

# Impacts of Flood Damage on Airborne Bacteria and Fungi in Homes after the 2013 Colorado Front Range Flood

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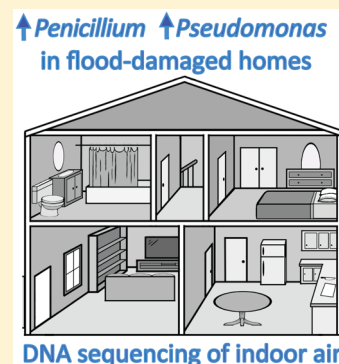
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## S Supporting Information

**ABSTRACT:** Flood-damaged homes typically have elevated microbial loads, and their occupants have an increased incidence of allergies, asthma, and other respiratory ailments, yet the microbial communities in these homes remain under-studied. Using culture-independent approaches, we characterized bacterial and fungal communities in homes in Boulder, CO, USA 2–3 months after the historic September, 2013 flooding event. We collected passive air samples from basements in 50 homes (36 flood-damaged, 14 non-flooded), and we sequenced the bacterial 16S rRNA gene (V4-V5 region) and the fungal ITS1 region from these samples for community analyses. Quantitative PCR was used to estimate the abundances of bacteria and fungi in the passive air samples. Results indicate significant differences in bacterial and fungal community composition between flooded and non-flooded homes. Fungal abundances were estimated to be three times higher in flooded, relative to non-flooded homes, but there were no significant differences in bacterial abundances. *Penicillium* (fungi) and Pseudomonadaceae and Enterobacteriaceae (bacteria) were among the most abundant taxa in flooded homes. Our results suggest that bacterial and fungal communities continue to be affected by flooding, even after relative humidity has returned to baseline levels and remediation has removed any visible evidence of flood damage.



## INTRODUCTION

A historic flooding event occurred in the Colorado Front Range (Southern Wyoming to central Colorado, USA) from September 9–15, 2013, damaging or destroying approximately 19 000 homes in the area<sup>1</sup> ([www.colorado.edu/resources/front-range-floods/assessment.pdf](http://www.colorado.edu/resources/front-range-floods/assessment.pdf)). Heavy rainfall resulted from a nearly stationary low-pressure system that pulled unusually high levels of tropical moisture from the Pacific Ocean and the Gulf of Mexico, which stalled over the Front Range.<sup>2</sup> Record rainfall was observed in Boulder, CO, where 42.9 cm fell over 7 days, compared to average annual precipitation levels of 52.5 cm, with record or near-record rainfalls occurring throughout the Front Range during that week.<sup>3</sup> Boulder Creek, which normally flows at approximately 1.5 m<sup>3</sup> per second, reached a flow rate of approximately 140 m<sup>3</sup> per second during the flooding event.<sup>2,4</sup> After the flooding event, more than 7 600 Boulder County residents applied for assistance associated with temporary housing, home repairs, and business recovery.<sup>4</sup>

Water damage in flooded homes has been known to increase mold contamination,<sup>5–9</sup> and exposure to mold can lead to adverse health effects, including asthma, allergies, and other

respiratory problems.<sup>10–12</sup> For example, mold growth was visible in 44% of homes studied in New Orleans, LA, after hurricanes Katrina and Rita,<sup>9</sup> and airborne spore concentrations were elevated in flooded areas, particularly indoors.<sup>8</sup> Bacterial endotoxins (soluble lipopolysaccharides from Gram-negative cell walls),<sup>8</sup> which correlate with adverse health effects, including flu-like symptoms,<sup>13</sup> also tend to be found in higher concentrations in flooded homes.<sup>8,14,15</sup> Fungi shown to be common in homes after flooding include *Aspergillus*, *Cladosporium*, *Penicillium*, *Trichoderma*, and *Paecilomyces*.<sup>8,14–16</sup> Studies of specific bacterial taxa in flood-damaged homes are limited, but diverse Staphylococcae have been detected in indoor air from flood-damaged homes,<sup>17</sup> as have *Streptomyces*.<sup>18,19</sup>

Such studies of bacteria and fungi found in flood-damaged homes have historically relied upon culturing, e.g., counting the

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number of colony-forming units,<sup>14</sup> direct microscopic counts,<sup>6</sup> and/or morphological analyses of fungal spores via microscopy.<sup>15</sup> It is now well appreciated that approximately 99% of microorganisms cannot be cultured,<sup>20</sup> and it has been shown that culture-dependent approaches miss the majority of microorganisms in indoor air from flooded homes.<sup>6</sup> In addition, previous studies of mold in flooded homes have indicated an inability to differentiate species of *Penicillium* and *Aspergillus* using spore counting techniques, due to morphological similarities, and an inability to identify many fungi at higher levels of taxonomic resolution.<sup>8</sup> Molecular approaches, including DNA sequencing of target genes for organism identification and quantitative PCR for abundance estimates, bypass these biases and are becoming the norm for microbial ecological studies across a range of ecosystems, including the built environment.<sup>21–28</sup>

After a home sustains flood damage, typical remediation efforts include the disposal of damaged furnishings and belongings, removal and replacement of drywall and flooring, drying (typically through some combination of sump pumps, fans, space heaters, dehumidifiers, and/or ventilation), and treating surfaces with a biostatic agent.<sup>6,8,10,14</sup> Remediation has been shown to result in a reduction of doctor-diagnosed allergies,<sup>10</sup> but studies investigating the effectiveness of remediation at reducing airborne mold and/or bacterial concentrations have yielded conflicting results. For example, some studies have shown that indoor microbial concentrations remained elevated in remediated homes,<sup>6,11</sup> while others have reported a reduction in indoor air microbial concentrations after remediation.<sup>14,10</sup> In a study of eight flooded homes, the highest spore concentrations were measured in a home that did not undergo remediation.<sup>8</sup>

In this study, we used molecular and microbial ecological approaches to investigate the effects of flooding and subsequent remediation on bacterial and fungal communities in homes after the 2013 Colorado Front Range flood. While most previous studies of microbial communities in flooded homes have compared indoor air to nearby outdoor air, presumably due to a lack of access to non-flooded homes in close proximity for comparison, the circumstances of the Front Range flood allowed us to directly compare indoor air from flooded and non-flooded homes in the same area, including homes in the same neighborhood. Approximately 2–3 months after the flooding event, after all indoor water had been removed and most remediation efforts had been completed, we collected indoor air samples from 50 homes (36 flooded homes and 14 homes with no reported flooding) in Boulder, CO for microbial analyses. We addressed three basic questions: (1) How does flooding impact the types of bacteria and fungi in homes 2–3 months after the flooding event? (2) Are there any differences in the absolute abundances of bacteria and fungi in flooded, relative to non-flooded homes? (3) If there are observable effects of flooding on microbial community composition and/or abundance, can they be correlated with environmental variables?

## MATERIALS AND METHODS

**Volunteer Recruitment and Information about the 50 Homes in the Study.** For logistical reasons, all homes were located on the south side of Boulder, CO, within an area roughly 5 km × 7 km in size. Volunteers were recruited through neighborhood and business e-mail lists. Apart from logistical constraints, the only requirement was the presence of a living

space (finished or unfinished) below ground level (e.g., a basement or below-ground split-level). Crawl spaces were not sampled. All participants gave approval to participate in the study, in accordance with University of Colorado, Boulder IRB Protocol No. 12-0624. We enrolled 36 flooded and 14 non-flooded homes in the study (50 homes total). We defined flooding as any amount of floodwater on the floor of the lowest level of the home (not including any crawl spaces), including a puddle in one case. One home with a water-damaged garage but no flooding in the basement was considered non-flooded. Both flooded and non-flooded homes were often in close proximity in the same neighborhood, as flooding was not localized to specific regions of the sampled area (for a sense of the distribution of flood-damaged homes in Boulder, CO, see a map of FEMA individual assistance applications, [https://www-webapps.bouldercolorado.gov/pds/flood/Council\\_Update\\_Dec3\\_FEMA\\_Registered\\_by\\_Grid\\_FEMA.pdf](https://www-webapps.bouldercolorado.gov/pds/flood/Council_Update_Dec3_FEMA_Registered_by_Grid_FEMA.pdf), accessed July 23, 2014).

In the 36 flooded homes, the depth of water ranged from less than 5 cm (15 homes) to 2.5 m (4 homes had >30 cm of water), with an average depth of 18 cm. In 35 of the 36 homes, at least some portion of the floodwater was sourced from groundwater (28 homes reported flooding from groundwater exclusively), and 3 homeowners indicated some flooding from creek and/or river overflow, while 6 homeowners reported some sewage flooding. All remediation practices were consistent with those documented in other studies of flooded homes (see the Introduction).<sup>14</sup> At the time of sampling, which began approximately 2 months after the flood and continued for 1 month, we did not observe evidence of residual water or moisture in any of the homes, and we only noticed a moldy odor in one home. There was no visible microbial growth or direct evidence of flooding in any home, apart from ongoing renovations (new drywall, fresh paint, etc.) in some homes. We did not directly check for microbial growth in duct systems, but we did not observe any visible evidence of mold during sampling.

**Sample and Metadata Collection.** We scheduled an initial ~30 min visit to each home in mid-November, 2013 (approximately 2 months post-flood) to begin sample collection. During that visit, the homeowner filled out a questionnaire about home and occupant characteristics, including self-reported health data and, if applicable, the extent of flooding and remediation (Tables S1 and S2 in the Supporting Information). We installed three sterile, empty Petri dishes (no growth media or adhesives were added) as passive air samplers to collect settled dust, largely based on a previously published protocol.<sup>25</sup> To minimize confusion with other studies that have used the words “Petri dish” or “Petri plate” to mean a media-containing vessel for growing microorganisms, we will henceforth refer to our Petri plates as “passive air samplers” or “passive samplers.” Each passive sampler was attached to a cardboard square with a nylon monofilament line tied to each corner, which hung from the ceiling via a thumbtack (see picture, Figure S1 in the Supporting Information). Passive samplers hung approximately 2–2.5 m above the floor and a minimum of 30 cm below the ceiling and at least 30 cm away from any windows or central air vents in the below-ground level (typically, the basement) in each home. Within a given home, all passive samplers were hung at the same height and in the same room. If there was more than one room in the basement, the passive samplers were placed in the largest room. The basement or below-

ground level was chosen because (1) we expected to encounter the most significant differences in microbial community composition in the flooded area, (2) we wanted to minimize accidental disruption of our sampling equipment and minimize inconvenience to the homeowners, and (3) differences in home use (cooking, cleaning, occupancy, etc.) might have resulted in confounding variables if samplers had been exclusively placed on the main level. One battery-powered iButton Hygrochron Temperature/Humidity Logger (Maxim Integrated, San Jose, CA) was also installed in each home to measure temperature (°C) and relative humidity (%) throughout the sampling period, and references to these data loggers will henceforth be shortened to “T/RH logger(s).” The T/RH logger for each home was attached to the cardboard underneath one of the passive samplers. During the first visit, if the home had a heating, ventilating, and/or air conditioning (HVAC) system, we also removed the homeowner’s HVAC filter and replaced it with a new one for the collection of whole-home air. Because of differences in HVAC systems, filter sizes and types varied (it was not practical to control for HVAC system or HVAC filter type for this study). The passive air samplers, T/RH loggers, and new HVAC filters remained in place for approximately 4 weeks (range 20–35 days, average 29 days), with removal occurring in mid-December, 2013. For downstream analyses, real-time temperature and relative humidity data were averaged over the sampling period for each home.

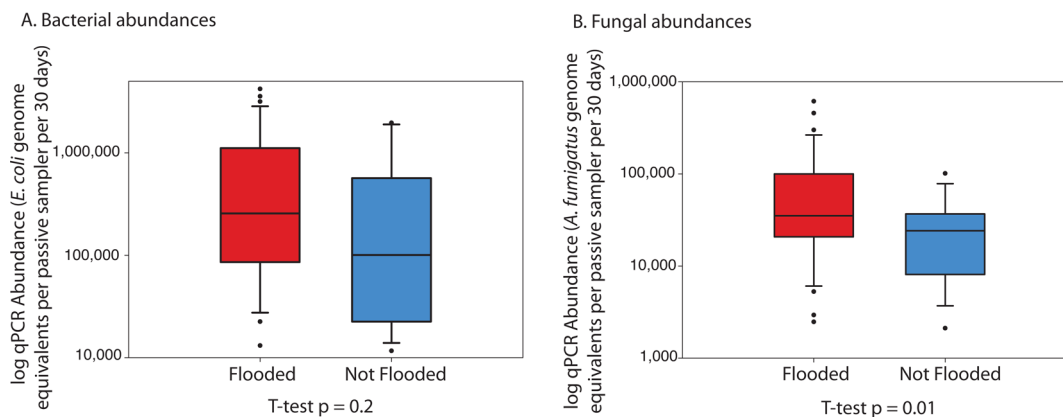
**DNA Extraction, PCR, and Sequencing.** All passive samplers (150 samplers in total) were prepared for DNA extraction, as follows: sterile, dual-tipped BBL CultureSwabs (Becton, Dickinson and Company, Franklin Lakes, NJ) were first applied to each Petri plate dry, such that all visible debris was transferred to one side of the swabs. The swabs were then flipped over, and 40  $\mu\text{L}$  of sterile, PCR-grade water was added in four drops (10  $\mu\text{L}$  each) to each Petri plate, and the full plate was reswabbed with the other side of the swabs to ensure removal of as much material as possible. Under sterile conditions, one of the two swab tips from each sample was placed in a single well of a MoBio PowerSoil-htp 96-well Soil DNA Isolation Kit (MoBio Inc., Carlsbad, CA), as described previously.<sup>29,30</sup> Each HVAC filter (41 HVAC filters in total) was swabbed and then placed in a 96-well DNA extraction plate under sterile conditions. For a description of HVAC filter swabbing techniques and a general discussion of sampling approaches in the context of this study, please see the Supporting Information. DNA was extracted according to the manufacturer’s instructions (MoBio Inc., Carlsbad, CA), with previously described modifications.<sup>31</sup>

PCR amplification was performed in triplicate for both bacteria and fungi for each DNA sample from the passive samplers (3 passive samplers per home) and the HVAC filters. To facilitate multiplexed sequencing, barcoded 515F and 806R primers with Illumina adapters and linkers were used to amplify the V4-V5 region of bacterial and archaeal 16S rRNA genes,<sup>32</sup> and barcoded fungal ITS1F (5'-CTTGGTCATTTAGAG-GAAGTAA-3') and ITS2 (5'-GCTGCGTCTTCATC-GATGC-3') primers with appropriate Illumina adapters and linkers were used to amplify the first internal transcribed spacer region (ITS1).<sup>33</sup> PCR reactions were performed with 1 $\times$  5 PRIME Hot Master Mix (5 PRIME Inc., Bethesda, MD), as previously described.<sup>34,35</sup> Negative controls were included to test for contamination. Triplicate PCR reactions were pooled for each sample (DNA from each passive sampler underwent three PCR reactions, which were pooled, but each sample

remained separate at this stage), and amplicon concentrations were measured with a PicoGreen dsDNA assay (Life Technologies, Grand Island, NY). We then pooled 300 ng of DNA per sample from each 96-well plate, cleaned the amplicons with the UltraClean PCR Clean-Up Kit (MoBio Inc., Carlsbad, CA), and then combined all pools (191 bacterial samples, 191 fungal samples, and 2 DNA extraction controls) for DNA sequencing at a final yield of 2  $\mu\text{g}$  of DNA, with a ratio of fungal to bacterial amplicon DNA of 1.5:1. The combined pool was sequenced on the Illumina MiSeq platform at the University of Colorado Next Generation Sequencing Facility. All sequencing data will be available for download from [datadryad.org](http://datadryad.org) upon publication, searchable by the citation for this article.

**Sequence Processing.** Sequences were demultiplexed, and forward and reverse 16S rRNA gene reads were merged. Owing to the variable length of the ITS1 region and the 150 bp read length (which precluded the merging of all forward and reverse read pairs), only forward reads were analyzed for fungi. All resulting sequences were quality-filtered, and singletons were removed via a combination of QIIME,<sup>36</sup> UPARSE,<sup>37</sup> and in-house python scripts. Sequences were then dereplicated, and a database of single representative sequences for each operational taxonomic unit (OTU) in the data set was generated via UCLUST (version 7)<sup>38</sup> clustering at 97% nucleotide identity, and then reads from the entire data set were mapped back to the representative bacterial or fungal database to generate one OTU table for bacteria and one for fungi. Taxonomy was assigned to each OTU via the Ribosomal Database Project taxonomic classifier for bacteria<sup>39</sup> and by sequence comparison to the UNITE fungal ITS database for fungi (version 5.0).<sup>40</sup> Fungal sequences with less than 75% identity to a known sequence in the UNITE database were removed from further analysis. We rarefied each OTU table to 3000 sequences per sample for bacteria and 4000 sequences per sample for fungi. All mitochondria and chloroplast OTUs were removed from the 16S rRNA gene OTU table prior to downstream analyses, as were OTUs at 10% or higher relative abundance in the DNA extraction controls for both bacteria and fungi.

**Quantitative PCR (qPCR).** DNA from all passive air samples was used for qPCR to determine the abundances of bacteria and fungi across samples, assuming that we removed all microbial biomass from the Petri plates for DNA extraction. We did not perform qPCR on HVAC filter samples, as the swabs from those filters were not quantitative. We used universal bacterial and fungal primers to estimate the total abundances of all bacteria and all fungi in the passive air samplers. We used the same 515F/806R bacterial 16S rRNA gene primers for sequencing, except the qPCR primers did not contain the adapters required for Illumina library construction. On the basis of reported *in silico* and laboratory fungal primer tests,<sup>41,42</sup> we used FF390/FR1 18S rRNA gene primers for fungal qPCR. These primers were designed and tested for soil and wood-associated fungi and, to our knowledge, this is the first application of these primers to air samples. As standards, the concentration of genomic DNA from *E. coli* K-12 (for bacteria) and *Aspergillus fumigatus* (for fungi) was assessed via a Quant-iT PicoGreen dsDNA assay (Invitrogen Life Technologies, Grand Island, NY), and seven 10-fold dilutions were used to generate a standard curve for each 96-well qPCR plate. The use of these standards means that our results are conservatively reported in *E. coli* or *A. fumigatus* genome equivalents, but the results should be interpreted as estimates of the total number of



**Figure 1.** Quantitative PCR abundance measurements of bacteria and fungi in basement air. Quantitative PCR (qPCR) measurements of bacterial abundances (A) and fungal abundances (B), log scale, in flooded and non-flooded homes. Note the difference in Y-axis scale between panels A and B. Results are presented in genome equivalents (A, *E. coli*; B, *Aspergillus fumigatus*) per passive sampler per 30 days. The line through the middle of each boxplot represents the median, the box is the 25th to 75th percentile, and the whiskers (lines) indicate the maximum and minimum values, apart from outliers, which are indicated with points beyond the whiskers.

bacterial or fungal cells. Each qPCR reaction included 1.25  $\mu\text{L}$  of the appropriate F and R primers (2.5  $\mu\text{L}$  total, 10  $\mu\text{M}$  starting concentration), 12.5  $\mu\text{L}$  2 $\times$  qPCR mix (Absolute QPCR SYBR Green Mix, no ROX, Fermentas Inc., Boston, MA), 5  $\mu\text{L}$  of PCR-grade water, and 5  $\mu\text{L}$  of template DNA. Triplicate qPCR reactions were run for each dilution of the appropriate standard and for each passive air sample on a Mastercycler ep realplex thermocycler (Eppendorf, Hamburg, Germany) in 96-well plates. Cycling conditions were as follows: 95  $^{\circ}\text{C}$  15 min, 40 cycles of (94  $^{\circ}\text{C}$  45 s, 50  $^{\circ}\text{C}$  1 min, 72  $^{\circ}\text{C}$  1.5 min), 72  $^{\circ}\text{C}$  10 min, 4  $^{\circ}\text{C}$  hold. Comparisons to the appropriate standard curve gave the estimated genome copy number in each qPCR reaction, and we report the estimated genome copy number on each passive sampler, normalized by the number of days that we sampled at the home. Results are presented as genome equivalents per passive air sampler (*E. coli* genome equivalents for bacteria and *A. fumigatus* genome equivalents for fungi) per 30 days (i.e., we took the measured qPCR abundance for each home, divided it by the number of days of sampling at that home, and then multiplied by 30 days).

**Statistical Analyses.** All statistical analyses were conducted in R,<sup>43</sup> unless otherwise indicated. Rarefied OTU tables were Hellinger-transformed, and Bray–Curtis dissimilarity matrices were generated for sample comparisons. For comparisons of flooded and non-flooded bacterial and fungal communities, Bray–Curtis dissimilarities from the three passive samplers for each home were averaged to best account for the replicate samples (i.e., the community dissimilarity to each sample in the study was averaged separately across the three passive samplers from each home). Unless otherwise indicated, these averaged dissimilarity matrices were used for community analyses of passive air samplers. We visualized results using nonmetric multidimensional scaling (NMDS) ordination plots and used permutational ANOVA (PERMANOVA) as implemented in PRIMER (version 6)<sup>44,45</sup> to test for significant differences in bacterial and fungal community composition between groups of samples (e.g., flooded vs non-flooded, HVAC filters vs passive samplers, sample groupings according to homeowner questionnaire data, etc.). For results deemed significant by PERMANOVA, we used the vegan R package “capscale” function to generate constrained ordinations to improve the visualizations.<sup>46</sup>

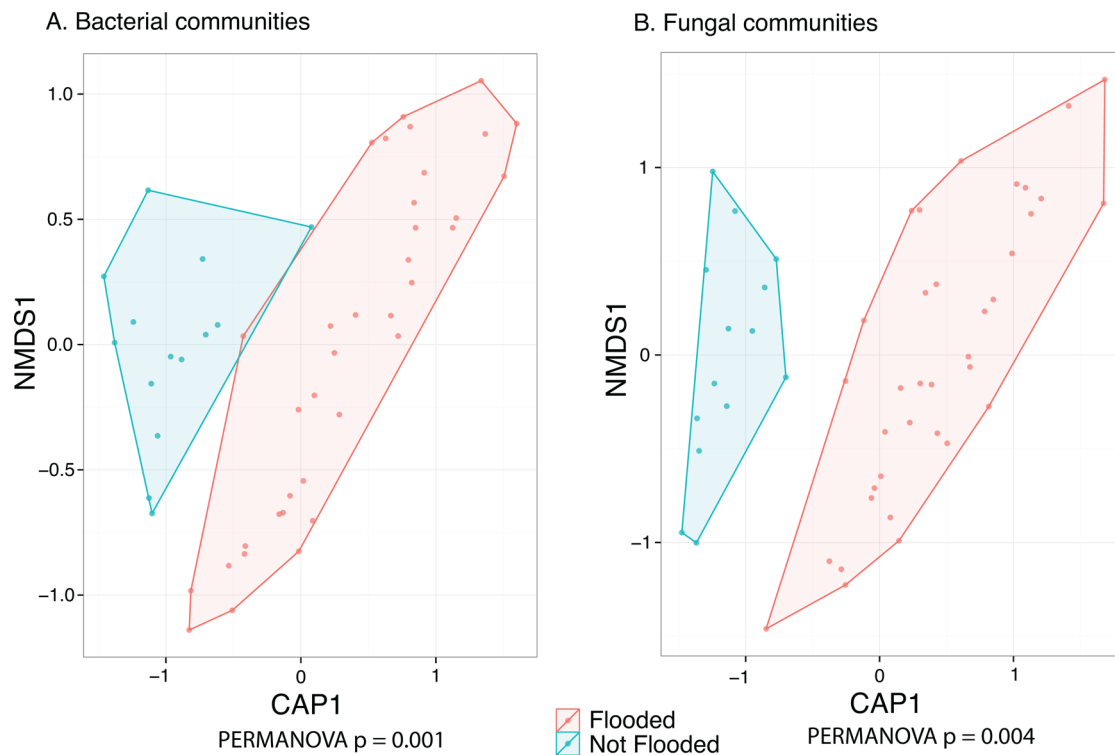
To identify taxa that had higher relative abundances in flooded, relative to non-flooded homes, we used the “wilcox.test” (Mann–Whitney) function in the vegan R package<sup>46</sup> on OTU tables at the family level for bacteria and at the order level for fungi. Within those larger taxonomic groups, we identified species that were relatively more abundant in flooded, relative to non-flooded homes by comparing the average proportional abundance of each species-level OTU identified in flooded homes to the average abundance of the same OTU identified in non-flooded homes. Species that were at least 1.5 times more abundant in flooded, relative to non-flooded homes, are mentioned in the text.

We used two-tailed students’ *t* tests to test for significant differences in environmental variables and qPCR estimates of overall bacterial and fungal concentrations between flooded and non-flooded homes. We also used Mantel tests implemented in PRIMER (version 6)<sup>44</sup> to compare bacterial and fungal community composition (Bray–Curtis dissimilarity matrices) to environmental variables and to qPCR data (Euclidean distance matrices).

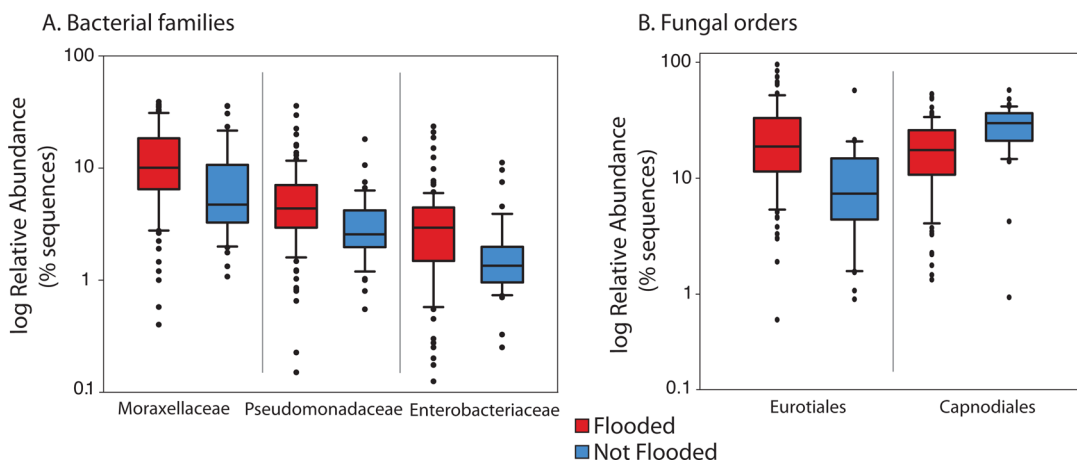
## RESULTS

**Quantitative PCR Abundance Estimates.** On the basis of the quantitative PCR (qPCR) results, we found that bacterial DNA concentrations were, on average, 76% higher on the passive samplers from flooded homes, compared to non-flooded homes (Figure 1A). These differences in bacterial concentrations between flooded and non-flooded homes were not significant (Student’s *t* test,  $p = 0.2$ ). However, on average, there was a significantly higher concentration of fungi in flooded, relative to non-flooded homes ( $p = 0.01$ ). There was approximately 210% more fungal DNA in flooded homes than in non-flooded homes (Figure 1B).

**Bacterial and Fungal Community Composition.** In a comparison of community composition across all samples in our study, we observed significant differences in both bacterial and fungal communities between HVAC filters and passive samplers (Figure S2 in the Supporting Information, permutational ANOVA  $p = 0.001$  for both bacteria and fungi). As such, we analyzed community data from HVAC filters separately from passive sampler data. On HVAC filters, no significant differences in bacterial or fungal community composition were



**Figure 2.** Comparisons of bacterial and fungal communities in basement air of flood-damaged and non-flooded homes. Constrained ordinations of bacterial (A) and fungal (B) communities on passive basement air samplers, separated by flood status (flooded or not flooded). CAP1 = constrained ordination axis 1, NMDS1 = nonmetric multidimensional scaling axis 1. Each point represents one home, plotted based on the averaged Bray–Curtis dissimilarities across the three passive air samplers from each home. Points closer together have more similar bacterial (A) or fungal (B) communities.



**Figure 3.** Bacterial and fungal taxa with significant differences in relative abundance between flooded and non-flooded homes. Three bacterial families (A) and two fungal orders (B) with significant differences in relative abundance between flooded and non-flooded homes, as calculated by Mann–Whitney tests. For each taxonomic group, the relative abundance, or the percent of sequences in each sample belonging to that taxonomic group, is presented for flooded and non-flooded homes (log scale). The line through the middle of each boxplot represents the median, the box is the 25th to 75th percentile, and the whiskers (lines) indicate the maximum and minimum values, apart from outliers, which are indicated with points beyond the whiskers.

detected between flooded and non-flooded homes (permutational ANOVA  $p = 0.127$  for bacteria,  $p = 0.141$  for fungi). However, significant differences in both bacterial and fungal community composition were detected between flooded and non-flooded homes recovered on the passive air samplers (Figure 2, permutational ANOVA  $p = 0.001$  for both bacteria and fungi). Because passive samplers showed significant differences between flooded and non-flooded homes, while

HVAC filters did not, all of our remaining community analyses focus on data from the passive samplers.

We identified an average of 421 bacterial and 131 fungal operational taxonomic units (OTUs) per sample, including an abundance of bacteria commonly associated with humans and indoor environments (e.g., Prevotellaceae, Comamonadaceae, and Staphylococcaceae).<sup>23,24,27</sup> Given the observed community differences between flooded and non-flooded homes, we sought

to determine which bacterial and fungal taxa were more abundant in flooded, relative to non-flooded homes. We identified taxa that were present in the majority of samples from flooded homes and significantly more abundant in flooded, relative to non-flooded homes. Of those taxa, we removed from consideration those at less than 1% average relative abundance in flooded homes. We initially focused on differences at the family level in bacteria and at the order level in fungi, and then, within those groups, we identified the most differentially abundant species. These analyses allowed us to identify those taxa that were present in the majority of flooded homes, at higher relative abundance in flooded homes, and abundant overall in flooded homes.

Bacterial taxa that were relatively more abundant in flooded homes are shown in Figure 3A. The Pseudomonadales (including both the Moraxellaceae and Pseudomonadaceae families) were more abundant in flooded, relative to non-flooded homes. On average, these two groups of Pseudomonadales represented approximately 16% of sequences identified at the family level in flooded homes, and they were on average 1.5 times more abundant in flooded, relative to non-flooded homes. Enterobacteriaceae, particularly from the genus *Pantoea*, were also approximately 1.5 times more abundant in flooded homes, relative to non-flooded homes. The Pseudomonadaceae were dominated by species of *Pseudomonas*, including *P. veronii*, *P. viridiflava*, *P. fragi*, and *P. stutzeri*. The Moraxellaceae (also in the Pseudomonadales order) were dominated by *Acinetobacter* species, particularly *A. johnsonii* and *A. rhizosphaerae*.

The Eurotiales were the most relatively abundant fungi in flooded homes (Figure 3B). On average, the Eurotiales represented approximately 19% of sequences identified at the order level in flooded homes and were nearly twice as abundant in flooded, relative to non-flooded homes. Within the Eurotiales order, *Penicillium* species, including *P. spinulosum*, *P. bialowiezense*, and *P. decumbens*, were at highest abundance in flooded, relative to non-flooded homes. Also at higher abundance in flooded homes were an unidentified *Aspergillus* species and *Eurotium repens*. Interestingly, the Capnodiales fungal order, and specifically *Cladosporium*, was found to be 52% more abundant in non-flooded, relative to flooded homes. It should be noted that these results are not a result of lower fungal biomass in the non-flooded samples because an equimolar amount of amplicon DNA was added to the sequencing reaction for each sample. Also, there were no statistically significant differences in the number of sequences generated per sample or richness (the number of bacterial or fungal species detected for each sample) between flooded and non-flooded homes.

**Temperature, Relative Humidity, and Other Environmental Variables.** Over the course of the study, the average temperature ranged from 12–25 °C in flooded homes and from 12–23 °C in non-flooded homes. The average relative humidity was between 22 and 54% in flooded homes and between 20 and 53% in non-flooded homes. No significant differences were observed for average temperature or average relative humidity between flooded and non-flooded homes (*t* test  $p = 0.54$  for average temperature,  $p = 0.99$  for average relative humidity). Mantel tests did not reveal significant correlations between bacterial or fungal community composition and any of the measured environmental variables (average, maximum, and minimum temperature and relative humidity).

We also used Mantel tests to compare sample groupings based on bacterial and fungal community composition to

sample groupings based on home characteristics that were reported as continuous variables. For example, we compared Bray–Curtis microbial community dissimilarity matrices to Euclidean dissimilarity matrices based on the depth of flooding, the degree of flood-related damage, the age of the home, and the number of inhabitants. Neither bacterial nor fungal community composition was correlated with any of these variables. For home and homeowner characteristics that were not measured as continuous variables, such as presence/absence of moldy odors, the use of antimicrobial agents and other remediation practices, the source of flood waters, and occupant-reported health effects, we used NMDS plots and PERMANOVA to compare sample groupings. No significant sample groupings were found for any of those variables.

## DISCUSSION

The detection of Eurotiales fungi, particularly several species of *Penicillium*, at relatively high abundance in flooded relative to non-flooded homes is consistent with previous studies, which have often shown *Penicillium* to be one of the more abundant fungal taxa in water-damaged homes.<sup>8,14–16</sup> However, the Capnodiales order, particularly the genus *Cladosporium*, was shown to be relatively more abundant in non-flooded homes in this study, in contrast to previous studies, which have shown it to be abundant in flooded homes.<sup>8,16</sup> This highlights the need to consider appropriate controls (non-flooded homes, in this case) for comparison where possible, and future studies might also consider targeted qPCR to resolve differences in absolute abundance at high taxonomic resolution.<sup>47</sup>

DNA sequencing-based resolution of *Penicillium* groups at the species level revealed *P. spinulosum*, *P. bialowiezense*, and *P. decumbens* to be particularly more abundant in flooded homes, compared to non-flooded homes. Considering that absolute abundances of fungi measured via qPCR were higher in flooded homes, it is reasonable to assume that absolute abundances of these *Penicillium* species were also higher in flooded homes. Because respiratory ailments have previously been correlated with exposure to water-damaged structures,<sup>10,48,49</sup> a study of the effects of *P. spinulosum* spores, isolated from the indoor air of water-damaged buildings, was conducted on mice but revealed minimal health effects.<sup>50</sup> A study of *P. bialowiezense* from indoor dust revealed many isolates capable of growth on or with other fungi,<sup>51</sup> and previous studies of closely related isolates showed growth on or in many indoor sites, including carpet,<sup>52</sup> drywall,<sup>53</sup> insulation,<sup>54</sup> and potted plants,<sup>55</sup> suggesting many potential sources for these airborne organisms. *P. decumbens* has been used to degrade lignocellulose on an industrial scale and is generally known to be an efficient plant cell wall degrader.<sup>56</sup> Though admittedly speculative, this lifestyle would be consistent with growth on wooden structural beams, which likely experienced water damage during flooding and may have been treated with biostatic agents but were not removed. In general, it is clear that there are many possible source environments for the abundant *Penicillium* species in flooded homes in this study, including “hidden molds” in unremediated wall areas.

Previous studies of bacteria in flooded homes are limited and do not generally suggest that specific taxonomic groups are more common in flood-damaged homes, though endotoxin data suggest that Gram-negative bacteria may be more abundant in homes after a flood.<sup>8,14,15</sup> Consistent with these results, Pseudomonadaceae and Enterobacteriaceae, the two most abundant bacterial families in flooded homes in our study,

are Gram-negative. The detection of specific species of *Pseudomonas*, including *P. veronii*, *P. viridiflava*, *P. fragi*, and *P. stutzeri*, at high relative abundance in flooded homes is consistent with a variety of potential source environments, including soil,<sup>57</sup> river water,<sup>58</sup> and fungus-associated environments.<sup>59</sup> Some *Pseudomonadaceae* are known to produce and live in biofilms. This lifestyle could make these organisms better adapted to resist the biostatic agents applied to kill microorganisms in water-damaged homes, as demonstrated under laboratory conditions.<sup>60</sup> The abundant *Acinetobacter* species in flooded homes, including *A. johnsonii*, have been shown to form aggregates,<sup>61</sup> which could result in similar resistance to antimicrobial treatments, and they have been found in a variety of environments that are consistent with their abundance in indoor air from flooded homes, including river water<sup>62</sup> and indoor air from a turkey farm.<sup>63</sup> The *Pantoea* genus, the most abundant group of Enterobacteriaceae in flooded homes, includes organisms isolated from a wide variety of environments, including plants, water, soil, humans, and animals.<sup>64</sup> Overall, these results suggest that the abundant bacteria in flooded homes are potentially better adapted to resist antimicrobial treatments, and they could come directly from flood waters, and/or they may have grown in response to flood conditions.

In terms of absolute abundances (concentrations), only fungi were significantly more abundant in flooded, relative to non-flooded homes, as measured by qPCR. Similar absolute bacterial abundances in flooded and non-flooded homes suggest that the total numbers of bacterial cells were not (or were no longer) elevated in flooded homes, despite significant differences in bacterial community composition. Consistent with culture- and epifluorescence microscopy-based fungal abundance estimates from Colorado homes flooded by the Arkansas River,<sup>6</sup> absolute fungal abundances in this study were estimated to be approximately 2–3 times higher in flooded homes a few months after the flood. In a study conducted 2 years after hurricane Katrina in New Orleans, LA, fungal abundances were still elevated in flooded homes, although they had decreased, relative to measurements made shortly after the flood.<sup>65</sup> Together, our results suggest that, despite extensive remediation efforts, fungal concentrations in the air of flooded homes did not return to baseline (non-flooded) levels.

Interestingly, temperature and relative humidity did not correlate significantly with flooding in this study. Boulder is a semiarid region with humidity levels in outdoor air that are typically quite low (52% on average),<sup>66</sup> so it is perhaps not surprising that residual moisture from the flood would have been removed prior to our sampling, which began 2 months after the flood. Many of the homeowners completed active water removal within a few days of the flooding event. Still, these results show that the lingering effect on bacterial and fungal community composition was not due to a prolonged increase in relative humidity from the flood within living spaces. Additionally, previous studies have shown water activity to be related to fungal growth on surfaces.<sup>67</sup> It may be that water activity, or a surrogate for this parameter, equilibrium relative humidity near surfaces, is more related to fungal abundance in the air.<sup>68</sup>

A comparison of bacterial and fungal communities on HVAC filters to those collected by passive samplers revealed a significant difference in community composition between the two types of samplers, irrespective of flooding conditions. HVAC filters actively collected particulate matter from the

whole home, along with some particulate matter from outdoor air,<sup>69</sup> whereas our passive samplers only collected settled dust from basement air approximately 2.1 m above the floor. As such, our HVAC filters and passive samplers likely sampled different indoor air and different (but perhaps overlapping) aerosol size fractions. Sampling on HVAC filters could have resulted in mechanical microbial lysis, as has been shown for other active aerosol sampling approaches.<sup>70</sup> Whether such lysis could have selectively changed the overall community composition is unknown, but one species of Gram-negative bacteria subjected to active aerosol sampling was shown to lyse more easily than a species of Gram-positive bacteria.<sup>70</sup> These studies suggest that differences in source air and sampling approach may explain some of the differences in microbial community composition that we observed between HVAC filters and passive basement air samplers. Still, since we only detected differences in microbial community composition on passive basement air samplers and not on active whole-home air samplers, it is possible that differences in microbial community composition may have been largely restricted to the flooded level and would not have been detected in other locations within these homes.

In hindsight, and in recommendation for future studies, the placement of additional passive air samplers on the main level of the home, instead of the collection of swabs from HVAC filters, would have been useful for direct comparisons to the basement passive samplers. However, these additional samplers may have been more invasive for our volunteer homeowners, and differences in the sizes and uses of space on the upper levels would likely have presented confounding variables for the interpretation of our results. In general, we found the passive air samplers to be inexpensive, easy to install, and conducive to massively parallel indoor air studies that require sampling air from tens of homes at the same time, and we would support the use of such samplers for future studies of this scale. Ideally, we would have collected outdoor air samples using the same methods, but practically, this was not possible, as we could not control for the influence of wind, precipitation, animals, etc. on our passive samplers outdoors. Outdoor air sampling techniques that have been used for other microbial ecological studies (e.g., vacuum filtration<sup>31</sup>) were not practical at the scale of this study, which required simultaneous sampling at 50 homes for 1 month. However, considering that some non-flooded homes in this study were in the same neighborhood as flooded homes, we assume that outdoor air microbial community composition would not likely have explained the differences in indoor air community composition that we observed between flooded and non-flooded homes.

This study provides the first insight at high taxonomic resolution into bacterial and fungal communities in homes following a flood, including direct comparisons of indoor air in flooded and non-flooded homes in close proximity. Differences in bacterial and fungal community composition in flooded homes 2–3 months after the flooding event suggest a persistent impact of flooding, despite nearly all of the homes having completed or nearly completed remediation. We identified specific bacterial and fungal taxa that were associated with flooded homes, some of which are consistent with previous results, and some of which provide new insight into the microbial ecology of flooded homes and the air that the occupants may be breathing. The fact that we found some consistencies in microbial community composition across flooded homes suggests that there are some commonalities to

the sources and/or environmental drivers of microbial community composition in flooded homes. Overall, these results highlight the need for further study, including (1) long-term monitoring of homes post-flood to assess the temporal scales on which differences in microbial exposures occur, (2) assessments of whether these long-term differences in microbial exposures have a continued health impact, (3) investigations of whether water activity or equilibrium relative humidity in the flooded area is related to microbial exposure, and (4) consideration of alternative and/or additional remediation strategies to mitigate long-term flood-associated changes in the bacteria and fungi present in indoor air.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Discussion of swabbing techniques; Figure S1, a photograph of a passive air sampler installed in a basement; Figure S2, a comparison of whole-home air and basement air bacterial and fungal communities; Tables S1 and S2 showing home and occupant characteristics. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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