Hurricane María drives increased indoor proliferation of filamentous fungi in San Juan, Puerto Rico: a two-year culture-based approach

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The extensive flooding caused by Hurricane María in Puerto Rico (PR) created favorable conditions for indoor growth of filamentous fungi. These conditions represent a public health concern as contamination by environmental fungi is associated with a higher prevalence of inflammatory respiratory conditions. This work compares culturable fungal spore communities present in homes that sustained water damage after Hurricane María to those present in dry, non-flooded homes. We collected air samples from 50 houses in a neighborhood in San Juan, PR, 12 and 22 months after Hurricane María. Self-reported data was used to classify the homes as flooded, water-damage or dry non-flooded. Fungi abundances, composition and diversity were analyzed using tools originally develop for the data generated by molecular biology approaches (*MicrobiomeAnalyst* and *METAGENassist* platforms). Our results showed no significant differences in indoor fungal concentrations (CFU/m^3) during the first sampling period in both culture media studied (MEA and G25N). During the second sampling period fungal levels were 2.6 times higher in flooded homes (Median = 408) when compared to dry homes (Median = 233), respectively (p-value < 0.005). Fungal profiles showed enrichment of *Aspergillus* species inside flooded homes compared to outdoor samples during the first sampling period (FDR-adjusted p-value = 0.05). In contrast, during the second sampling period, the indoor fungal composition consisted primarily of non-sporulated fungi, most likely basidiospores, which are characteristic of the outdoor air in PR. Together, this data highlights that homes that suffered water damage not only have higher indoor proliferation of filamentous fungi, but their indoor fungal populations change over time following the hurricane. Ultimately, after nearly two years, the indoor and outdoor fungal communities converged in this sample of

naturally ventilated homes.

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41 **ABSTRACT**

42   The extensive flooding caused by Hurricane María in Puerto Rico (PR) created favorable
43   conditions for indoor growth of filamentous fungi. These conditions represent a public health
44   concern as contamination by environmental fungi is associated with a higher prevalence of
45   inflammatory respiratory conditions. This work compares culturable fungal spore communities
46   present in homes that sustained water damage after Hurricane María to those present in dry, non-
47   flooded homes. We collected air samples from 50 houses in a neighborhood in San Juan, PR, 12
48   and 22 months after Hurricane María. Self-reported data was used to classify the homes as
49   flooded, water-damage or dry non-flooded. Fungi abundances, composition and diversity were
50   analyzed ~~using~~ tools originally develop for the data generated by molecular biology approaches
51   (*MicrobiomeAnalyst* and *METAGENassist* platforms). Our results showed no significant
52   differences in indoor fungal concentrations (CFU/m^3) ~~during the first sampling period~~ in both
53   culture media studied (MEA and G25N). During the second sampling period fungal levels were
54   2.6 times higher in flooded homes (Median = 408) when compared to dry homes (Median = 233),
55   ~~respectively~~ (p-value < 0.005). Fungal profiles showed enrichment of *Aspergillus* species inside
56   flooded homes compared to outdoor samples during the first sampling period (FDR-adjusted p-
57   value = 0.05). In contrast, ~~during the second sampling period,~~ ~~the~~ indoor fungal composition
58   consisted primarily of non-sporulated fungi, most likely basidiospores, which are characteristic of
59   the outdoor air in PR. Together, this data highlights that homes that suffered water damage not
60   only have higher indoor proliferation of filamentous fungi, but their indoor fungal populations
61   change over time following the hurricane. Ultimately, after nearly two years, ~~the~~ indoor and
62   outdoor fungal communities converged in this sample of naturally ventilated homes.
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64   **Keywords:** Hurricane María, mold spores, fungi, mycobiome, aeroallergens, indoor air, indoor
65   environment, *Aspergillus*, built environment, flooded homes, water-damage

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80   **INTRODUCTION**

81   Fungi are ubiquitous eukaryotic organisms of environmental and medical importance ~~as~~
82   ~~environmental fungal exposures are~~ associated with adverse health effects in humans (Kendrick,
83   2011; Cannon et al., 2018). Fungi reproduce by spore production, many of which are dispersed

84   in the air with fungal spore sizes ranging from approximately 0.65 µm to > 20 µm (Portnoy,
85   Barnes & Kennedy, 2008; Hamilos, 2010; Li et al., 2011; Kwon-Chung & Sugui, 2013; Claub,
86   2015). Fungal spores and fragments less than 2.5 m can deposit in the alveoli and trigger asthma
87   in sensitized individuals (Joubert et al., 2020). In contrast, large fungal spores and clusters of
88   small fungal spores can deposit in the upper airway triggering allergic rhinitis (Joubert et al.,
89   2020). Environmental fungal contamination, including high airborne fungal spore concentrations
90   in the air, are associated with inflammatory respiratory conditions such as asthma and allergic
91   rhinitis (Lewis et al., 2019). In addition, for people already suffering from asthma, exposure to
92   fungal spores simultaneously with other allergens (animal dander, dust mites, cockroaches) may
93   cause dangerous exacerbations of existing asthma (Gautier & Charpin, 2017). Approximately,
94   339 to 500 million people worldwide are affected by asthma and allergic rhinitis, respectively
95   (Kołodziejczyk & Bozek, 2016; Global Asthma Network, 2018). In Puerto Rico (PR), asthma
96   prevalence in children and adults is higher than in the United States (2017: 15.5% vs 7.5% for
97   children and 12.2% vs. 9.1% for adults) (Ortiz-Rivera, 2018).

98   Due to its tropical location, frequent ~~hurricanes and extreme~~ rain events, PR has a high
99   concentration of fungal spores in the air. Very high levels observed in PR exceed 110,000 spores/
100 m^3, 2.2 times higher than those observed in the other National Allergy Bureau (NAB) of the
101 American Academy of Allergy Asthma and Immunology mold levels stations (AAAAI, 2021).
102 Outdoor fungal spore concentrations typically reach their maximum levels in PR during the rainy
103 months of September to November (Quintero, Rivera-Mariani & Bolaños-Rosero, 2010). The
104 fungi found in outdoor ambient air are typically split between Basidiomycetes (60%) and
105 Ascomycetes (40%) (Rivera-Mariani et al.; AAAAI, 2021). *Pleurotus ostreatus, Chlorophyllum*
106 *molybdites* and *Ganoderma applanatum* are some of the basidiomycetes common in the air of PR
107 (Rivera-Mariani et al., 2011). Ascomycetes include filamentous *Aspergillus* species (*A.*
108 *fumigatus*, *A. terreus*, *A. niger*, *A. flavus*) which are the most frequent causative agents of human
109 aspergillosis, lung inflammation and among the most frequent triggers of asthma and allergic
110 rhinitis (Portnoy, Barnes & Kennedy, 2008; Hamilos, 2010; Kwon-Chung & Sugui, 2013).
111 Indeed, a recent study in PR found that increases in outdoor fungal spore concentrations were
112 associated with increases in asthma-related health claims, confirming that fungal spores are
113 triggers of asthma (Lewis et al., 2019). Most Puerto Rican homes have natural air ventilation;
114 therefore, spores and pollen present outdoors can easily penetrate indoors through open windows
115 and doors. Even though the 2017 outdoor fungal spore season was lower than expected as a result
116 of Hurricane María, the fungal spore season for 2018 was the highest ever recorded in the 16-year
117 history of data collection at the San Juan station most likely due to the accumulation of organic
118 debris in the aftermath of Hurricane María (AAAAI, 2021; Bolaños-Rosero, 2021).

119   ~~The~~ essential conditions for indoor growth of filamentous fungi include high moisture and
120 humidity, warm temperature, poor ventilation or air circulation, and the presence of organic
121 source materials (Institute of Medicine, 2004; WHO, 2009). Taking these factors into
122 consideration, the extensive flooding caused by Hurricane María in PR had the potential to
123 provide a favorable environment for fungal proliferation indoors in homes with water damage.
124 Several studies have evaluated the effects of hurricanes on airborne fungi in the United States,
125 with the most common fungi isolated being *Aspergillus, Penicillium* and *Cladosporium* (CDC,

126 2006; Chew et al., 2006; Rao et al., 2007; Schwab et al., 2007; Rabito et al., 2008; Saporta &
127 Hurst, 2017; Gargano et al., 2018; Chow et al., 2019; Kontoyiannis et al., 2019). Although PR
128 has been affected by more than 15 hurricanes and tropical storms since the 1980's, the effect of
129 these natural disasters on the concentration and composition of indoor airborne fungi ~~have~~ not yet
130 been studied. Here, we ~~provide a critical and quantitative analysis of the~~ airborne fungal
131 concentrations in the months following Hurricane María in PR. Our hypothesis is that homes
132 which sustained flooding or water damage will have higher fungal diversity and abundance of
133 fungi related with damp indoor environments. In addition, since the sampled homes are
134 predominately naturally ventilated, we hypothesize that fungal taxa in the indoor environment of
135 dry non-flooded homes may reflect the taxa in the outdoor environment. We believe this study
136 will provide the basis for understanding the effect of fungal growth and species composition as a
137 result of a flooding event in PR. The results from this study lay the foundation to design
138 mitigation strategies to minimize adverse fungal exposures after flooding events to better control
139 lung inflammation and asthma in PR and other tropical countries.
140
141 **MATERIALS AND METHODS**
142
143 *Study Population.* After obtaining Institutional Review Board (IRB) approval from the University
144 of Puerto Rico - Río Piedras Campus (IRB protocol 1718-058), 50 residences from the area of
145 Santurce (San Juan, PR) were selected to participate in this study (**Fig. 1**). Many homes located in
146 this area sustained flooding during and after Hurricane María's passage through the island. Of the
147 50 homes, 10 were located in a non-flooded area and 40 were located in a flooded area according
148 to a reference map generated by the Federal Emergency Management Agency (FEMA). In
149 addition to considering this classification, participants were surveyed and asked to describe in
150 more detail the level of water damage. This reported data allowed the classification of each
151 household in greater detail: 13 houses were dry non-flooded, 24 houses had water-damage
152 (received water from above, roof and/or windows), and 13 houses were flooded (received water
153 from below, through doors and crevices). We acknowledge that the sample size for the different
154 categories was small, but we were working with recruitment under very difficult circumstances.
155 We thus have two types of classifications for each home: (1) FEMA based: whether the homes
156 were located in a flooded or non-flooded area according to FEMA (dry area or flooded area) and
157 (2) Self-reported damage reported by study participants (dry, water-damage or flooded). After
158 obtaining written informed consent, participants were asked to complete questionnaires ~~in order~~
159 to gather information on the demographics, health of home occupants (respiratory and
160 psychological), home characteristics (ventilation, number of windows, construction materials)
161 and the physical impact of Hurricane María on the home (water-damage, remediation activities,
162 and the presence of visible mold). For the purpose of this study, we focus on evaluating the
163 relationship between the self-reported degree of water damage and viable fungal communities
164 present in samples collected from the indoor and outdoor environment of 50 homes in PR.
165
166 *Air Sampling & Fungal Culturing.* We collected indoor and outdoor samples during two different
167 sampling periods: August - September 2018 and June-July 2019, 12 and 22 months, respectively,

168   after Hurricane María. For the air sample collection, we used the MicroBio MB2 Bioaerosol
169   Sampler (Cantium Scientific, Dartford, UK). This volumetric instrument collects airborne
170   bioaerosol contaminants by aspirating air through a removable stainless-steel head (cover) which
171   contains 220, 1 mm holes. This design creates a laminar airflow that impels the microorganisms,
172   in this case the fungal spores, onto the surface of the culture media. A pilot study using different
173   sampling volumes allowed us to set the optimal total air sampling volume at 60 liters (flow rate
174   of 60L/35 seconds) which allowed us to count and identify to genus level the viable fungi without
175   overgrowth on the Petri plate. Prior to Hurricane María, the standard sampling volume used in a
176   previous study was 150 liters, but due to record-high outdoor fungal spores levels reported in
177   2018, the sampling volume was decreased to 60 liters (Bolaños-Rosero et al., 2013). At each
178   home, indoor samples were collected at a height of 1 meter from four indoor locations (living
179   room, kitchen, bedroom and bathroom) and an outdoor sample was collected in the front yard of
180   the residences. Before sample collection, interior fans were turned off and the number of open
181   and closed windows were counted. We used two types of culture media, Malt Extract Agar
182   (MEA) and 25% Glycerol Nitrate Agar (G25N; K_2HPO_4 0.375 g, Czapek concentrate 3.75 ml,
183   yeast extract 1.75 g, glycerol 125 ml, agar 6 g, distilled water 375 ml). MEA is a nutritive
184   growth media used for the isolation of general fungi ~~since it is suitable for most fungal growth~~
185   ~~while the~~ G25N is a selective growth media for xerophilic (dry tolerant) fungi, which grow at low
186   water activity or availability (Wu, Su & Ho, 2000; De León et al., 2018). After sampling, ~~the~~
187   Petri plates were incubated at 25 ± 2°C for up to 2 weeks. Visible fungal colonies were counted
188   using a stereomicroscope as early as 24-48 hours after sampling with MEA, taking precautions to
189   avoid mixing of the fungal colonies such as keeping the inverted plates horizontally. This process
190   was repeated daily for 7 days in the case of MEA and for 14 days in the case of G25N. Culturable
191   fungi were reported in colony forming units per cubic meter (CFU/m^3) using the MicroBio PC
192   Reporter software and data from the fungal colony count with positive-hole correction and the
193   total volume of air drawn through the MicroBio MB2 Bioaerosol sampler for each sample
194   collected. ~~The~~ positive-hole correction accounts for the probability of more than one viable
195   particle could be entering through a sampling hole, merging with other microorganisms at the
196   same impaction site and producing a single colony(Macher, 1989). In addition to determining
197   culturable fungi at each participating home, we also measured relative humidity levels both
198   indoor and outdoor at each home. We also compiled precipitation data from the National Weather
199   Service Forecast Office (NWSFO) and outdoor fungal spore levels from the San Juan AAAAI
200   station for both sampling periods to characterize the ambient fungal background levels during the
201   sampling events (NWSFO, 2014; AAAAI, 2021).
202
203   *Fungal Identification.* ~~After fungal colonies were counted,~~ we performed slide mounts to analyze
204   the colonies under the microscope and identify them to the genus level. First, we used the
205   stereomicroscope to evaluate how many possible distinct fungi we had in our sample. Prior to
206   preparing the slide mounts, we isolated the most predominant fungi present for further
207   experiments. Then, we used fungi tape and lactophenol cotton blue staining to prepare the slide
208   mounts. Following this, we observed the fungal slide mounts under the microscope and the fungi

209   were identified to the genus level by evaluation of morphological characteristics such as spore

210   shape, size, color and arrangement (Samson et al., 2019).

211

212   *Statistical Tests and Data Analyses.* Data on fungal colonies concentrations and metadata

213   variables were analyzed in RStudio© Version 1.2.1335 (R Foundation for Statistical Computing,

214   Vienna, Austria) (RStudio-Team, 2020). When analyzing the indoor data, we used the value of

215   the calculated median of the indoor measurements per home. To evaluate differences in indoor

216   relative humidity by degree of water damage (Dry, Water-Damage, Flooded), we performed the

217   non-parametric Kruskal Wallis test. We also used the non-parametric test Kruskal-Wallis to

218   analyze differences in fungal colonies concentrations as a function of different self-reported

219   degree of water damage (dry, water-damage, flooded). We considered a p-value less than 0.05

220   statistically significant. To evaluate the contribution of outdoor fungal levels to indoor fungal

221   levels, we performed a Spearman Correlation analysis and a Linear Regression analysis with

222   RStudio for graphs and Intellectus Statistics for interpretation (Intellectus Statistics; RStudio-

223   Team, 2020). For the purposes of performing alpha and beta diversity analyses and visualization

224   of the plots, we used *MicrobiomeAnalyst* and *METAGENassist* which are web-based platforms

225   used for the analysis of microbiota data (Arndt et al., 2012; Dhariwal et al., 2017). Biodiversity

226   census, either culture-based or culture-independent, require a consensus on diversity metrics.

227   That is, standardized methods for the measurement of biodiversity at different scales exist, such

228   as Chao1 observed species metrics or diversity indexes of Shannon or Simpson, initially used to

229   monitor biodiversity (Roswell, Dushoff & Winfree, 2021). Here, we cataloged the grown molds

230   (culture-based approach) and used the species table of abundances to calculate alpha and beta

231   diversity estimates. For data filtering, processing and normalization, we used a low-count filter

232   based on 10% prevalence and no data rarefaction was performed. Normalization was done row-

233   wise, log transformed on the total sum, in *METAGENassist* so that the data could follow a

234   Gaussian distribution and samples could be more comparable to each other. Statistical analyses

235   for alpha diversity, were done using compare_alpha_diversity.py in QIIME which uses a non-

236   parametric t-test with Monte Carlo permutations. For Beta-diversity analyses, PCoA, Jensen-

237   Shannon Divergence and permutational MANOVA (PERMANOVA) were used as ordination,

238   distance and statistical methods, respectively. Taxonomic bar plots were created using percentage

239   abundance and a taxa resolution of the top n taxa, with n = 10. To identify significant taxa

240   signatures at genus/species level between two different groups, a Linear Discriminant Analysis

241   (LDA) Effect Size (LEfSe) algorithm for biomarker discovery was used (Segata et al., 2011).

242   This method allowed us to identify statistically significant fungal features that differ between the

243   indoor and outdoor environments of homes classified by self-reported degree of water damage,

244   expressed as LDA values shown in boxplots and using either Original (uncorrected) or FDR

245   (False Discovery Rate)-adjusted p-value < 0.05). In addition, we compared the fungal profile

246   (alpha, beta, abundance and biomarker analysis) between dry and wet (water-damage + flooded)

247   homes.

248

249   **RESULTS**

250

251   During the first sampling period, a total of 50 homes were sampled (**Fig. 1**). For the
252   second sampling period, a total of 35 homes completed the follow-up sampling event (70%
253   retention rate). During the first sampling period in 2018, 10 homes were in a non-flooded zone
254   and 40 were in a previously flooded zone as per FEMA's classification scheme following
255   Hurricane María (**Table 1**). For the second sampling period in 2019, 8 of 35 homes were in a dry
256   FEMA zone and 27 in a previously flooded area according to FEMA. The self-reported data on
257   flooding conditions provided by participants yielded a more detailed assessment of actual
258   flooding and water damage conditions within the study homes. It is worth noting that the FEMA
259   classification does not take into account details that can influence the extent of the flooding (e.g.
260   some homes were on second floors and were not flooded even though they were located in an
261   area categorized by FEMA as flooded). Of the 50 homes sampled in 2018, 13 houses were
262   reported by household members to be dry, 24 had water-damage, and 13 had been subjected to
263   flooding. Of the 35 homes sampled in 2019, 11 houses were reported by household members to
264   be dry, 15 had water-damage, and 9 had been subjected to flooding. All the homes were normally
265   found to be naturally ventilated via open doors and windows. Fans and/or an occasional air
266   conditioner were also present in some homes but these were turned off during air sample
267   collection.
268
269   No significant differences in median relative humidity levels inside dry, water-damage
270   and flooded homes were observed during the first sampling period (**Fig. 2A**). In contrast, the
271   indoor environment of flooded homes had significantly higher relative humidity levels during the
272   second sampling period when compared to dry homes (p-value = 0.007) (**Fig. 2B**).
273
274   Since outdoor fungal spore concentrations are important in the context of tropical homes
275   with natural ventilation, we reviewed these data for the sampling periods of our study. Outdoor
276   levels of fungal spores, reported at the San Juan AAAAI station, were higher during the first
277   sampling period (an average of 86,478 spores/m^3 and 103,762 spores/m^3 in August and
278   September of 2018, respectively) when compared to the second sampling period (an average of
279   44,615 spores/m^3 and 38,632 spores/m^3 in June and July of 2019, respectively). Similar results
280   were obtained for outdoor culturable fungi (CFU/m^3) measured outside the study homes with
281   1.88- and 1.12-times higher levels during the first sampling period when compared to the second
282   sampling period in G25N and MEA, respectively (data not shown, not statistically significant).
283   As outdoor San Juan fungal spore concentrations follow a seasonal pattern related to seasonal
284   precipitation, the precipitation levels during the sampling periods were also compared (Quintero,
285   Rivera-Mariani & Bolaños-Rosero, 2010). Precipitation levels were higher in August 2018 (first
286   sampling period, total monthly precipitation of 6.29") and in July of 2019 (second sampling
287   period, total monthly precipitation of 6.72") (NWSFO, 2014).
288
289   We evaluated the relationship between outdoor and indoor fungal concentration (CFU/
290   m^3). For indoor levels we used the culturable measurements median of the indoor locations at
291   each home. For the first sampling period, 12 months after Hurricane María, a significant positive
292   correlation was observed between outdoor and indoor fungal concentration (CFU/m^3) (*r* =

293   0.47, $p < .001$, 95% CI = [0.22, 0.66]). This correlation indicates that as outdoor fungal colonies
294   increase, indoor fungal colonies also ~~tend~~ to increase. ~~The results of the~~ linear regression model
295   ~~were significant,~~ $F(1,48) = 60.79$, $p < .001$, $R^2 = 0.56$, ~~indicating that~~ approximately 56% of the
296   variance in indoor fungal colonies ~~is explainable by the outdoor levels~~ (**Fig. 3A**). For the second
297   sampling period, 22 months after Hurricane María, a significant positive correlation was still
298   observed between outdoor and indoor (living room) fungal colonies ($r = 0.71$, $p < .001$, 95% CI =
299   [0.49, 0.84]). The correlation coefficient between outdoor and indoor was 0.71, indicating a large
300   effect size. The results of the linear regression model were also significant, $F(1,33) =$
301   11.40, $p = .002$, $R^2 = 0.26$, with approximately 26% of the variance in indoor fungal colonies
302   explained by outdoor fungal colonies (**Fig. 3B**).
303
304   We evaluated indoor culturable fungi concentrations ~~in the study homes~~ as a function of
305   self-reported degree of water damage. During the first sampling period in 2018, no significant
306   differences in culturable fungi concentration were observed in both culture media evaluated (**Fig.**
307   **4A and 4B**). For the second sampling period, indoor CFU/m^3 increased with degree of water
308   damage for both culture media (**Fig. 4A and 4B**). Both flooded (Median = 758) and water-
309   damage homes (Median = 408) had 2.6 and 1.4 times higher CFU/m^3 when compared to dry non-
310   flooded homes (Median = 233) in the G25N culture media (**Fig. 4A**), although only statistically
311   significant between flooded and dry homes (p-value < 0.005). Results from the MEA culture
312   media (**Fig. 4B**) during the same period showed 2 and 1.6 times higher CFU/m^3 in flooded
313   (Median = 716) and water-damage homes (Median = 583) when compared to dry, non-flooded
314   homes (Median = 358), although only statistically significant between flooded and dry homes (p-
315   values < 0.01).
316
317   ~~We also analyzed the culture-based data using web-based platforms originally designed~~
318   ~~for visualizing molecular data.~~ Beta-diversity analysis using PCoA showed statistical differences
319   (p-values < 0.006) between fungal communities as a function of self-reported degree of water
320   damage during the first sampling period (**Fig. 4C**). ~~Results from~~ alpha-diversity analysis using
321   the Chao1 richness estimator showed no significant differences (p-value = 0.38415) in richness
322   ~~by self-reported degree of~~ water damage (**Fig. 4D, Supplementary Table 1**).
323   ~~We then compared the relative abundance of fungal taxonomic features at the genus level~~
324   ~~by sampling location (indoor or outdoor) and self-reported degree of water damage during both~~
325   ~~sampling periods (first and second) (**Fig. 5A and 5B**).~~ During the first sampling period, the
326   fungal taxa present in the indoor environment of dry homes had similar relative abundance levels
327   to those present in the outdoor environment and were dominated by non-sporulating fungi (**Fig.**
328   **5A**). ~~The~~ water-damage homes were still dominated by non-sporulating fungi, but there was an
329   increase of *Aspergillus* taxa in the indoor environment. Specifically, during the first sampling
330   period, the indoor environment of flooded homes was enriched by *Aspergillus* species when
331   compared to the outdoor samples. During the second sampling period, enrichment of *Aspergillus*
332   taxa in flooded homes was not observed (**Fig. 5B**). Both indoor and outdoor environments were
333   dominated by non-sporulating fungi regardless of the degree of water-damage in the homes
334   sampled. Linear discriminant analysis (LDA) together with effect size measurement (LEfSe)

335   method ~~was used in order to~~ ~~identify~~ statistically significant fungal features at the genus/species
336   level (**Fig. 5C and 5D**). Higher LDA scores represent increased abundance of fungal biomarkers
337   in the samples classified by sampling location (indoor versus outdoor) and self-reported degree of
338   water damage (dry, water-damage, flooded). During the first sampling period, enrichment of non-
339   sporulating fungi was identified in the outdoor samples of dry homes, whereas *Aspergillus*
340   *tamari* and *Rhodotorula* were significantly enriched in the outdoor samples of water-damage and
341   flooded homes, respectively (**Fig. 5C**). In contrast, *Aspergillus* was significantly enriched in the
342   indoor environment of flooded homes during the first sampling period. During the second period
343   no significant features were found.
344
345   ~~Finally, we analyzed the abundance levels of fungal taxa individually indoors versus~~
346   ~~outdoors (~~**Fig. 6**~~).~~ During the first sampling period, *Aspergillus* spp. and *Penicillium* spp. were
347   more abundant in indoor samples, whereas non-sporulating fungi and *Cladosporium* spp. were
348   more abundant outdoors (**Fig. 6A**). For the second sampling period, Trichoderma was more
349   abundant indoors while *Aspergillus,* non-sporulating fungi and *Eurotium amstelodami* were more
350   abundant outdoors (**Fig. 6B**).
351
352   ~~We also evaluated the indoor and outdoor fungal profile of dry and wet (water-damage +~~
353   ~~flooded) homes (~~**Fig. 7**~~).~~ Beta-diversity analysis using PCoA showed differences in the fungal
354   communities of dry homes grouped by sampling location (indoor and outdoor) and sampling
355   period (first and second) (**Fig. 7A**). No differences in group clustering were identified in samples
356   from wet homes (**Fig. 7B**). ~~Results from~~ alpha-diversity analysis using the Chao1 richness
357   indicated no significant differences (p-value = 0.1288) in the number of unique taxa in the dry
358   homes (**Fig. 7C, Supplementary Table 2**) and a significant increase in the taxa present in the
359   indoor samples from the second sampling period in wet homes (p-value = 0.002) (**Fig. 7D**).
360   *Aspergillus* spp., *Cladosporium* spp., non-sporulating fungi and *Penicillium* spp. were the most
361   abundant fungi present in the indoor environment of both dry and wet homes during both
362   sampling periods (**Fig. 8**). Non-sporulating fungi and *Penicillium* spp. abundance inside dry
363   homes decreased whereas *Aspergillus* spp. and *Cladosporium* spp. abundance increased during
364   the second sampling period when compared to the first sampling period (**Fig. 8A and 8C**). The
365   same trend was observed for wet homes (**Fig. 8B and 8D**). *Aspergillus* spp. and non-sporulating
366   fungi were identified as biomarkers of dry homes, both with an FDR-adjusted p-value = 0.005
367   (**Fig. 9**). *Aspergillus* spp. and non-sporulating fungi were more abundant in the indoor and
368   outdoor environments of the second and first sampling periods, respectively (**Fig. 9A and 9B**).
369
370   **DISCUSSION**
371
372   Fungal quantification and fungal community characterization after hurricanes are
373   important to develop guidelines for post-flood recovery efforts. In this work, we analyzed
374   culturable fungal levels in homes located within a neighborhood in San Juan (PR) affected by
375   Hurricane María's extensive flooding. Culturable fungi levels (CFU/m^3) were higher inside
376   flooded homes 22 months following Hurricane María as compared to the levels measured inside

377   dry non-flooded homes (**Fig. 4B**). This result is consistent with previous studies conducted in the
378   aftermath of hurricanes which have typically found higher fungal growth and fungal components
379   (spores, endotoxins and glucans) inside homes and areas that sustained moderate to severe water-
380   damage and/or flooding (Chew et al., 2006; Riggs et al., 2008; Barbeau et al., 2010). ~~This rise in~~
381   ~~fungal concentrations could be explained by the fact that~~ water damage and flooding may
382   increase humidity and moisture levels inside the affected home, including wetting furniture and
383   household construction materials, ~~thus creating~~ favorable conditions for fungal growth ~~indoors~~.
384
385   Since humidity ~~is a contributing factor for~~ fungal proliferation, we measured relative
386   humidity levels in the homes during the different sampling periods (first and second). ~~In terms of~~
387   ~~humidity,~~ almost two years after Hurricane María we found higher relative humidity levels in
388   inside flooded homes; however, this trend was not evident during the first year after Hurricane
389   María (**Fig. 2A and 2B**). The ground and surrounding areas of flooded homes were inundated,
390   during and after Hurricane María by rain and phreatic water (underground water). Although not
391   measured through in this research, this could have also impacted relative humidity levels in the
392   flooded homes that are also located in shallow water table areas. During the first sampling period,
393   no differences in relative humidity levels were observed possibly due to closeness of the
394   sampling to the hurricane and flooding events.
395
396   We analyzed our culture-based data using different tools typically applied to molecular-
397   based data. These analyses and visualizations provided additional insight into the fungal
398   communities present in our samples as a function of different variables (degree of water damage,
399   indoor versus outdoor sampling location). We found significant differences in fungal composition
400   and structure when analyzing beta-diversity by self-reported degree of water exposure during the
401   first sampling period (p-value < 0.001), meaning that the distribution of the fungal species
402   recovered varied between the categories analyzed (**Fig. 4C**). However, there were no significant
403   differences in ~~species richness according to~~ alpha diversity (**Fig. 4D**). Meaning there were no
404   differences in the number of species within the categories studied. Results from abundance
405   taxonomic bar plots highlighted the shift from fungal communities present in the outdoor
406   environment (typically non-sporulating fungi) to enrichment of taxa previously associated with
407   damp environments and human disease (**Fig. 5A and 5B**). Non-sporulating fungi refer to fungi
408   that fail to produce spores on culture media, therefore identification through microscopy
409   techniques is not possible. Non-sporulating fungi are most likely members of the Basidiomycota
410   phylum, which compose approximately 60% of the fungal spores present in the outdoor air of PR
411   (Rivera-Mariani et al.; AAAAI, 2021). Thus, the indoor environment of dry non-flooded homes
412   appears to reflect the outdoor environment (**Fig. 5A**). This result was expected, since the study
413   homes, as with most homes in PR, are naturally ventilated allowing for fungal spores present in
414   the outdoor air to enter the homes through doors and windows. In contrast, water-damage homes
415   yielded an increase in abundance of *Aspergillus* taxa which eventually dominated the indoor
416   environment of flooded homes (**Fig. 5A and 5C**). Approximately 180 species of *Aspergillus*
417   exist, with *A. fumigatus, A. terreus, A. niger, A. flavus* being the most frequent causative agents
418   of human fungal infection, including aspergillosis, asthma and allergic rhinitis (Sugui et al., 2014;

419   Seyedmousavi et al., 2018). Moreover, *Aspergillus* taxa ~~have been previously~~ associated with
420   damp environments (Miller, Haisley & Reinhardt, 2000; Chew et al., 2006; Solomon et al., 2006;
421   Rao et al., 2007; Schwab et al., 2007; Riggs et al., 2008; Bolaños-Rosero et al., 2013; Flores et
422   al., 2013). The shift in fungal taxa from the first to the second sampling period in water-damage
423   and flooded homes (from *Aspergillus* species to non-sporulating fungi predominance) indicates
424   that 22 months after Hurricane María, there has been a recovery in the fungal communities to the
425   taxa normally present in the tropical environment of Puerto Rico (**Fig. 5B**). *Rhodotorula* was
426   found significantly enriched in the outdoor environment of flooded homes (**Fig. 5C**). This
427   unicellular red-orange pigmented yeast is part of the Basidiomycota division and has been
428   previously associated with water-damaged materials and moisture damage in buildings (Andersen
429   et al., 2011; Adams et al., 2020).
430
431   Finally, the most abundant fungi recovered during the first sampling period from both the
432   indoor and outdoor environments of dry and wet homes were *Aspergillus*, *Cladosporium*,
433   *Penicillium* and non-sporulating fungi. The first three fungi correspond with findings of other
434   studies, while non-sporulating fungi represent the characteristic fungi from the tropical outdoor
435   environment of PR (**Fig. 6A, 8 and 9**) (Miller, Haisley & Reinhardt, 2000; Chew et al., 2006;
436   Solomon et al., 2006; Rao et al., 2007; Schwab et al., 2007; Riggs et al., 2008; Bolaños-Rosero et
437   al., 2013; Flores et al., 2013).
438
439   Our study provides a unique and valuable data set. First, we evaluated fungal
440   concentration and composition during two sampling periods one and two years in the aftermath
441   of Hurricane María. Even one year after Hurricane María, the homes sampled had differences in
442   fungal communities according to the degree of water damage. Almost 2 years after the hurricane,
443   we found that fungal communities indoor began to recover and resemble the outdoor fungal
444   communities. Most of the studies that evaluated fungal growth and spore levels after a Hurricane
445   were carried out within 6 months after the natural disaster. Second, we collected our data through
446   culture-based methods which allows analysis of viable fungi present in the sampled environment.
447   We acknowledge the limitations of this approach for fungal identification, however useful
448   information was still gained regarding indoor fungal amplification and diversity. Third, we used
449   two different culture media for the recovery of fungal isolates, MEA and G25N allowing for the
450   characterization of a wide range of fungi from hydrophilic to xerophilic fungi, respectively. Our
451   results showed that G25N is more useful for the collection of fungal isolates that usually amplify
452   (indoor concentrations greater than the outdoors) under damped or flooded conditions. Since
453   G25N allows slow and discrete colony growth, all fungi can grow without a dominating genus
454   overgrowth occupying the culture plate surface as it happens in MEA. However, the use of both
455   culture media is recommended to recover as many fungal species as possible. We also considered
456   the levels of outdoor fungal spores reported by the San Juan AAAAI station, which were highest
457   during the first sampling period and contributed to the indoor fungal concentration, agreeing with
458   an outdoor-indoor air continuum. Also, the fungal isolates obtained from these samples agreed
459   environmental fungi from PR obtained through the San Juan AAAAI station. Lastly, we applied
460   tools originally develop for molecular-based data for the analysis of our culture data gaining

461   additional insights into fungal communities' composition, richness, abundance and biomarkers
462   significantly enriched in our samples. By applying tools originally develop for data generated by
463   molecular biology approaches for the analysis of culture-based data, we ~~were able to further~~
464   ~~understand our results and identify~~ fungal features characteristic between dry and flooded homes.
465   Particularly, we ~~were able to identify~~ a significant shift from *Aspergillus* taxa to non-sporulating
466   fungi inside flooded homes between the first and second sampling period.
467
468   In summary, this data highlights dynamic changes in indoor fungal populations,
469   characterized by different types of fungi between dry and flooded homes. More specifically, dry
470   homes were dominated by fungi present in the outdoor environment highlighting the naturally
471   ventilated types of homes common in PR. On the other hand, flooded homes presented
472   amplification of *Aspergillus* species. Additionally, our study results indicate that in naturally
473   ventilated homes, the outdoor fungal levels have a significant effect on indoor fungal levels. This
474   brings attention to the outdoor environment. Thus, after-flood cleanup and remediation at a
475   community scale may help address this issue. Since tropical homes are characterized by an
476   outdoor-indoor air continuum (**Fig. 3A and 3B**), it is extremely important to maintain clean
477   outdoor surroundings after the disaster. In this study, we observed high quantities of water-
478   damage materials, wood and trees debris outside many of the homes sampled. These organic
479   materials could have also contributed to outdoor filamentous fungi proliferation that eventually
480   entered the homes via open windows and/or doors. ~~In order~~ to apply these suggestions in the
481   context of low-income communities, assistance from local government is warranted. Disaster
482   relief for flooding victims, including debris removal from the surroundings, covered-expenses
483   home repairs, materials and/or furniture replacement, ~~provide~~ cleaning materials and basic
484   education on flood preparedness and recovery. Furthermore, to prevent indoor fungal
485   proliferation, it's recommended to maintain a clean indoor environment after flooding events.
486   Building problems should be repaired and water-damaged materials must be cleaned, dried and/or
487   removed.[15] ~~Studies have shown that~~ remediation of mold-damaged materials, through cleaning or
488   replacement, decrease or eliminate mold growth (Solomon et al., 2006; Riggs et al., 2008;
489   Jayaprakash et al., 2017). It is important to identify indoor moisture sources such as roof, walls
490   and plumbing leaks, condensation, floods and high relative humidity. An additional
491   recommendation is to keep indoor relative humidity levels below 60%, ideally between 30% and
492   50%, ~~in order~~ to inhibit mold growth(EPA). Moreover, dehumidifiers and High-Efficiency
493   Particulate Air (HEPA) filters ~~are tools that~~ help control fungal growth and spore levels,
494   respectively (Mazur & Kim, 2006). ~~These findings are helpful for developing guidelines for post-~~
495   ~~flood recovery efforts as well as strategies to mitigate respiratory diseases in tropical~~
496   ~~environment.~~
497

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640

Table 1(on next page)

Number of homes categorized by self-reported degree of water-damage.

The total number of samples for each sampling period, total number of fungal colonies observed, and total number of unique fungal species present are summarized for each category.

1

Self-Reported Degree of Water Damage	Sampling Period	Number of Homes	Number of Samples	Number of Fungal Colonies	Number of Fungal Species
Dry	First	13	124	4,229	40
	Second	11	108	1,925	33
Water- Damage	First	24	240	10,624	42
	Second	15	150	7,136	35
Flooded	First	13	121	3,606	38
	Second	9	90	4,893	31

2
3

Figure 1

Map of the homes sampled in San Juan, Puerto Rico (n = 50).

Blue, yellow and red dots corresponds to Dry, Water-Damage and Flooded homes, respectively.
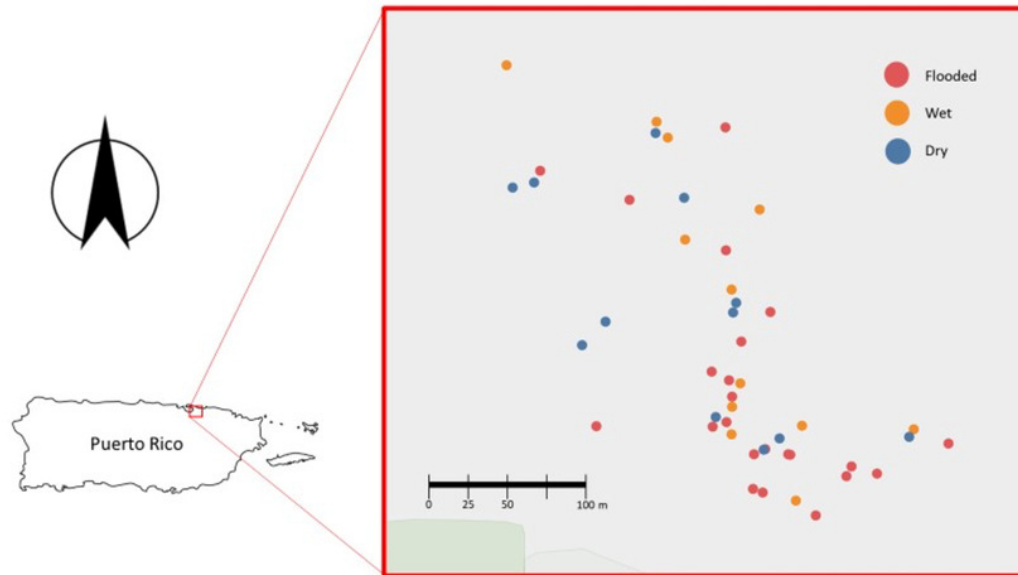
Figure 2

Indoor relative humidity levels (%) in homes as a function of self-reported degree of water damage (Dry, Water-Damage, Flooded).

A) First Sampling (n = 50 homes). B) Second Sampling (n = 35).
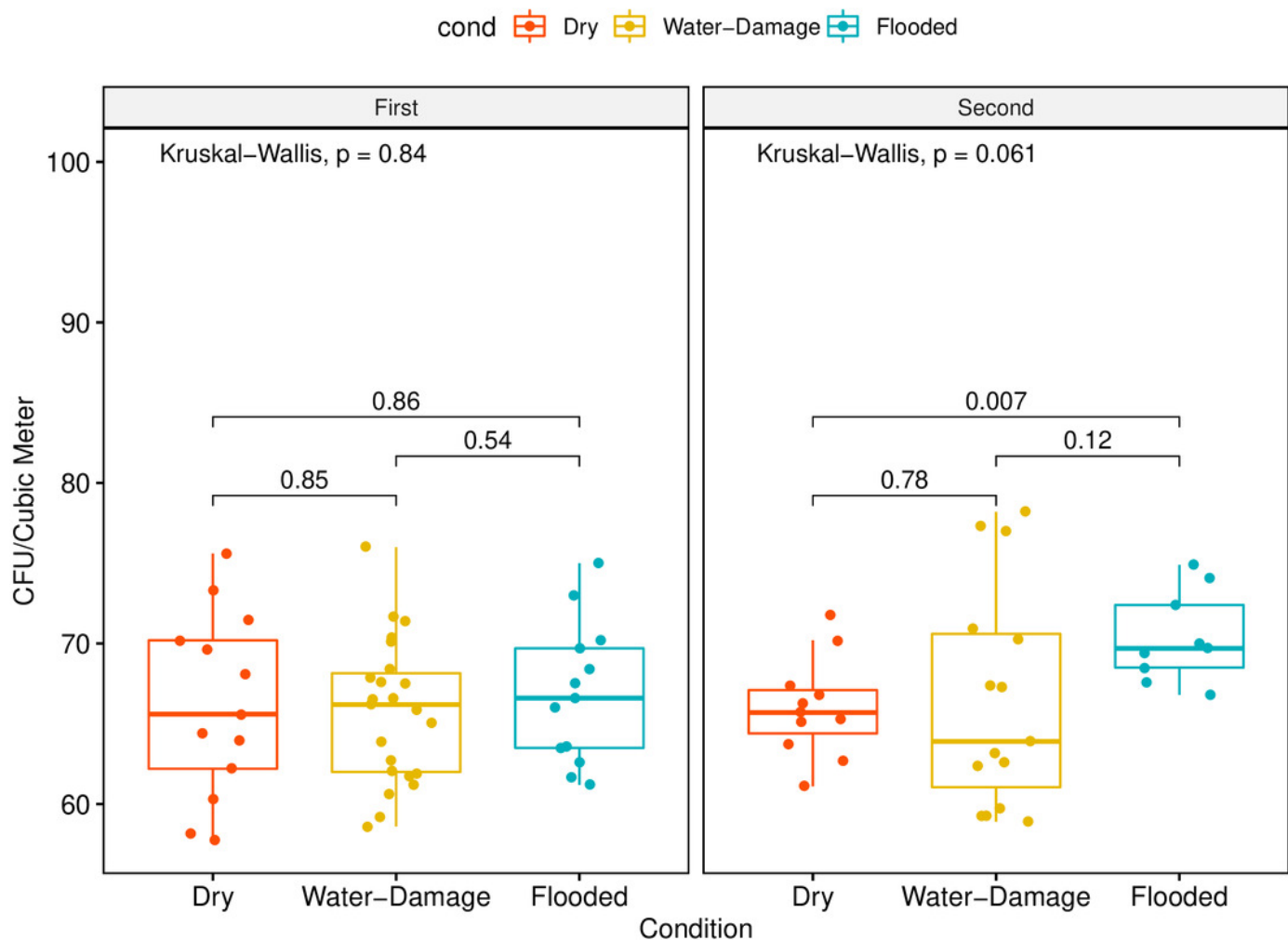
Figure 3

Linear Regression Indoor Fungal Spores and Outdoor Fungal Spores per cubic meter

(m^3) of air in G25N Culture Media.

A) First sampling (2018) and B) Second sampling (2019).

A)

B)
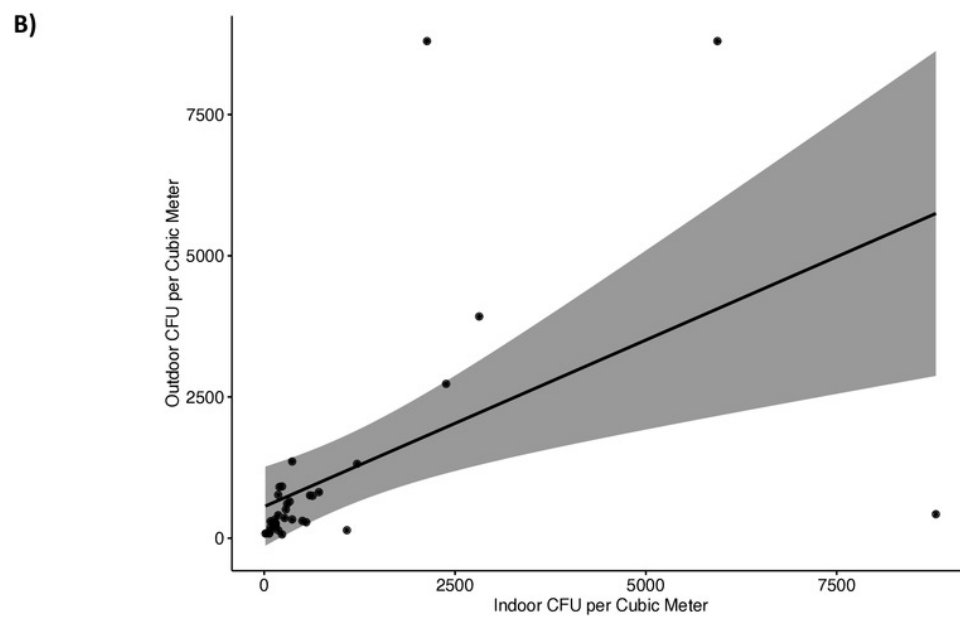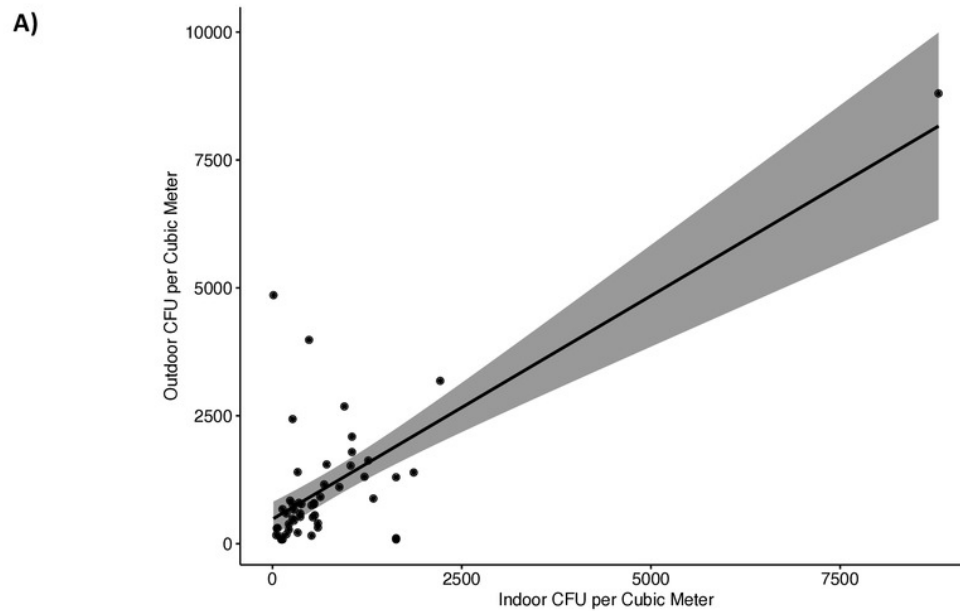
Figure 4

Fungal concentration and profile by self-reported degree of water damage (Dry, Water-Damage, Flooded).

A) G25N culture media. B) MEA culture media. C) Beta-diversity analysis for first sampling (2018). D) Alpha-diversity analysis for first sampling (2018). A, B) Red, Yellow and Blue boxplots correspond to Dry, Water-Damage and Flooded homes, respectively; first sampling n = 50 homes and second sampling n = 35 homes; Kruskal-Wallis test, p-value <0.05 was considered significant. C) Principal coordinate analysis (PCoA) performed with Jensen-Shannon Divergence. D) Chao1 richness estimate.
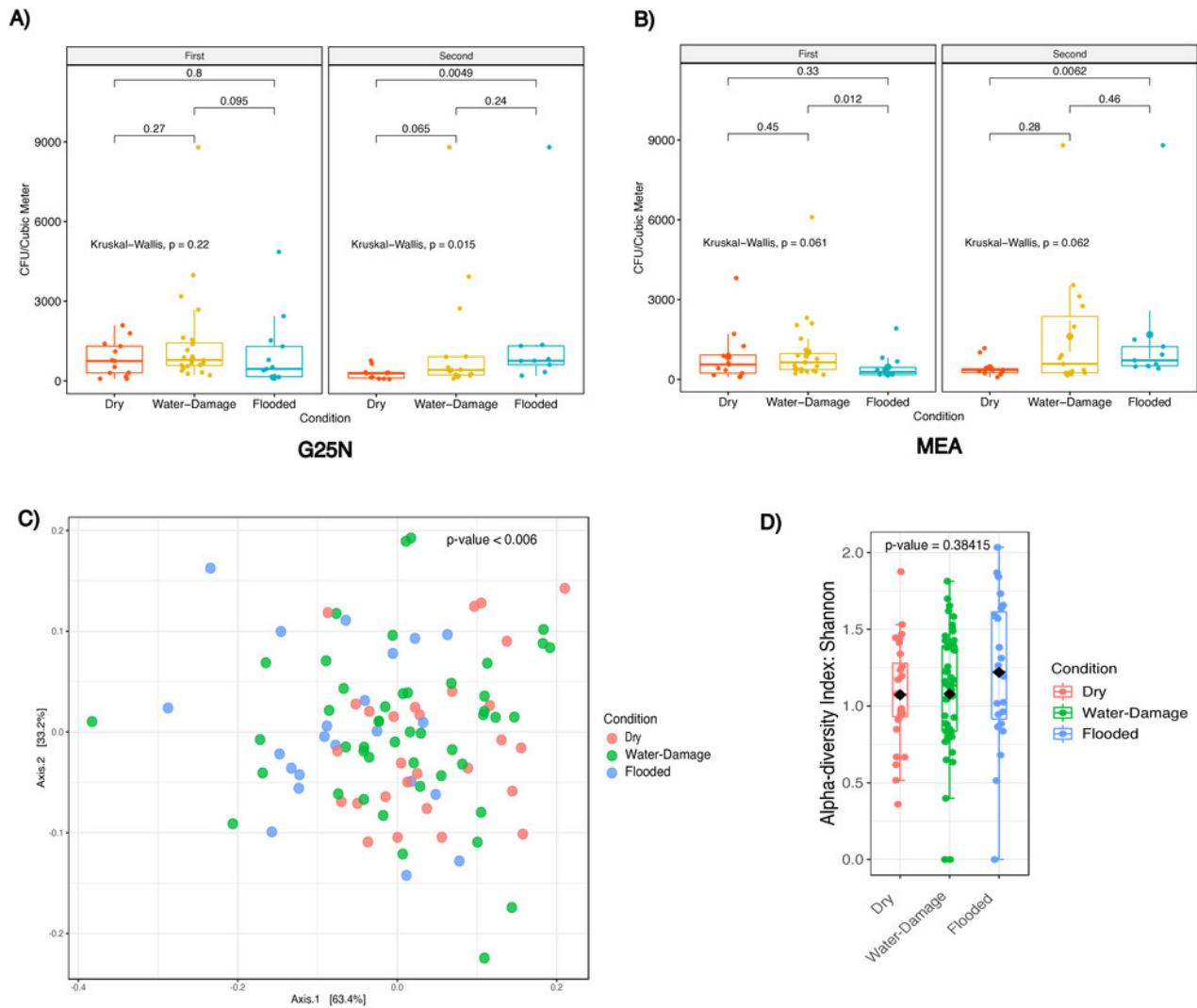
Figure 5

Abundance taxa bar plots and histogram of Linear Discriminant Analysis (LDA) Effect Size (LEfSe) by sampling location (Indoor, Outdoor), self-reported degree of water-damage (Dry, Water-Damage, Flooded) and sampling period (First, 2018 and Second, 2019).

A) Abundance taxa bar plots during first sampling (2018). B) Abundance taxa bar plots during second sampling (2019). C) LDA during first sampling (2018), FDR-adjusted p-value = 0.05. Log LDA score computed for differentially abundant taxa (genus level) with cut-off LDA score >2.0.

A)
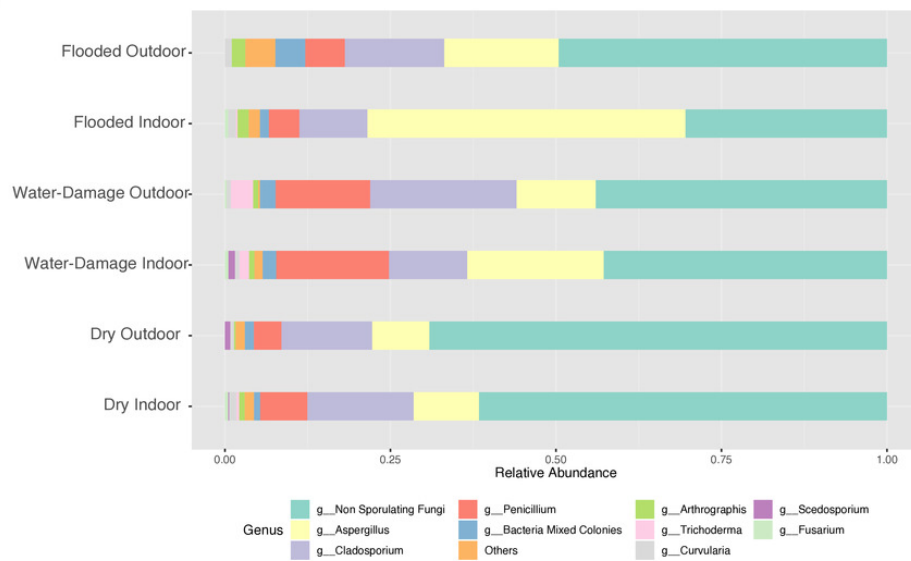
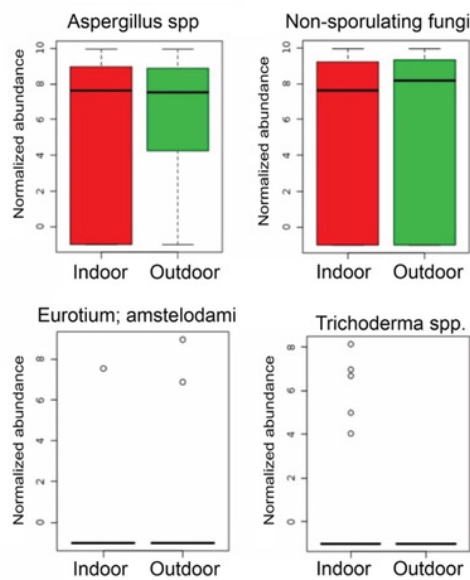**B)**
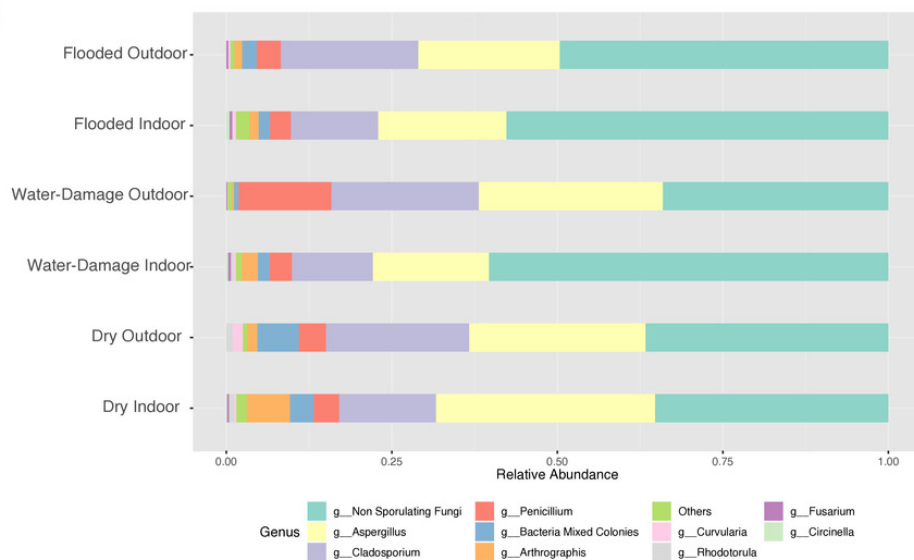
C)
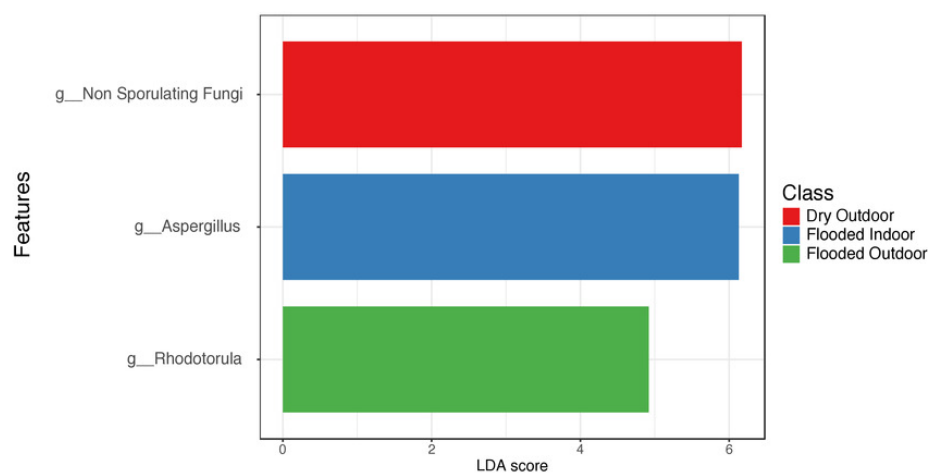
Figure 6

Individual fungi normalized abundance boxplots by sampling location (Indoor, Outdoor).

A) Individual fungi abundance boxplots during first sampling (2018). B) Individual fungi abundance boxplots during second sampling (2019). A, B) Red and Green boxplots corresponds to indoor and outdoor samples, respectively.
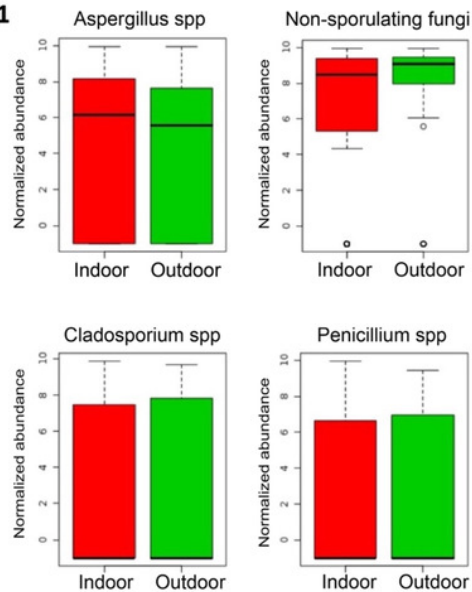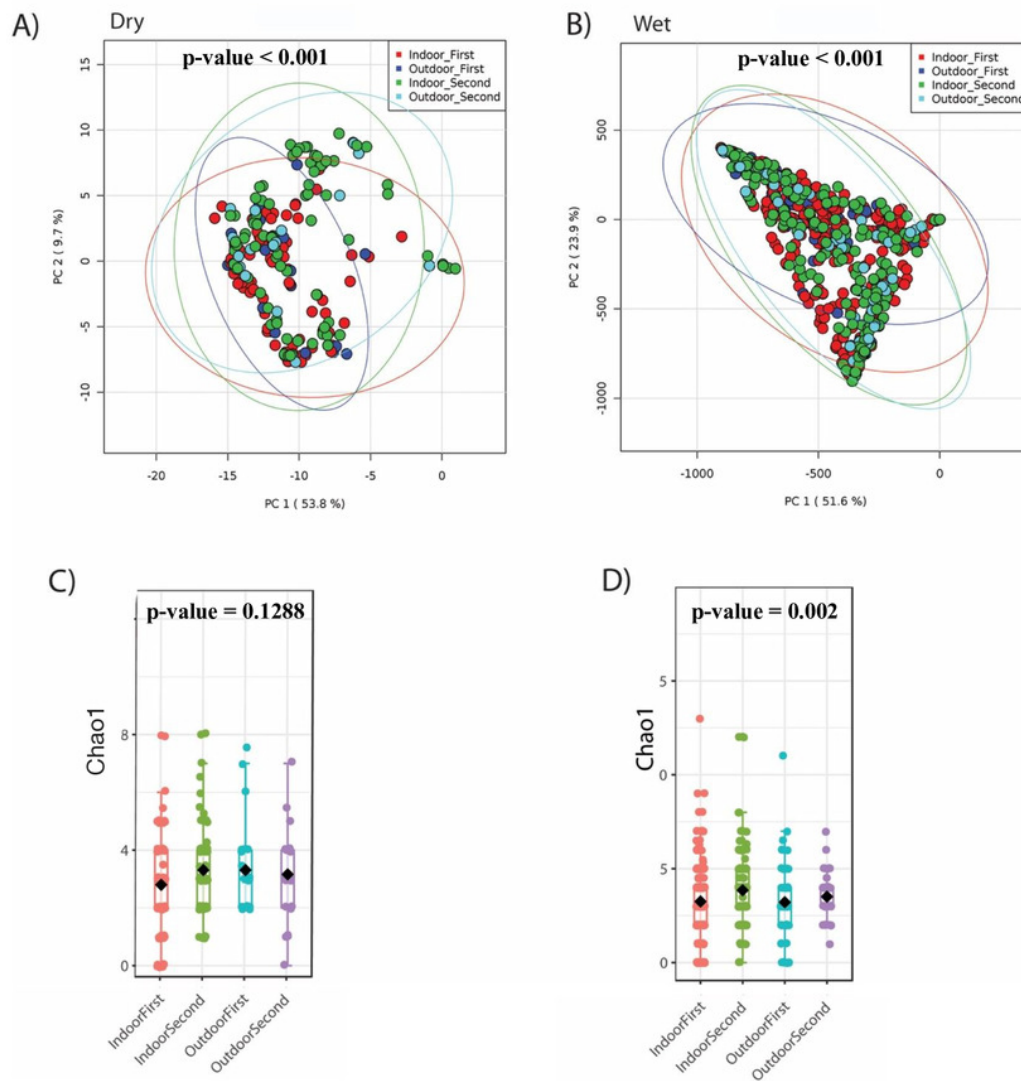
Figure 7

Fungal profile of dry and wet (Water-Damage and Flooded Homes Combined) homes during both sampling periods (First and Second).

A) Beta-diversity analysis for dry homes. B) Beta-diversity analysis for wet homes. A, B) Red, Dark Blue, Green and Light Blue dots corresponds to indoor samples from first sampling, outdoor samples for first sampling, indoor samples from second sampling and outdoor samples from second sampling; respectively. Principal coordinate analysis (PCoA) performed with Jensen-Shannon Divergence. C) Alpha-diversity analysis for dry homes. D) Alpha-diversity analysis for wet homes. C, D) Red, Green, Blue and Purple boxplots corresponds to indoor samples from first sampling, indoor samples from second sampling, outdoor samples for first sampling, and outdoor samples from second sampling; respectively. Chao1 richness estimate.
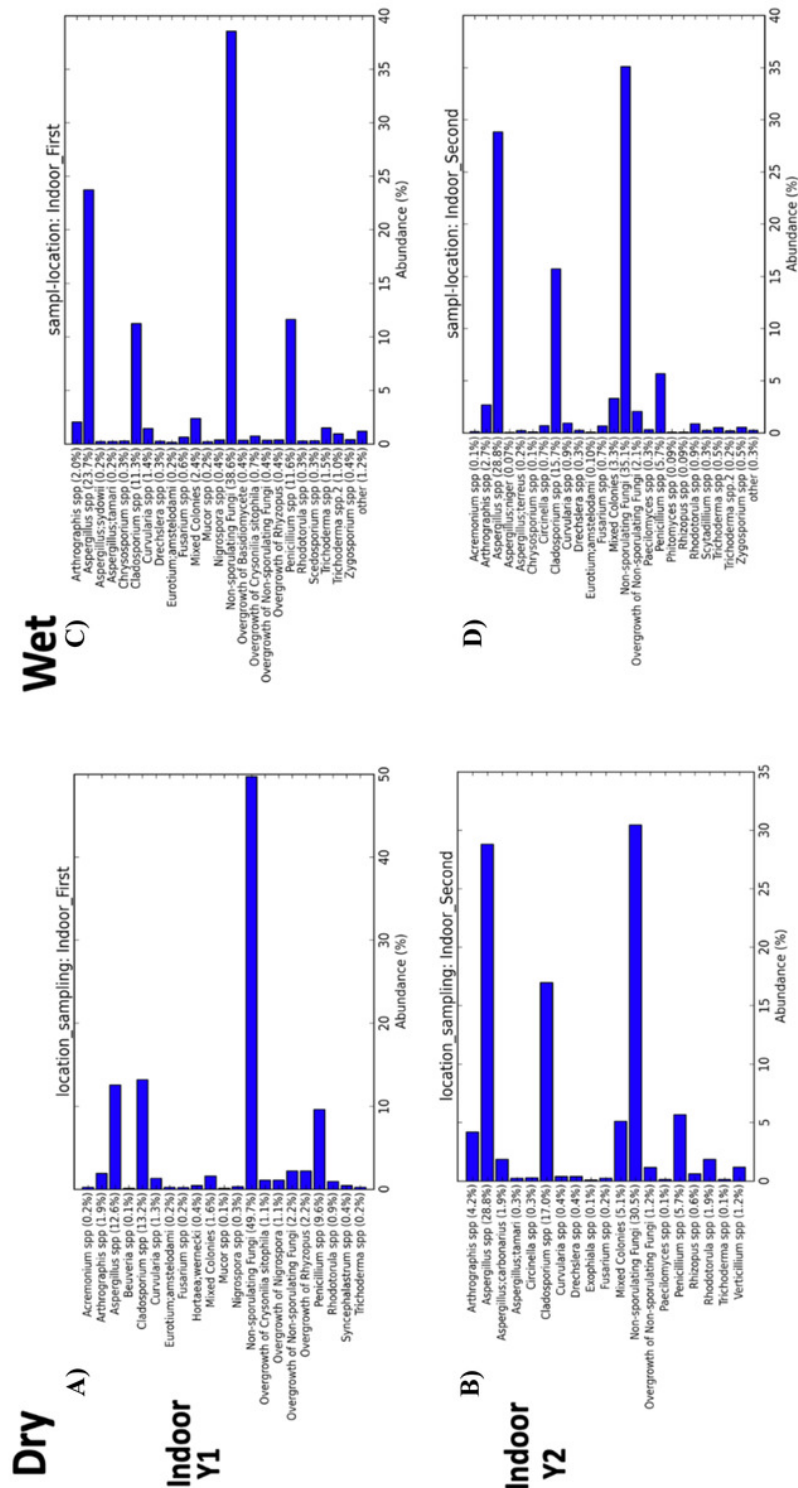
Figure 8

Indoor fungal isolates abundance percent by sampling period (First, Second).

A) Samples from indoor environment of dry homes during the first sampling period. B) Samples from indoor environment of wet homes during the first sampling period. C) Samples from indoor environment of dry homes during the second sampling period. D) Samples from indoor environment of wet homes during the second sampling period.

Figure 9

Fungal dry biomarkers by sampling location (Indoor, Outdoor) and sampling period (First, Second).

A) *Aspergillus* spp. abundance levels. B) Non-sporulating fungi abundance levels. A, B) Red, Dark Blue, Green and Light Blue boxplots corresponds to indoor samples from first sampling, indoor samples from second sampling, outdoor samples for first sampling, and outdoor samples from second sampling; respectively.