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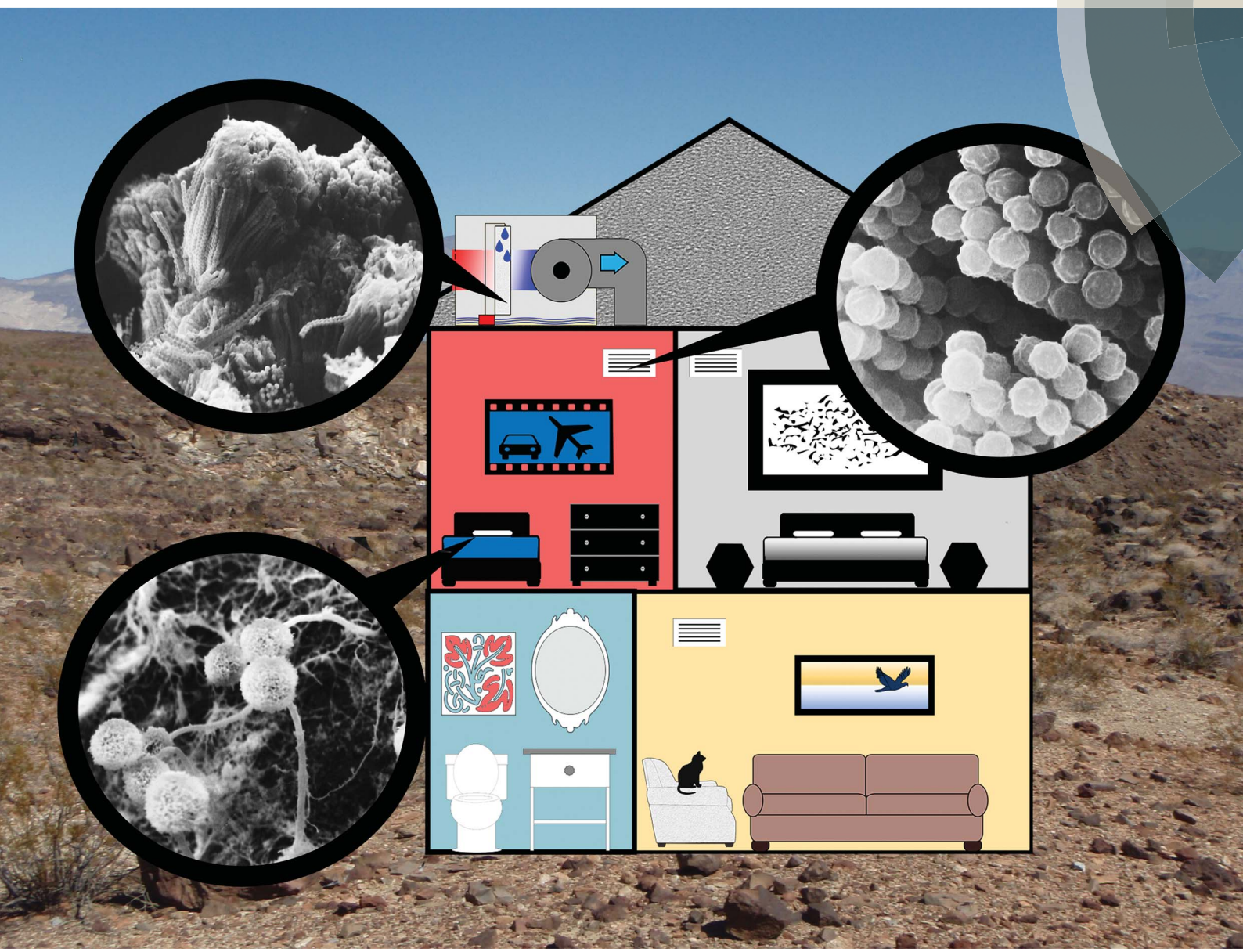

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Microbial rRNA sequencing analysis of evaporative cooler indoor environments located in the Great Basin Desert region of the United States†

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Recent studies conducted in the Great Basin Desert region of the United States have shown that skin test reactivity to fungal and dust mite allergens are increased in children with asthma or allergy living in homes with evaporative coolers (EC). The objective of this study was to determine if the increased humidity previously reported in EC homes leads to varying microbial populations compared to homes with air conditioners (AC). Children with physician-diagnosed allergic rhinitis living in EC or AC environments were recruited into the study. Air samples were collected from the child's bedroom for genomic DNA extraction and metagenomic analysis of bacteria and fungi using the Illumina MiSeq sequencing platform. The analysis of bacterial populations revealed no major differences between EC and AC sampling environments. The fungal populations observed in EC homes differed from AC homes. The most prevalent species discovered in AC environments belonged to the genera *Cryptococcus* (20%) and *Aspergillus* (20%). In contrast, the most common fungi identified in EC homes belonged to the order Pleosporales and included *Alternaria alternata* (32%) and *Phoma* spp. (22%). The variations in fungal populations provide preliminary evidence of the microbial burden children may be exposed to within EC environments in this region.

Environmental impact

Ribosomal RNA gene sequencing of fungal ITS and bacterial 16S loci has been used to evaluate the microbial diversity of indoor and outdoor environments. Using next generation sequencing approaches, this method of microbial exposure assessment can provide a more thorough evaluation of microbial contaminants in an environment compared to traditional methods of assessment. This study evaluated the fungal and bacterial burden within homes utilizing traditional air conditioners or evaporative coolers in the Great Basin Desert region of the United States. While no great difference in bacterial populations was observed between the two environments, evaporative cooler homes revealed more hydrophilic fungal species, specifically Pleosporales, capable of producing major fungal allergens. This could contribute to pediatric asthma and increased skin test reactivity observed in children residing in these environments.

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Introduction

Microbial-derived bioaerosols have been demonstrated to be associated with adverse respiratory health effects in exposed individuals.[1–7] In particular, fungal bioaerosols can be a burden to public health as personal exposure in contaminated indoor and occupational environments has been associated with an increased risk of respiratory morbidity, including asthma, allergic sensitization and hypersensitivity pneumonitis.[5,8,9] Several factors contribute to the propagation of fungal contaminants in indoor environments, including dampness and humidity.[10] Occupants are often unaware that such conditions can result in or exacerbate existing health effects. It has been observed that most people spend almost 90% of their time indoors.[11] The amount of time spent at home is likely to be increased for young children and elderly populations and this could result in increased susceptibility to personal exposure to indoor fungal bioaerosols.

It was recently reported that skin test reactivity to fungal and dust mite allergens was significantly increased in children living in homes cooled by evaporative coolers in the Great Basin Desert region.[12] Unlike traditional air conditioners (AC) that use refrigeration to cool the home, evaporative coolers (EC), also known as swamp coolers, use water vapor to cool the indoor environment. They consist of a water tank, pump, and cooling pad and cool the home by passing warm air across the cooling pad kept moist by the water pump. Arid climates are thought to limit the growth of many fungi due to the lack of humidity in the environment; however, the increased humidity[13] and moisture produced by an EC may make an indoor home environment more susceptible to fungal contamination within EC handling units as well as on the surface of indoor building materials. Although differences in fungal populations between EC homes and AC homes have been previously reported in a study located in Arizona, culture-dependent methods of assessment were used to evaluate viable fungal populations.[14] Using these traditional methods of assessment, fungi derived from the orders Eurotiales, Helotiales and Pleosporales were shown to be prevalent within these indoor environments, generally in greater abundance in EC homes.

Exposure assessment studies of the airborne fungal burden in indoor and occupational environments in the Great Basin Desert area of the United States have remained limited. Similarly, the influence of ECs on indoor fungal populations in this region has not been determined. Traditional methods to assess fungal exposure have included viable culture or the enumeration and quantification of collected fungal bioaerosols on a filter or tape. These methods are often time consuming and require mycology expertise to subjectively identify the resulting fungal colonies and/or fungal propagules. More recently, molecular-based platforms have been developed to address these limitations. Within the last decade, a quantitative polymerase chain reaction (qPCR) method using species-specific primer and probe sequences was developed by Vesper *et al.*[15] to assess the prevalence of 36 common hydrophilic and xerophilic fungal contaminants. While this method has provided quantitative datasets, it is limited to those species included in the panel. Recently, molecular methods that utilize genomic sequencing, including Sanger sequencing, 454 pyrosequencing, and most recently, Illumina MiSeq next generation sequencing, have been developed. The most commonly sequenced loci to assess fungal burden using these approaches has included the Internal Transcribed Spacer (ITS) regions of the ribosomal RNA (rRNA) gene, which are highly variable among species.[16] Due to the numerous fungal ITS sequences annotated in nucleotide databases, it is possible to identify the complete spectrum of fungi based on the sequenced fingerprint that is produced using these molecular approaches.

Bacterial populations can additionally be assessed using these genomic sequencing technologies. Using a similar approach as is used for fungi, bacterial 16S rRNA genes can be sequenced and used to identify Gram-negative, as well as Gram-positive, bacterial species in an indoor or occupational environment.[17,18] These sequencing-based approaches, in addition to the analysis of Gram-negative bacterial endotoxin, can provide additional insight into bacterial burden and potential microbial sources associated with adverse respiratory health outcomes.

The objective of this study was to utilize molecular-based approaches and, for the first time, assess the complete spectrum of fungal and bacterial bioaerosols in air samples derived from EC compared to AC environments in a desert region of the United States. Fungal-specific qPCR has been used to assess the fungal populations in these air samples and these datasets are compared to data collected using the Illumina MiSeq next generation sequencing platform. In addition, Gram-positive and Gram-negative bacterial populations in the two environments have been evaluated and compared to determine if the type of cooling system influences the microbial populations identified within these homes of atopic children located in the Great Basin Desert region of the United States. These new methods will allow for a comprehensive bioaerosol assessment of EC and AC indoor environments in this unique desert region of the United States.

Experimental methods

Air and swab sample collection

Air samples were collected in the fall of 2013 from homes of children participating in a cohort study of pediatric allergy and/or asthma (University of Nevada, Reno IRB#2013B030) that were either cooled using a standard central AC unit ($n = 11$) or an EC unit ($n = 10$). The homes were located within a 15 mile radius of the University of Nevada, Reno School of Medicine with the exception of one AC sample collected 43 miles away and one EC sample collected 50 miles away. The EC cooling pads in these homes were typically replaced once a year. In this study, air and dust samples were collected from the homes at the end of the season prior to cooling pad replacement. The heating, ventilating and air conditioning (HVAC) system of the home was turned off for a minimum of 1 hour prior to collection of air samples. Air samples were collected from the subject's bedroom after being vacated for a minimum of ten minutes. Airborne samples were collected from the cooling duct closest to the subject's bed. After removing the vent cover to the air duct, an M-TRAP® air sampling cassette (Assured Bio Labs, Oak Ridge, TN) was positioned three inches from the vent opening. Sampling began at the time the HVAC system was turned on. Air samples were collected for 10 minutes utilizing a Zefon Z-Lite IAQ Air Pump (Zefon International, Ocala, FL) that was calibrated to collect at 15 liters per minute. Outdoor air samples ($n = 3$) were also collected from areas within 30 miles of UNR School of Medicine representing urban, suburban and rural/desert environments. When accessible, EC water pans ($n = 7$) were swabbed with cotton transport swabs (Fisher Scientific, Houston, TX) at the water line. After collection, all samples were placed in individual plastic bags. Swabs were stored at −20 °C and M-TRAPs® were stored at 4 °C until shipped on ice for analysis.

Genomic DNA extraction

Air samples. M-TRAP® filters ($n = 24$) were placed in a 2 mL screw-cap microcentrifuge tube with 0.3*g* of 212–300 μm acid-washed glass beads. A spore suspension of *Geotrichum candidum* (10 μL) was added to serve as an internal positive control along with 350 μL Tissue Lysis Buffer from a High Pure PCR Template Preparation Kit (Roche Applied Sciences, Penzberg, Germany). Samples were lysed in a Roche Magna Lyser for 14 seconds at 6000 rpm followed by centrifugation for 1 minute at 13 200 rpm. The M-TRAP® filter and any remaining buffer was transferred to a Zymo-Spin IV column (Zymo Research, Irvine, CA). Samples were then centrifuged for 1 minute at 7000 rpm and the flow-through was transferred to a High Pure filter column from the Roche High Pure PCR Template Kit after 200 μL of binding buffer was added. The samples were then washed and eluted from the column as recommended by the manufacturer.

Swab samples. Genomic DNA (gDNA) was extracted from cotton swab samples ($n = 7$) using the High Pure PCR Template Kit (Roche) as previously described.[19,20] Swabs were cut into small sections and placed in a 2 mL screw-cap microcentrifuge tube containing 0.3*g* of 212–300 μm acid-washed glass beads (Sigma, St. Louis, MO). The tubes were placed in liquid nitrogen for 30 seconds followed by 30 seconds in a bead beater (BioSpec Products, Bartlesville, OK). This process was repeated two times before adding 650 μL of Tissue Lysis Buffer provided in the kit and processing in the bead beater for 30 seconds. An additional 200 μL of lysis buffer and 20 μL of CelLytic B Cell Lysis Reagent (Sigma) was added and the tubes were incubated at 37 °C for 15 minutes. After two rounds of centrifugation at 20 000 × *g* for 1 minute, the supernatants were transferred to new microcentrifuge tubes. The kit's Binding Buffer (400 μL) and 80 μL of proteinase K solution were added and the tubes were incubated for 10 minutes at 70 °C. The samples were transferred to a High Pure filter column and were washed and eluted as recommended by the manufacturer. An extraction reagent blank was included in the analysis as a control.

Ribosomal DNA amplification, cloning and sequencing

Bacterial and fungal gDNA samples were submitted to Research and Testing Laboratory (RTL; Lubbock, TX) for next-generation Illumina MiSeq sequencing of the bacterial 16S and fungal ITS1 rRNA genes. Samples were amplified for sequencing in a two-step process. The forward primer was constructed with the Illumina i5 sequencing primer and the ITS1F primer (CTTGGTCATTTAGAGGAAGTAA) for fungi or the 357F primer (CCTACGGGAGGCAGCAG) for bacteria. The reverse primer was constructed with the Illumina i7 sequencing primer and the ITS2R primer (CCTCCGCTTACTTATATGCTT) for fungi or the 926wR primer (CCGTCAATTYMTTTRAGTTT) for bacteria. Amplifications were performed in 25 μL reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, CA), 1 μL of each 5 μM primer, and 1 μL of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, CA) under the following thermal profile: 95 °C for 5 min, then 25 cycles of 94 °C for 30 s, 54 °C for 40 s, 72 °C for 1 min, followed by one cycle of 72 °C for 10 min and 4 °C hold. Products from the first stage amplification were added to a second PCR based on qualitatively determine concentrations. Primers for the second PCR were designed based on the Illumina Nextera PCR primers. The second stage amplification was run the same as the first stage except for 10 cycles.

Amplification products were visualized with eGels (Life Technologies, Grand Island, NY). Products were then pooled equimolar and each pool was size selected in two rounds using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, IN) in a 0.7 ratio for both rounds. Size selected pools were then quantified using the Quibit 2.0 fluorometer (Life Technologies) and loaded on an Illumina MiSeq (Illumina, Inc. San Diego, CA) 2 × 300 flow cell at 10 pM.

Sequences were subject to quality checking to remove failed sequence reads, sequences with low quality tags, and sequences that were less than half the expected amplicon length. Paired sequences were merged using the PEAR Illumina paired-end read merger[21] and subject to a RTL internal trimming algorithm. Sequences were then clustered into operational taxonomic units (OTUs) at a 4% divergence using the USEARCH clustering algorithm.[22] After OTU selection using the UPARSE OTU selection algorithm,[23] chimera checking was performed using the UCHIME chimera detection software.[24] Taxonomic identifications were made by comparing the OTU sequences against a database of high quality sequences derived from the NCBI database using a combination of a USEARCH global search algorithm and an internally developed python program.

For swab samples, bacterial and fungal rDNA were amplified using methods described previously.[19,20,25,26] Bacterial 16S rDNA was amplified using the primer pair p8FPL/p806R[25] and Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) using 5 μL gDNA template. PCR was performed in an Eppendorf Mastercycler Pro (Hamburg, Germany) using PCR conditions adapted from McCabe *et al.*:[25] initial denaturation at 95 °C for 4 min; 33 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and primer extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. Fungal rDNA was amplified using the primer pair Fun18Sf/ITS4 and Platinum Taq DNA polymerase using 5 μL gDNA template as previously described.[19,20,26] PCR reactions were run in triplicate for each sample and were combined and purified using a PCR Purification Kit (Qiagen, Valencia, CA). Amplicons were cloned into the pDRIVE vector using a PCR cloning kit (Qiagen). Cloned plasmids were then used to transform chemically competent *Escherichia coli*. Positive clones (48/sample for bacterial analysis, 24/sample for fungal analysis) were identified and cultured as previously described.[20] Glycerol stocks were sent to Genewiz, Inc. (South Plainfield, NJ) for Sanger sequencing analysis.

Bacterial 16S regions and fungal ITS1 and ITS2 regions were sequenced in both directions allowing for sequencing of the entire region. Sequence chromatogram files (.ab1) were downloaded, trimmed and assembled using Geneious R7 Software (Biomatters Ltd, Auckland, New Zealand) as previously described.[20] Sequences were clustered into OTUs using MOTHUR v1.32.1 software using a 97% similarity cutoff.[27] Taxonomic identification was determined by performing a BLASTn search using Geneious R7

Software against NCBI's database using a representative sequence of each OTU.

Dust sample collection and endotoxin analysis

Dust samples ($n = 20$) from the subject's bedrooms were collected with a ReadiVac hand-held vacuum in a cloth bag secured to the intake. The subject's bed was stripped of all bedding and the mattress, along with the floor within a one foot perimeter around the bed when accessible, was vacuumed. Dust samples were collected and stored in a zip-lock bag for analysis. Fine dust from was retrieved from 11 of the 20 samples. Endotoxin potency (EU: endotoxin unit) of each sample was determined using kinetic chromogenic *Limulus* amoebocyte lysate (LAL; Associates of Cape Cod, Inc., Falmouth, MA) assay method as previously described.[28,29] Results were reported as EU per milligram of dust.

Statistical analysis

All analyses were performed using the *R* statistical package unless otherwise noted. Chao1 richness, Shannon diversity and Bray–Curtis dissimilarity indices were calculated for AC, EC and outdoor sample groups. Pairwise comparisons of Chao1 indices among sample groups were calculated using a Wilcoxon rank sum test. An analysis of dissimilarity was conducted by comparing Bray–Curtis indices among sample groups using permutational multivariate analysis of variance (PERMANOVA) pairwise comparisons using adonis.[30,31] For endotoxin analysis of dust samples, statistical significance between groups was determined using a single factor analysis of variance (ANOVA) in SigmaPlot (Systat Software Inc.). A p value of <0.05 was considered statistically significant.

Results

Characteristics of the indoor environments

General characteristics of each home sampled are described in Table 1. Most homes sampled in each group were detached homes located in suburban neighborhoods within a 15 mile radius of the University of Nevada, Reno School of Medicine. The AC and EC homes had no visible water damage or mold growth. Temperature and humidity readings were taken at the vent opening after the HVAC system had been turned off prior to air sampling. The average indoor temperature at the vent opening was significantly higher in AC homes (72.6 °F/22.6 °C) than EC homes (66.3 °F/19.1 °C). Conversely, no significant difference in indoor humidity was observed in AC (32.7%) *versus* EC homes (35.8%). No significant differences in outdoor temperature or humidity during AC *versus* EC air sample collection were reported.

Table 1 Characteristics of AC and EC home environments

Home characteristics		Air conditioner	Evaporative cooler
Number of homes sampled		11	9
Number of rooms in home		7.9 (±2.1)	6.7 (±2.0)
Type of home:	Detached	90.9%	77.8%
	Row house	9.1%	0%
	Mobile home	0%	22.2%
Residential pets		90.0%	66.7%
Pests (mice, rats, cockroaches)		30.0%	11.1%
Smoker in home		11.1%	33.3%

Bacterial populations in air and swab samples

Extracted gDNA from air samples ($n = 24$) was analyzed by Illumina MiSeq sequencing for bacterial prevalence. Species richness and diversity estimates were calculated for the sample groups (Table 2). Outdoor, AC, and EC environments showed similar species richness with no statistically significant differences between groups. In contrast, species diversity was greater among AC and EC samples than outdoor air samples (Table 2). Bray–Curtis indices demonstrated low dissimilarity among outdoor air samples but high dissimilarity among samples within the AC and EC groups. Although the sample number in the outdoor air group was low, statistical differences in dissimilarity were observed between outdoor air and AC and EC groups ($p = 0.008$ and $p = 0.007$, respectively). Differences in dissimilarity between AC and EC groups were not observed ($p = 0.129$). The number of sequences identified in each group were also compared. Outdoor air samples had a 4–5 times greater number of bacterial sequences identified per sample than both EC and AC environments, which were comparable. Outdoor air ($n = 3$) was dominated by the Gram-negative species, *Bacillus pseudomycoides*, with 99.8% of sequences identified derived from this organism. In contrast, the species identified in the sampled indoor environments were divided among three major bacterial phyla. AC environments were predominantly composed of the Gram-negative phylum Proteobacteria (58.38%), and Gram-positive phyla Firmicutes (18.73%) and Actinobacteria (17.06%) (Fig. 1A). Similarly, the predominant species identified in EC environments belonged to the phyla Proteobacteria (55.93%), Actinobacteria (21.63%) and Firmicutes (14.43%) (Fig. 1A). No differences were observed at the phyla level when air samples from AC and EC environments were compared. Although there was not a substantial difference in the Gram-negative bacterial populations detected between AC (64.16%) and EC (63.14%) homes, the amount of endotoxin detected in dust collected from these homes was significantly higher in EC environments (Table 3).

Swab samples ($n = 7$) collected from EC water pans were analyzed by Sanger sequencing to determine the bacterial populations present. Sequences were clustered into 117 OTUs for taxonomic identification. 116 OTUs were identified as bacterial species while 1 OTU belonged to the kingdom Plantae. The swab sample bacterial populations were similar to those observed in the EC air samples. The dominant species were placed in the phyla Proteobacteria (56.91%) and Actinobacteria (18.23%) (Fig. 1B).

Fungal populations in air and swab samples

Extracted gDNA from air samples ($n = 22$) were analyzed for fungal prevalence. Two air samples did not contain enough

Table 2 Species richness and diversity indices for outdoor, AC and EC environments

		Chao-1 richness	Shannon diversity	Bray–Curtis distance
		Mean (min, max)	Mean (min, max)	Mean (min, max)
Bacteria	Outdoor air ($n = 3$)	48.92 (29.5, 87.7)	0.0137 (0.0011, 0.0336)	0.1710 (0.0347, 0.2553)
	Air conditioner ($n = 11$)	51.82 (17.0, 94.0)	2.63 (1.17, 3.41)	0.8267 (0.5717, 0.9539)
	Evaporative cooler ($n = 10$)	44.56 (10.5, 102.0)	2.29 (1.16, 3.78)	0.8921 (0.5307, 0.9999)
Fungi	Outdoor air ($n = 3$)	197.8 (160.2, 237.3)	2.25 (1.86, 2.49)	0.3633 (0.2964, 0.3973)
	Air conditioner ($n = 9$)	33.86 (15.0, 102.0)	1.39 (0.68, 2.51)	0.9778 (0.8313, 1.0000)
	Evaporative cooler ($n = 10$)	25.46 (12.5, 49.5)	1.10 (0.50, 1.72)	0.9624 (0.3804, 1.0000)

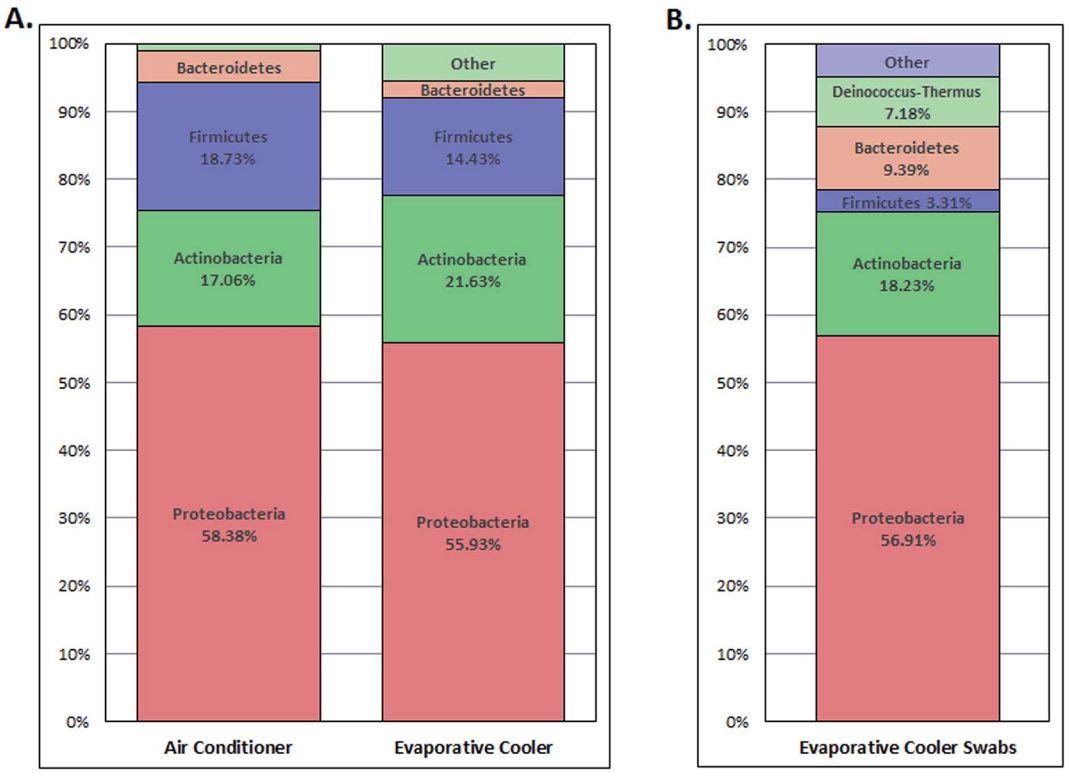

Fig. 1 Bacterial phyla identified in (A) Illumina MiSeq analysis of air conditioner ($n = 11$) and evaporative cooler ($n = 10$) environments and (B) Sanger sequencing analysis of evaporative cooler swab samples ($n = 7$).

Table 3 Endotoxin analysis of indoor dust samples

Cooling unit		Endotoxin (EU mg^{-1})	Standard deviation	p value
Air conditioner	($n = 6$)	58.08	±48.92	$p = 0.039$
Evaporative cooler	($n = 5$)	162.25	±91.77	

genetic material for fungal ITS analysis. Fungal ITS1 regions were sequenced using the Illumina MiSeq next generation sequencing platform and analyzed for taxonomic identification (RTL). Fungal species richness and diversity estimates were calculated within the outdoor, AC, and EC sample groups (Table 2). Species richness was significantly higher in outdoor air compared to AC and EC environments ($p = 0.009$ and $p = 0.014$, respectively) while no significant difference in richness was observed between AC and EC environments ($p = 0.391$). Species diversity was also greater among outdoor samples compared to AC samples than EC samples (Table 2). Bray–Curtis indices demonstrated high dissimilarity among samples within the AC and EC groups with low dissimilarity observed in outdoor air samples. Dissimilarity between outdoor air and AC and EC groups was statistically significant ($p = 0.005$ and $p = 0.006$, respectively). Statistically significant differences in dissimilarity were also observed between AC and EC environments (0.028). Fungal prevalence between outdoor, AC and EC environments were also compared. On average, the number of sequences detected in EC samples were almost four times the number of sequences detected in AC samples. The outdoor

samples had seven times and two times more sequences than the AC and EC samples, respectively. The most predominant fungal order detected in outdoor air ($n = 3$) was Capnodiales (71%). Prevalent species identified within the order Capnodiales included *Davidiella* spp. (43%) and *Cladosporium* spp. (25%) (data not shown). This data was also captured in the mold-specific quantitative PCR panel in which the order Capnodiales, specifically *Cladosporium* spp., was the predominant fungal species detected (Table S1†).

Air samples from AC environments contained a heterogeneous population of fungi. The fungal species were dispersed evenly among the phyla Ascomycota (53%) and Basidiomycota (47%) (Fig. 2A). The most abundant Ascomycota order was Eurotiales, and included *Aspergillus* spp. that made up 21% of all fungi detected (Fig. 3 and Table 4). *A. penicillioides* was the most prevalent *Aspergillus* spp. identified and accounted for 11% of all fungi detected. The prevalent orders belonging to the phylum Basidiomycota included Tremellales that consisted primarily of *Cryptococcus* spp. (20% of all fungi), and Sporidiobolales consisting entirely of *Rhodotorula* spp. (13% of all fungi) (Fig. 3 and Table 4). Although Illumina analysis of air samples only detected 4% *Cladosporium* spp., the quantitative PCR panel suggested the predominant fungi in these samples was *Cladosporium herbarum* (Table S1†), similar to what was observed in the outdoor environment. The Eurotiales (*Aspergillus*/*Penicillium* spp.) were the remaining prevalent species detected in the qPCR analysis.

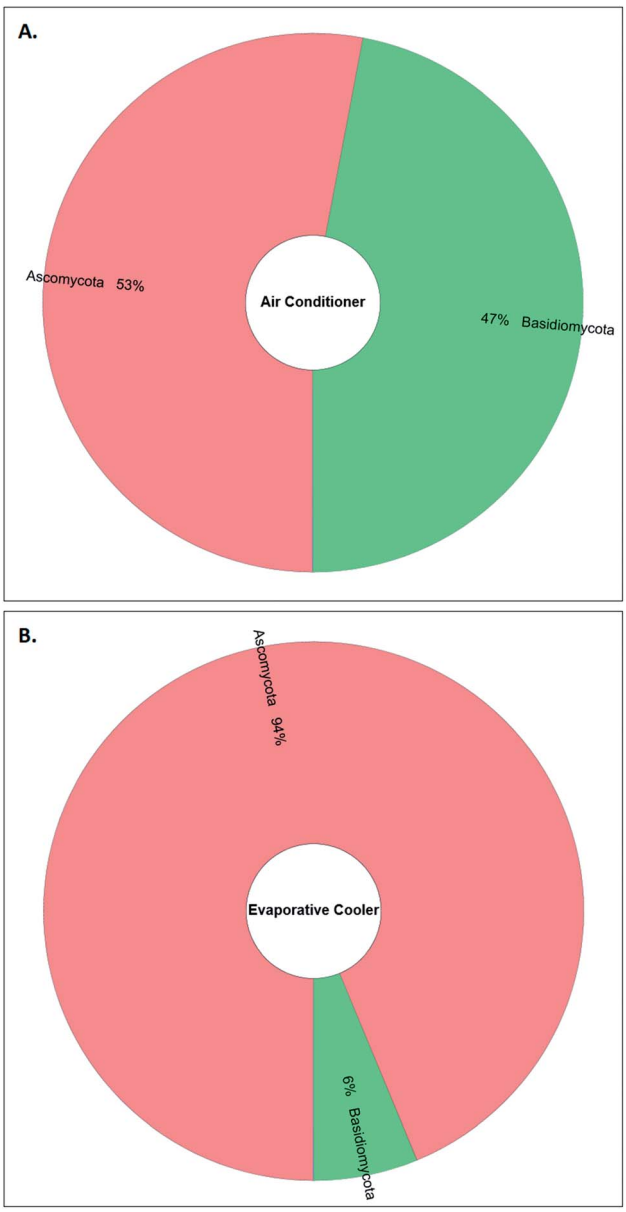

Fig. 2 Krona diagram depicting the fungal phyla identified in Illumina MiSeq analysis of (A) air conditioner ($n = 9$) and (B) evaporative cooler ($n = 10$) environments.

A shift in the fungal populations in air samples of homes utilizing ECs was observed. The majority of species detected belonged to the phylum Ascomycota (94%) while only a small fraction were identified as Basidiomycota (6%) (Fig. 2B). Although the most abundant species varied among samples, the most prevalent orders observed in these environments were the Pleosporales (68%), and to a lesser extent, Capnodiales (15%) (Fig. 3). The most prevalent species in the order Pleosporales included *Alternaria alternata* (32% of all fungi, 47% of Pleosporales) and *Phoma* spp. (22% of all fungi, 31% of Pleosporales) (Fig. 4A and Table 4). *Davidiella* spp. made up the majority of Capnodiales detected (12% of all fungi, 85% of Capnodiales) (Fig. 4B and Table 4).

Similar to outdoor and AC environments, the quantitative PCR analysis of EC environments demonstrated *C. herbarum* to be the most prevalent fungal species (Table S1†). The remaining fungi detected using this panel included *Aspergillus*/*Penicillium* spp. and *Aureobasidium pullulans*. Although the fungal populations observed in EC environments was not considerably different compared to AC homes, over three times the number of fungal spores were detected in EC environments using quantitative PCR analysis compared to AC environments.

Fungal gDNA extracted from the swab samples ($n = 7$) collected from EC water pans was limited. ITS amplification and Sanger sequencing revealed only 40 sequences that clustered into 16 OTUs. One of the OTUs belonged to the kingdom Protozoa. The remaining OTUs were placed in the fungal phyla Ascomycota (62%), Basidiomycota (24%), and Chytridiomycota (14%) (Fig. 5). Chytrids are typically classified as aquatic fungi and were not detected in the air of EC homes. The most prevalent fungal species detected was *A. pullulans* (22%) that belongs to the order Dothideales.

Discussion

Personal exposure to fungal bioaerosols has been shown to be associated with adverse respiratory health effects, including allergic sensitization and asthma.[5,8,9,32] Dampness in the indoor environment is a major risk factor for the proliferation of fungi on a variety of building materials and can result in high concentrations of fungal bioaerosols within the indoor environment.[5,10,33] To date, limited research has been conducted to investigate the role of an evaporative cooler, a system utilizing water vapor to cool the indoor environment, on the proliferation

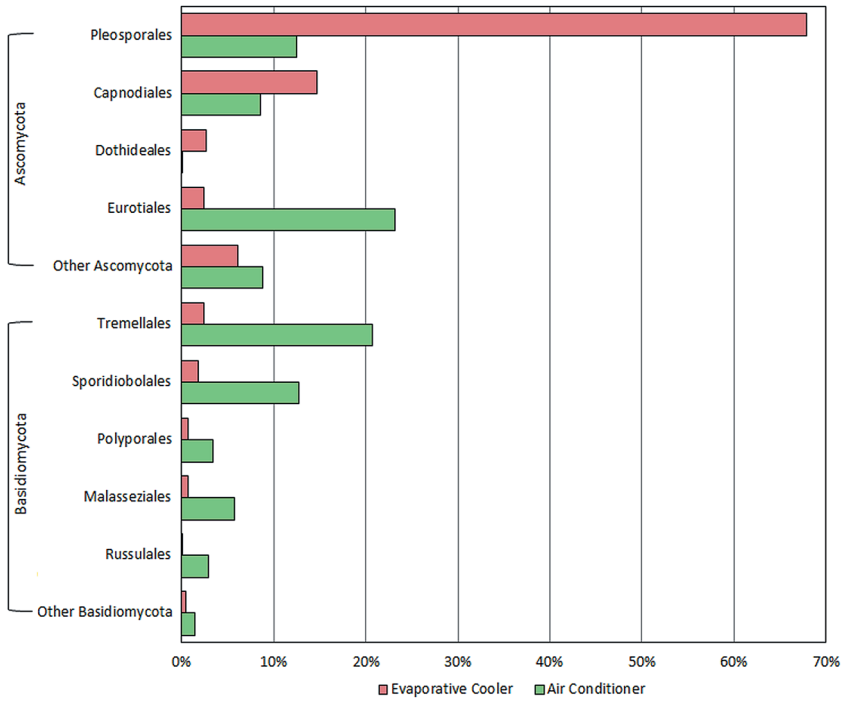

Fig. 3 Fungal order distribution of evaporative cooler ($n = 10$) *versus* air conditioner environments ($n = 9$) identified in Illumina MiSeq analysis. Values are representative of the percentage of fungal orders within each environment. Pink = evaporative cooler; green = air conditioner.

Table 4 Most prevalent genera identified in each environment using Illumina MiSeq technology

Genus	Count	Std. Dev.	Percent
Outdoor air ($n = 3$)			
Davidiella	161 609	±13 318.4	43.3%
Cladosporium	94 014	±13 378.8	25.2%
Ustilago	17 986	±8515.5	4.8%
Air conditioner ($n = 9$)			
Aspergillus	31 786	±9081.2	20.5%
Cryptococcus	31 257	±3642.2	20.2%
Rhodotorula	19 647	±5365.1	12.7%
Evaporative cooler ($n = 10$)			
Alternaria	195 448	±61 796.3	32.1%
Phoma	135 688	±33 877.1	22.3%
Davidiella	76 130	±14 532.9	12.5%

of fungi and indoor air quality (IAQ) within these environments. In this study, fungal populations were detected for the first time in homes cooled with either AC or EC systems using molecular-based approaches. These homes were participating in a pediatric allergy and asthma study located in the Great Basin Desert region of the United States. The results of the study demonstrated a substantial shift in fungal populations between AC and EC indoor environments.

Sequencing of fungal ITS and bacterial 16S rRNA gene loci has expanded the knowledge of microbial populations in indoor and occupational environments. Sequence-based approaches provide a comprehensive representation of indoor microbial populations, including both viable and nonviable organisms, compared to traditional methods of microbial exposure assessment, such as culture and nonviable microscopic-based approaches. While these molecular-based approaches have provided advancements in the field of microbial exposure assessment, there are still a number of limitations that are often overlooked. Extraction and primer biases, as well as other factors, like gene copy number, could significantly influence the ability to detect certain organisms and bias results toward a specific fungal phylum.[19,34,35] These approaches are also limited to what has been banked in sequence databases. Although sequencing-based approaches have known limitations, these methods currently provide reproducible datasets of the complete spectrum of indoor microbial bioaerosols compared to traditional-based methods of assessment.

Using these more robust techniques, a substantial difference in the fungal populations and diversity observed in EC *versus* AC indoor environments were observed. Overall, beta diversity analysis revealed higher fungal species diversity in AC *versus* EC environments. Analysis at the population level demonstrated that the phylum Ascomycota accounted for 53% of all fungal bioaerosols detected in AC homes with Basidiomycota making up the remaining 47%. In contrast, over 94% of fungi detected EC homes were placed in the phylum Ascomycota. Previous studies using culture-based approaches in an Arizona desert region of the United States demonstrated that *Asperpgillus* spp., *Bispora* spp., and *Alternaria* spp. were more prevalent in EC environments than AC environments.[14] The results of the current study further extend these findings by characterizing the complete fungal burden within these environments.

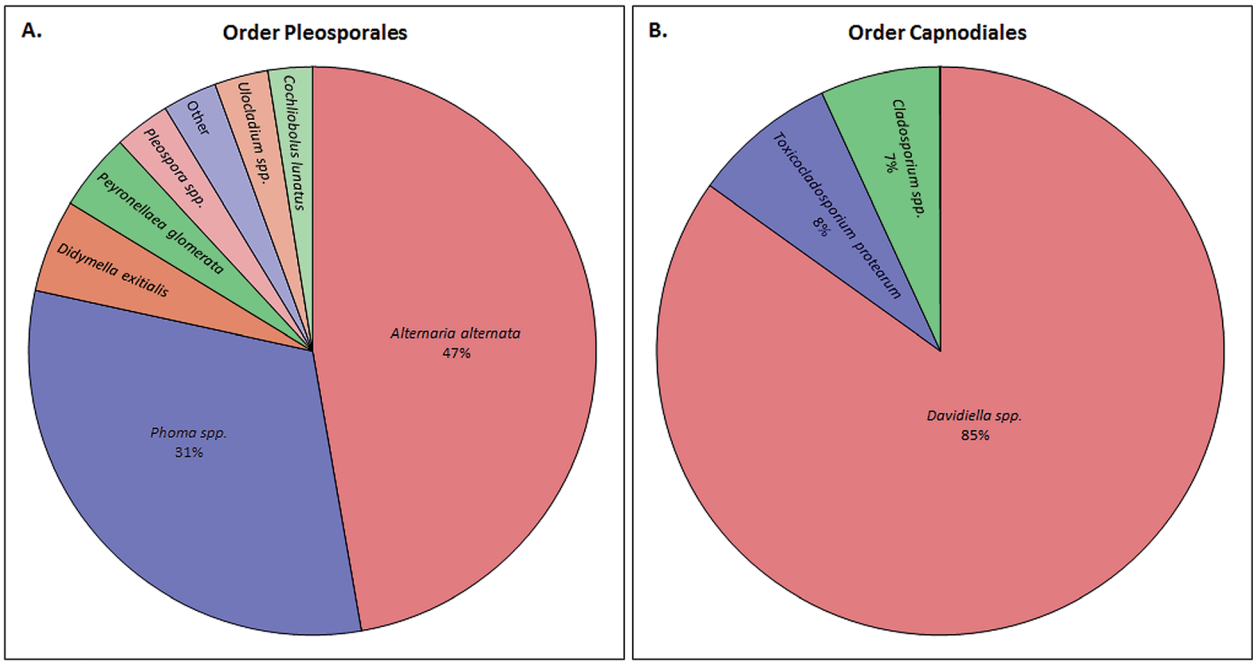

Fig. 4 Most prevalent species within the fungal orders (A) Pleosporales and (B) Capnodiales identified in evaporative cooler environments (n = 10) using Illumina MiSeq. Values are representative of the percentage of each species within each order.

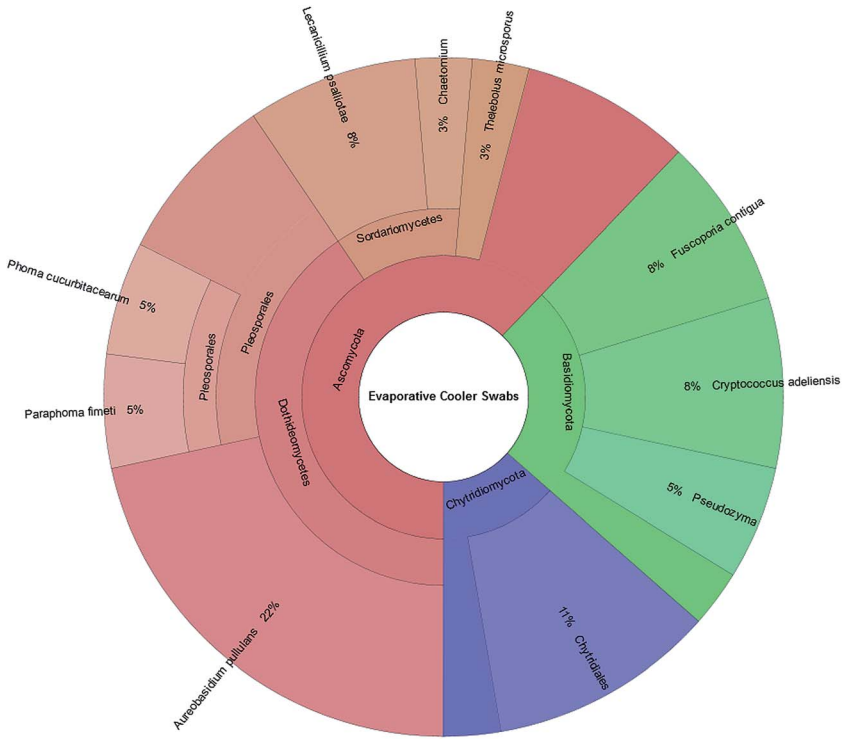

Fig. 5 Krona diagram depicting the fungal populations identified in Sanger sequencing analysis of evaporative cooler swab samples (n = 7).

Xerophilic fungi, including *Aspergillus* spp. and *Cryptococcus* spp., were the most common fungal genera detected in AC homes. In contrast, hydrophilic fungi, such as *A. alternata*, along with other members of the order Pleosporales, including *Phoma*, *Didymella and Ulocladium* species, were the most prevalent in EC environments. The fungal populations detected in swab samples from EC pans did not correlate to the populations detected in the air. Taking into account this observation and that the humidity within the EC homes was not greater than the AC homes at the time of collection, the source of the

differing fungal populations could be the EC cooling pad. The pads in these homes had gone through an entire season at the time of sample collection, a time over which many hydrophilic fungal species could propagate. These results support the hypothesis that homes that utilize evaporative cooling strategies in the Great Basin Desert region of the United States are susceptible to the proliferation of hydrophilic fungal bioaerosols placed in the fungal order Pleosporales. This is a particularly important risk factor for fungal sensitized populations as fungi placed in this order are among the most frequently encountered fungal sensitizers and contain allergens that are homologous with *A. alternata*.[36–38]

Although *A. alternata* was detected in most EC environments, the most prevalent species were placed in either the order Pleosporales or Capnodiales. Interestingly, Pleosporales species, such as *A. alternata*, were not detected using qPCR. A recent molecular biodiversity study has demonstrated that indoor *A. alternata* isolates have a high level of gene flow across the continental United States.[39] These data suggest that an ITS region amplified from gDNA derived from environmental samples could be identified as *A. alternata* in nucleotide databases but may not be amplified using *A. alternata*-specific primers used in the qPCR panel. It is hypothesized that the ITS sequence of the *A. alternata* strains detected in Illumina MiSeq may have varied by several nucleotides from the strain used to develop *A. alternata*-specific primers. This represents a potential limitation to qPCR-based approaches and demonstrates the importance of sequencing-based approaches to identify inter-strain variability within the ITS loci.

The fungal species, *A. alternata* and *C. herbarum* are among the most common species that are associated with fungal allergic sensitization.[40] Members of the family *Pleosporaceae*, as well as *Davidiella* spp., a telomorph of *Cladosporium* spp., were readily detected in EC environments. *Alternaria* spp., along with other species belonging to the order Pleosporales, including *Pleospora* and *Ulocladium* species, produce the major allergen Alt a 1 or a similar homolog, sometimes in greater quantities than *A. alternata*.[36–38,41] Alt a 1 has been shown to be recognized by human immunoglobulin *E* in over 80% of *Alternaria*-sensitized individuals[42,43] and previous studies have demonstrated that 85% of *Alternaria*-sensitized individuals had positive skin test reactivity to recombinant Alt a 1.[44,45] Individuals in EC environments are likely exposed to Alt a 1, or another homologous allergen, increasing susceptibility to fungal sensitization. In addition to Alt a 1, enolase is a highly conserved allergen recognized by *Alternaria*, as well as, *Cladosporium*-sensitized patient sera.[40,44–46] Cross-reactivity to fungal enolase has been previously reported[40,41] and represents an additional burden to personal fungal exposure as a subjects sensitized to such an allergen could develop an allergic response to several other fungi they may encounter in the environment.

Gram-positive as well as endotoxin-producing Gram-negative bacteria were also identified in AC and EC environments. Air samples from both AC and EC homes contained large populations of Gram-negative Proteobacteria as well as Gram-positive Actinomycetes. Although personal exposure to these organisms in the indoor environment can result in adverse health effects,[1–7] no substantial differences in the bacterial burden between AC and EC environments were observed in the metagenomic datasets. In contrast, a significant difference in the level of endotoxin detected in the dust of EC compared to AC homes was observed. Endotoxin levels in EC homes were almost three-fold higher than those of AC homes. Inhalation exposure to endotoxin has been previously shown to exacerbate respiratory morbidity, including chest tightness and bronchoconstriction.[3,47,48] In addition, exposure to both endotoxin and fungi have been shown to have synergistic adverse respiratory health effects in occupants of water-damaged buildings.[28] More recently, endotoxin exposures have been associated with enhanced asthma severity and respiratory wheeze within indoor environments.[7,48,49] While endotoxin exposure may not be the cause of allergic asthma in the pediatric population residing in EC environments, personal exposure could potentially exacerbate preexisting conditions due to exposures with other indoor air contaminants, such as fungi and dust mites.

Conclusions

For the first time, fungal ITS sequencing analysis of samples derived from EC *versus* AC environments in the Great Basin Desert region of the United States revealed substantial differences in fungal burden. The diversity of AC environments was significantly higher than EC environments. The fungal populations detected in the EC environments consisted mostly of species placed in the phylum Ascomycota while those detected in AC homes were divided among the major phyla Ascomycota and Basidiomycota. Among the Ascomycota detected in EC environments were several species capable of producing major fungal allergens, which were not prevalent in AC homes. In addition, endotoxin evaluation revealed significantly higher levels in EC *versus* AC environments. Using molecular-based approaches, this study describes a new comprehensive representation of bacterial and fungal bioaerosol populations present within EC and AC environments in the Great Basin Desert region. The variations in fungal populations observed could partially account for the increased skin test reactivity and disease severity reported in pediatric populations that live in EC environments located in this region.

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