).

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