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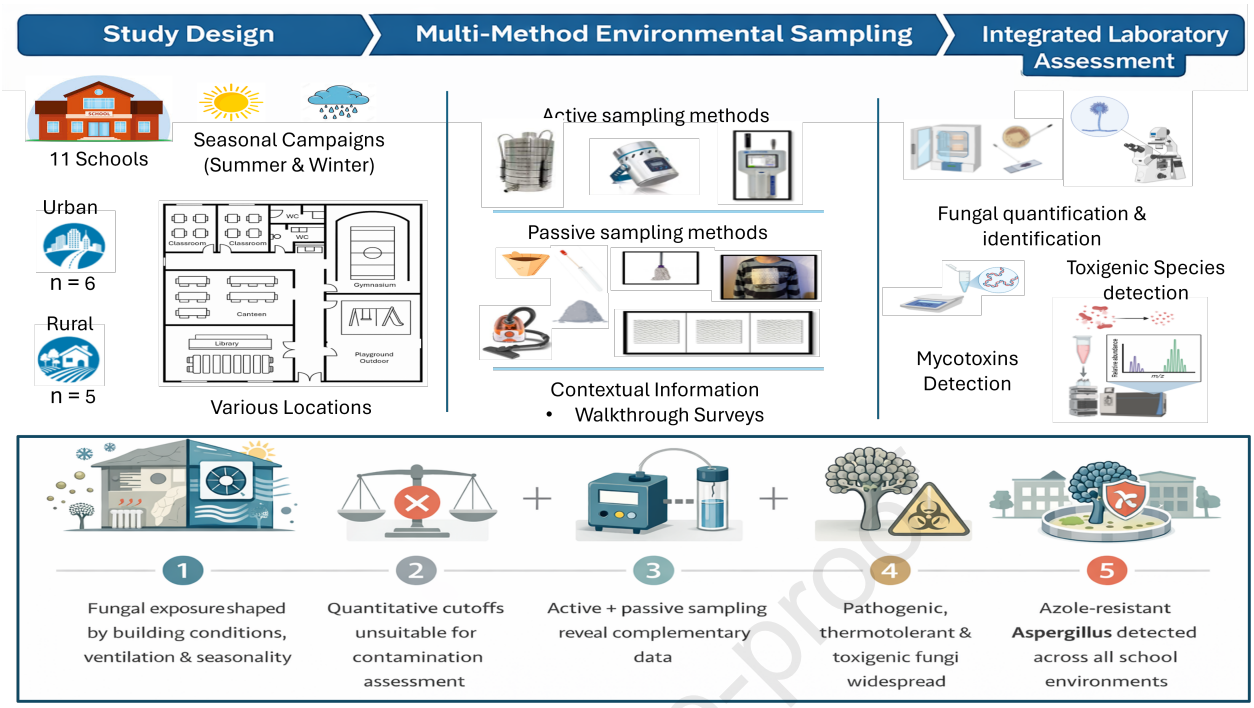

Beyond Quantitative Indicators: Comprehensive Characterization of Indoor Fungal Contamination in Portuguese Elementary Schools

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Abstract

Children are particularly vulnerable to environmental pollutants, making indoor air quality in schools a key health determinant. This study addresses regulatory gaps and the lack of standardized methods for assessing fungal contamination in Portuguese elementary schools, particularly regarding pathogenicity, toxigenicity, and antifungal resistance. Fungal contamination was assessed in 11 Portuguese elementary schools using surveys on building context and operation, active and passive air sampling, molecular detection of *Aspergillus* sections by qPCR, mycotoxin and azole resistance screening, and PM measurements. Results: Schools showed heterogeneous ventilation, with some relying on natural ventilation and others using mechanical systems. Airborne fungal contamination was dominated by respirable fractions, reaching 3.3×10^{2} CFU m^{-3}. Culture-based analyses revealed distinct fungal profiles, with *Cladosporium* sp. and *Penicillium* sp. frequently prevailing, while *Aspergillus* detection increased at higher incubation temperatures. Seasonal variability was observed, with contamination patterns differing between seasons. Rural and urban schools showed contrasting fungal distributions, with higher concentrations in rural schools, particularly in EDCs on DG18 at 27 °C (1.67×10^{3} CFU/m^{2}). Azole resistance screening detected resistant *Aspergillus*

sections across environments. In gymnasiums, the *Fumigati* section represented 100% of sections detected on ITZ in filters and EDCs. In classrooms, *Circumdati* and *Fumigati* accounted for 100% of sections detected on ITZ in EDCs, while the *Candidi* section represented 100% in filters. The *Fumigati* section also grew on VOZ and POZ in EDCs. Mycotoxins, including ochratoxin A, mycophenolic acid and sterigmatocystin, were detected in 1.93% of dust samples. Particle concentrations occasionally exceeded reference thresholds in warm (47.1% PM2.5; 58.8% PM10) and cold seasons (47.4% PM2.5; 23.1% PM10). Regulatory thresholds did not prevent the detection of relevant fungal hazards, including *Aspergillus* species and azole-tolerant growth. These findings show that quantitative indicators alone may not capture exposure complexity, highlighting the need for complementary data, and the limitations in the current legal framework.

1 - Introduction

Children constitute one of the most vulnerable populations to environmental pollutants due to their developing respiratory and immune systems, higher inhalation rates relative to body mass, and the significant amount of time spent in indoor spaces, such as schools (Cervantes et al., 2025; Norbäck et al., 2014). Consequently, indoor air quality (IAQ) in primary schools is a key determinant of children's health, well-being, and cognitive performance (Daisey et al., 2003; Mendell & Heath, 2005; Wargocki & Wyon, 2013). Educational buildings often host large numbers of individuals in confined spaces for prolonged periods, which can exacerbate the accumulation of pollutants and bioaerosols (Fouladi-Fard et al., 2023). Among these, airborne fungi represent a biologically complex and highly relevant component of IAQ due to their ability to release spores, fragments, and metabolites with allergenic, infectious, or toxigenic potential (Heseltine et al., 2009). Exposure to these fungal contaminants has been consistently associated with a range of adverse health outcomes, including the onset and exacerbation of allergic rhinitis and asthma, respiratory infections, and non-specific symptoms such as fatigue and mucosal irritation (Fisk et al., 2010; Groot et al., 2023; Mendell et al., 2011; Quansah et al., 2012).

The proliferation of fungi in the indoor school environment is a multifactorial process, influenced by parameters such as relative humidity, temperature, ventilation efficacy, building design, and cleaning and maintenance practices (Nevalainen & Seuri, 2005). This dynamic is further complicated by climate change, which acts as a modifying factor

by altering environmental conditions that govern fungal growth, sporulation, and secondary-metabolite production (Williams et al., 2024). Rising global temperatures, shifts in precipitation patterns, and an increased frequency of extreme weather events are projected to create conditions more conducive to indoor dampness and fungal colonisation, particularly in regions with Mediterranean climates such as Portugal (Environment, 2019; IPCC, n.d.).

Beyond traditional allergenic concerns, two emerging issues underscore the need for a detailed assessment of indoor fungi. First, the development of environmental antifungal resistance, especially among *Aspergillus fumigatus* strains due to agricultural and environmental azole use, highlights a important One Health issue where non-clinical settings may act as reservoirs for resistant pathogens (Bader et al., 2015; Chowdhary et al., 2017; Schoustra et al., 2019; Snelders et al., 2012; Verweij et al., 2016). Second, the potential for mycotoxin production by common indoor fungal genera represents a significant, yet still under-investigated, toxicological risk through inhalation exposure, a topic that remains largely unexplored in primary school settings (Habschied et al., 2021; Navale et al., 2021; Nielsen et al., 2004; S. Viegas et al., 2018).

Despite the growing evidence on these complex hazards, the regulatory framework for managing microbiological IAQ in Portugal lacks specific guidelines for primary schools. The primary legal instrument, Ordinance No. 138-G/2021, establishes reference values for microbiological parameters exclusively for commercial and service buildings, leaving the school environment without a specific and legally binding standard (DRP, n.d.). Crucially, the ordinance's approach is fundamentally limited as it relies primarily on a simple indoor/outdoor (I/O) total fungal count ratio (with a guideline value of ≤ 1) as the main compliance criterion (DRP, n.d.). While exceedance of this cut-off does trigger the requirement for further investigation, including the identification of fungal genera or species, the regulatory framework remains predominantly quantitative in nature (DRP, n.d.). As such, it fails to systematically integrate the qualitative and intrinsic hazards of fungal contamination into the assessment of compliance. In particular, the presence of species with known pathogenic potential (e.g., *Aspergillus fumigatus*, a WHO critical priority pathogen) or toxigenic capacity (e.g., *Stachybotrys chartarum*, certain *Aspergillus* sections and *Penicillium*) is not sufficient to determine non-compliance when total fungal counts remain below the prescribed threshold (Pitt, 2000; WHO, n.d.-b). By overlooking species-level characterization, antifungal resistance profiles, and mycotoxin

exposure, the current guideline does not adequately assess the microbiological and toxicological risks for vulnerable schoolchildren, representing a significant public health oversight.

While awareness is increasing, comprehensive data on indoor fungal contamination in primary schools remain scarce, particularly in Portugal and other Southern Europe countries (Chawla et al., 2023). Existing studies often focus selectively on either airborne culturable counts or dampness indicators, neglecting integrated analyses that concurrently evaluate environmental drivers, fungal biodiversity, resistance markers, and cytotoxic or toxigenic potential (Cervantes et al., 2025; Madureira et al., 2016). Therefore, this study aims to bridge these gaps. This environmental health study combines field sampling with laboratory-based analytical approaches, conducting a integrated assessmentof the fungal contamination in elementary schools across the Lisbon Metropolitan Area. To account for potential seasonal variability, the sampling campaign was conducted across two distinct seasons, acknowledging the well-documented influence of factors such as temperature, humidity, and ventilation patterns. Additionally, schools from both urban and rural settings were included to capture possible differences arising from contrasting environmental conditions, land-use characteristics, and outdoor fungal sources. We concurrently analyse key environmental parameters such as, fungal diversity (with emphasis on relevant species), the presence of azole-resistant *Aspergillus fumigatus*, and a panel of 38 mycotoxins in the schools´ indoor environments. This study therefore provides a integrated framework for assessing fungal contamination and associated risks in primary school indoor environments

2 - Materials and methods

2.1 Study area and schools' characterization

The study was conducted in the scope of InChildHealth project CE-ESTeSL-Nº. 116-2022 (https://inchildhealth.eu/) in 11 public elementary schools located in the Lisbon Metropolitan Area (Portugal), Schools were selected by convenience sampling based on geographic criteria within the metropolitan area, and recruited through school clusters. Inclusion was restricted to public first-cycle elementary schools. A walkthrough survey (WTS) was carried out to collect contextual information prior to sampling (Vorkamp et al., 2024). The survey followed a structured checklist adapted from established guidelines for assessing dampness, mould, and indoor air quality (Heseltine et al., 2009; C. Viegas, Almeida, et al., 2020). Data collected included building descriptors (construction year,

number of floors, presence of basements), use and occupancy (number of classrooms, pupils per class, number of staff members), ventilation type, cleaning practices, and time spent indoors (Supplementary material - Table S1). Information on visible dampness, maintenance routines, and neighbourhood context (e.g., proximity to traffic or green spaces) was also recorded. All WTS variables were later encoded as categorical covariates to contextualise environmental and microbiological data collected during the study.

2.2 Study Design

Two cross-sectional seasonal campaigns, summer (warm) and winter (cold), were conducted in each of the 11 schools, complemented by a longitudinal dust sampling programme with monthly electrostatic cloth (EDC) collections over one academic year. Sampling covered classrooms, canteens, libraries, gymnasiums, bathrooms, and an outdoor reference area. Sampling procedures were performed during regular school hours (6–8 h/day) while classes were being held (Figure 1). Both active and passive sampling methods were used. Active air sampling employed two impaction-based devices, a MAS-100 air sampler (Millipore, Billerica, United States) and an Andersen six-stage cascade impactor, operated simultaneously to collect airborne particles onto agar plates for quantitative analysis. Passive sampling methods included electrostatic dust cloths (EDC; Swiffer, Portugal), surface swabs (Frilabo, Portugal), electrostatic cloths attached to children's T-shirts (EDCT; Swiffer, Portugal), vacuumed settled dust (SD) using a HOOVER Brave BV71_BV10 A2 (United States), filters from vacuumed dust (Continente, Portugal), and mops used by school cleaning personnel (Figure 1). All samples were transported and stored refrigerated (0–4 °C) in sterile bags prior to analysis, following validated protocols among the consortium (Almeida & Viegas, 2021; Cervantes et al., 2022; Frankel et al., 2012; Noss et al., 2008; Whitby, 2022). Each EDC sample remained in place for 30 consecutive days, and further sampling details are provided in Supplementary Material - Table S2.

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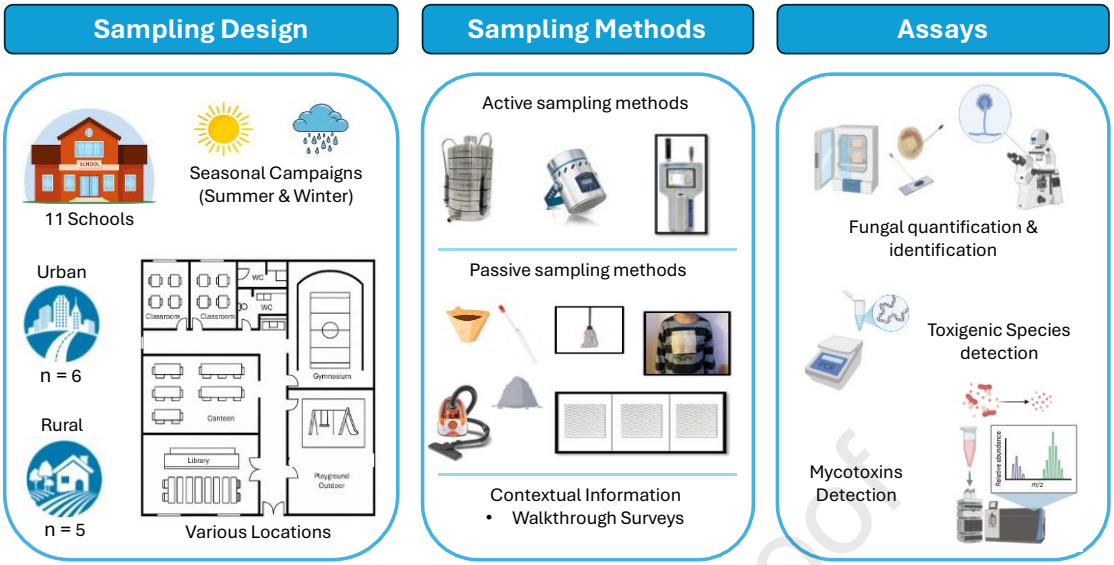


156 Figure 1 - Study Design

157

158 **2.3 Sample preparation**

159 Surface swabs were extracted with 1 mL of 0.1% Tween™ 80 saline solution (0.9%
160 NaCl) (Frilabo, Maia, Portugal) for 30 min at 250 rpm on an orbital shaker. Settled dust
161 filters and mop samples (2 cm^2) were extracted with 5 mL of the same solution under
162 identical conditions. Each EDC (weighted) was extracted using 10 mL of 0.1% Tween™
163 80 saline solution, while EDCTs (weighted) were extracted with 25 mL of the solution,
164 both for 30 min at 250 rpm at room temperature. All extracts were subsequently seeded
165 onto the selected media for microbial assessment (C. Viegas, Gomes, et al., 2022).

166

167 **2.4 Determination of the concentration and diversity of cultivable fungi**

168 Malt extract agar (MEA) supplemented with 0.05% chloramphenicol (Frilabo, Maia,
169 Portugal) and dichloran-glycerol agar (DG18) were used for general fungal growth,
170 150 µL of the extracts from all passive samples were seeded onto selected agar media.
171 All plates were incubated at 27 °C for 5–7 days. An additional DG18 plate was inoculated
172 for each sample and incubated at 37 °C for 5–7 days to support the isolation of potentially
173 pathogenic fungi. For azole resistance screening, 150 µL of the extracts from all passive
174 samples, excluding swabs, were inoculated onto Sabouraud dextrose agar (SDA) (Frilabo,
175 Maia, Portugal) supplemented with one of the following antifungal agents: 4 mg L^{-1}
176 itraconazole (ITZ), 2 mg L^{-1} voriconazole (VCZ), or 0.5 mg L^{-1} posaconazole (PSZ). A

non-supplemented SDA plate was used as a control. This protocol was adapted from the guidelines of the European Committee on Antimicrobial Susceptibility Testing, 2020. (EUCAST, 2020) (EUCAST, n.d.). As reference strains, an azole-susceptible *A. fumigatus* (ATCC 204305) was used as the negative control, and a pan-azole-resistant *A. fumigatus* isolate, provided by the National Health Institute Dr. Ricardo Jorge (INSA, IP), was used as the positive control. Plates were incubated at 27 °C for 3 to 4 days. Fungal quantification was expressed as colony-forming units (CFU) and CFU concentrations ($CFU \cdot m^{-3}$, $CFU \cdot m^{-2}$, $CFU \cdot m^{-2} \cdot day^{-1}$, or $CFU \cdot g^{-1}$), depending on the sample type. Fungal species identification was based on the observation of macroscopic and microscopic morphological features, performed by an expert mycologist (C. Viegas, Gomes, et al., 2022).

2.5 Detection of *Aspergillus* sections

For molecular detection of selected *Aspergillus* sections, fungal DNA was extracted from 8.8 mL of each passive sample extract using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, USA). Real-time PCR (qPCR) was performed on a CFX-Connect PCR System (Bio-Rad) following a standardized protocol: each reaction contained 1× iQ Supermix (Bio-Rad, Portugal), 0.5 μM of each primer, and 0.375 μM of TaqMan probe, in a final volume of 20 μL. The amplification consisted of 40 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s. Negative (non-template) and positive controls were included in each run (C. Viegas et al., 2017). Positive controls consisted of DNA from reference Aspergillus strains provided by the Reference Unit for Parasitic and Fungal Infections, Department of Infectious Diseases, National Health Institute Dr. Ricardo Jorge, IP. These strains were previously identified by sequencing the ITS, β-tubulin, and calmodulin regions (Supplementary material - Table S3).

2.6 Fungal legal compliance

Species-based compliance was determined according to (DRP, n.d.). Each identified species was classified as Common (limit 500 CFU/m³), Uncommon (limit 50 CFU/m³, except Acremonium spp. < 50), Toxinogenic (limit < 12 CFU/m³), or Pathogenic (limit 0 CFU/m³). An environment was considered non-compliant if: (a) any species exceeded its limit, (b) the total concentration of Common/Uncommon species exceeded 500 CFU/m³, (c) any Pathogenic species was detected, or (d) any Toxinogenic species reached or exceeded 12 CFU/m³. This classification was applied independently of the I/O ratio.

2.7 Detection of mycotoxins

A comprehensive screening was conducted on a total of 206 samples to assess the presence of mycotoxins. The sample set comprised, settled dust filter samples obtained via vacuuming (N=88), electrostatic dust cloths (N=62, electrostatic dust cloths in T-shirts (N=38), samples from Mops (N=18). Mycotoxin detection was carried out through high-performance liquid chromatography (HPLC) using the Nexera system (Shimadzu, Tokyo, Japan), coupled with a triple quadrupole-linear ion trap mass spectrometer (5500 Qtrap; Sciex, Foster City, CA, United States). The selection of mycotoxins analyse was based on their documented toxicological relevance and reported occurrence in indoor environments associated with fungal contamination (IARC, n.d.). The specific mycotoxins analyzed, along with their corresponding limits of detection (LOD), are detailed in Supplementary material - Table S4.

2.8 Particulate matter, temperature, and relative humidity

To contextualise indoor fungal dynamics and exposure patterns, particulate matter (PM), temperature, and relative humidity were measured using the Lighthouse Handheld Particle Counter HH3016-IAQ (Lighthouse Worldwide Solutions). Particle mass concentration (μg/m³) was estimated using particle counter operating in 'Mass Concentration Mode,' which calculates approximate mass density from particle counts according to the manufacturer's algorithm (LWS, 2011). (Heseltine et al., 2009). Temperature and relative humidity are key determinants of fungal growth, survival, and mycotoxin production, while particulate matter contributes to the transport, dispersion, and resuspension of fungal spores and fragments within indoor environments (Heseltine et al., 2009). These parameters therefore provide essential contextual information for interpreting fungal contamination and potential inhalation exposure. Measurements were performed with a flow rate of 2.83 L/min. Measurements lasted 7 minutes per location, including 14 cycles of 30 seconds, following a 2-minute stabilization period. Monitoring was performed at the level of the respiratory tract (approximately 1.2 m above the floor), whenever possible, during active class periods. Particle counts were recorded for six size ranges: 0.3, 0.5, 1.0, 2.5, 5.0, and 10.0 μm, in compliance with ISO 21501-4 standards (DRP, n.d.; ISO, n.d.).

2.9 Statistical analysis

The data were analysed using SPSS statistical software, version 29.0 for windows. The results were considered significant at the 5% significance level. To test the normality of the data, the Shapiro-Wilk test was used. To study the relationship between fungal contamination (in different culture media) and azole resistance (in different media), Spearman's correlation coefficient was used, since the assumption of normality was not verified. To compare fungal contamination and azole resistance between seasons and between the school context (rural/urban), the Mann-Whitney test was used, since the assumption of normality was not met. To compare fungal contamination and azole resistance between workplaces, the Kruskal-Wallis test was used, since the assumption of normality was not verified. To assess species diversity, Simpson and Shannon indices, given by $\text{Shannon Index (H)} = -\sum_{i=1}^{s} p_i \ln(p_i)$ and $\text{Simpson Index (D)} = \frac{1}{\sum_{i=1}^{s} p_i^2}$, were used, where pi is the proportion (ni/n) of individuals of one particular species found (ni) divided by the total number of individuals found (n).

3 – Results

3.1 Study area and school's contextual information

Most schools relied primarily on natural ventilation through open windows and doors (63,6%), while a few were equipped with mechanical or HVAC systems (36,4%). However, in most of these cases, the systems were not functioning or lacked regular maintenance, and no air purification or filtration devices were reported in any of the assessed schools. Daily floor and surface cleaning was performed after school hours in ten schools by in-house staff (91%) and in one by an external contractor. Cleaning was typically carried out using dry and wet mopping, with occasional use of disinfectants, though details on dilution or frequency were unavailable. Classroom microenvironmental conditions revealed additional factors potentially influencing indoor contamination. The presence of animals and plants was reported in 18.2% of classrooms, representing possible supplementary sources of biological particles, however, no consistent patterns or associations could be established between these factors and the observed contamination profiles. Occupancy density was relatively consistent, with an average of 28 children and one teacher per classroom. The assessed schools varied considerably in size, ranging from single-classroom facilities to institutions comprising up to 18 classrooms.

3.1 Viable fungal contamination

Active and passive data were analysed by season and setting; active samples exhibited little variation across settings, with differences evident only by season. The MAS-100 contamination results show that in the cold season, the bathroom (MEA 6.5x10^2 CFU.m-3; DG18 7.50x10^2 CFU.m-3), canteen (DG18 9.85x10^2 CFU.m-3), classroom (MEA 5.20x10^2 CFU.m-3; DG18 9.70x10^2 CFU.m-3), and gymnasium (MEA 5.50x10^2 CFU.m-3; DG18 6.3x10^2 CFU.m-3) had higher load than outdoor samples (MEA 4.8x10^2 CFU.m-3; DG18 9.0x10^2 CFU.m-3). For the warm season, only the canteen (MEA 9.47x10^2 CFU.m-3), classroom (MEA 7.65x10^2 CFU.m-3), and gymnasium (MEA 1.18x10^3 CFU.m-3) had higher load than outdoor samples (MEA 6.2x10^2 CFU.m-3) (Figure 2).

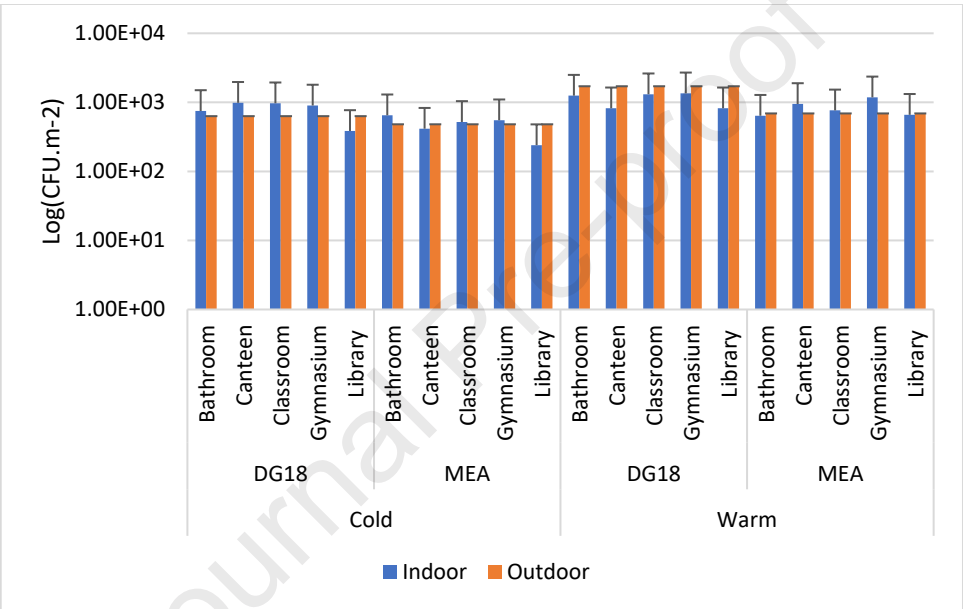


Figure 2 – Fungal contamination in school environments during warm and cold seasons, cultured on MEA and DG18 media. Values represent means with standard deviation.

Airborne fungal load was assessed across canteens, classrooms, and library settings during cold and warm seasons using Andersen 6-stage impactor sampling, with DG18 media incubated at 27°C and 37°C.

At 27°C, overall fungal load was higher compared to 37°C across all stages and environments. In the cold season sampling at 27°C, the highest counts were recorded at Stage 5 in classrooms (2.6x10^2 CFU.m-3) and canteens (2.4x10^2 CFU.m-3). Similarly, warm season samples showed higher load in canteens (3.3x10^2 CFU.m-3 at Stage 5) and libraries (2.81x10^2 CFU.m-3 at Stage 5). The classroom and library also displayed elevated counts at stages 4 and 5, corresponding to particle sizes of approximately 2.1 µm and 1.1 µm, respectively (Supplementary material - Figure S1).

At 37°C, contamination was lower. Cold season values were lower in the canteen and library, with minor counts observed in classrooms. During the warm season, the canteen showed a moderate rise in early stages (Stg2: $2.71x10^1$ CFU.m-3; Stg3: $2.9x10^1$ CFU.m-3), but overall fungal load remained lower compared to 27°C conditions (Supplementary material - Figure S1).

Among the passive samples, filters were the most contaminated matrix, followed by mops and EDC. Filters and EDCs showed contamination across all types of culture media, whereas mops showed contamination only on MEA.

In filter samples, the most contaminated area was the bathroom in all the culture media (MEA $2.0x10^3$ CFU.m-2; DG18 (27ºC) $2.75x10^3$ CFU.m-2; DG18 (27ºC) $1.25x10^3$ CFU.m-2), gymnasium (MEA $2.0x10^3$ CFU.m-2; DG18 (27ºC) $2.50x10^3$ CFU.m-2; DG18 (27ºC) $5.0x10^2$ CFU.m-2) and library (MEA $2.0x10^3$ CFU.m-2; DG18 (27ºC) $1.75x10^3$ CFU.m-2; DG18 (27ºC) $2.5x10^2$ CFU.m-2).

From the EDC, the most contaminated areas were the gymnasium (MEA $2.95x10^1$ CFU.m-2; DG18 (27ºC) $1.67x10^1$ CFU.m-2; DG18 (27ºC) $2.78x10^0$ CFU.m-2) and the classroom (MEA $1.39x10^1$ CFU.m-2; DG18 (27ºC) $1.39x10^1$ CFU.m-2; DG18 (27ºC) $5.55x10^0$ CFU.m-2).

For mop samples, the most contaminated area was the canteen (DG18 (27ºC) $2.0x10^3$ CFU.m-2) (Figure 3)

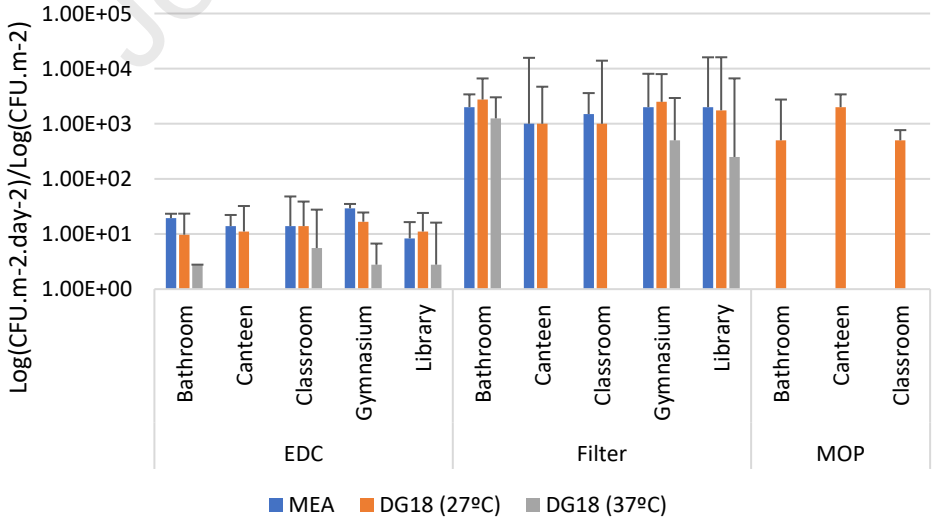


Figure 3 – Fungal contamination in passive samples (EDC, filters, and mops) cultured on MEA, DG18 (27°C), and DG18 (38°C). Values represent means with standard deviation.

Regarding EDCT personal samples, cold season rural area had more contamination on MEA (1.33x103 CFU.m-2.day-1), while warm season urban samples had higher contamination on DG18 (27ºC) (1.67x103 CFU.m-2.day-1). Only the warm season rural area had contamination on DG18 (37ºC) (1.67x102 CFU.m-2.day-1) (Supplementary material - Figure S2).

3.1.2 Fungal identification

The MAS-100 sampler revealed environment-specific fungal patterns. In cold seasons, *Cladosporium* sp. was the most prevalent in indoor environment, particularly canteens (DG18_27ºC: 79.71%) and classrooms (DG18_27ºC: 51.66–73.15%), while *Penicillium* sp. was prevalent in the librarys (DG18_27ºC: 61.70%). Outdoor samples showed high *Cladosporium* sp. (70.43–90.71%) and a lower prevalence of *Fusarium* sp. (≤3.79%). Warm seasons saw higher *Aspergillus* sp. in canteens (DG18_27ºC: 7.38–7.77%) and classrooms (DG18_37ºC: 50.00–66.64%), alongside persistent *Cladosporium* sp. in gymnasiums (DG18_27ºC: 86.69%) (Supplementary table – S5).

3.1.3 Legal Compliance

Although the indoor/outdoor ratio was compliant with the Portuguese Ordinance 138-G/2021, which is primarily based on quantitative fungal counts, the analysis of the fungal species present in the samples, including their toxigenic and pathogenic potential, as well as species mixtures, revealed relevant health-related concerns that are not explicitly addressed by the current legislation (Table 1 and Supplementary material - Table S6).

Table 1 – Air Sampling (MAS-100) Microbiological Compliance (I/O Ratio and Species Check) by Season and Environment for MEA and DG18 (27 °C).

		MEA_27ºC		DG18_27ºC	
Season	**School**	**I/O_Check**	**Species_Check**	**I/O_Check**	**Species_Check**
Cold	S1	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S2	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S3	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S4	✗ Non-Compliant	✗ NON-COMPLIANT	✗ Non-Compliant	✗ NON-COMPLIANT
	S5	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S6	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S7	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S8	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S9	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S10	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT

Season	School	MEA_27ºC I/O_Check	MEA_27ºC Species_Check	DG18_27ºC I/O_Check	DG18_27ºC Species_Check
Warm	S1	✓ Compliant	✗ NON-COMPLIANT		
	S2	✗ Non-Compliant	✗ NON-COMPLIANT	✗ Non-Compliant	✗ NON-COMPLIANT
	S3	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S4	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S5	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S6	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S7	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S8	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S8	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S9	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT
	S10	✓ Compliant	✗ NON-COMPLIANT	✓ Compliant	✗ NON-COMPLIANT

In cold seasons, the sections *Circumdati* (74.98%) and *Nidulantes* (36.67–60.02%) were the most prevalent in the Library and Classroom at DG18_27ºC. Warm-season Gymnasium samples in DG18_37ºC showed only *Fumigati* (100%), while Toilets presented *Nigri* (DG18_27ºC: 82.76%). Notably, *Candidi* emerged in warm Classroom DG18_37ºC (13.93%) (Figure 4).

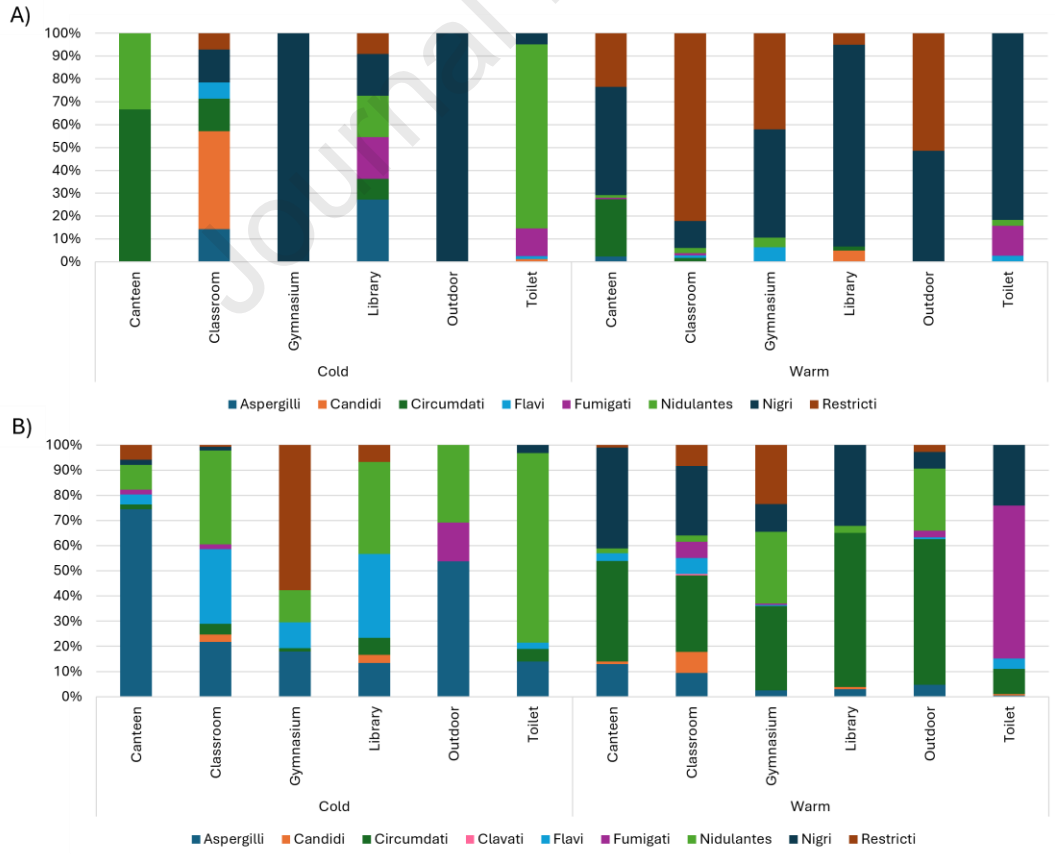


Figure 4 – *Aspergillus* sections prevalence on MAS-100 samples in MEA (A) and DG18 (B), in warm and cold seasons among school environments.

The Andersen sampler data demonstrated significant seasonal and stage-dependent variations in fungal genera distribution across environments. During cold seasons, *Cladosporium* sp. was the most prevalent genus in DG18_27ºC across all stages in the Canteen, with percentages ranging from 38.16% (Stage 2) to 67.69% (Stage 4). In the Classroom, *Cladosporium* sp. prevalence remained high (34.16–72.82%), though *Aspergillus* sp. showed increased contributions at DG18_37ºC, peaking at 90.48% (Stage 3). The Library exhibited a distinct profile, with *Penicillium* sp. dominating DG18_27ºC (41.59–61.70%) and *Aspergillus* sp. reaching 58.41% in Stage 6. In warm seasons, *Aspergillus* sp. contributions increased in the Canteen (DG18_27ºC: 8.84–20.04%) and Classroom (DG18_27ºC: 17.00–36.59%), while *Cladosporium* sp.-maintained prevalence in outdoor environments (e.g., Library: 72.20–87.64%) (Supplementary material - Table S7) *Aspergillus* sections distribution varied with temperature and stage. In cold seasons, the most prevalent section in Classroom environments at DG18_37ºC was *Fumigati* (Stages 1–4: 83.30–100%), while *Restricti* prevailed in the Canteen at DG18_27ºC (Stages 1–6: 23.11–79.17%). Stage 4 in the Canteen under DG18_37ºC showed exclusively *Nidulantes* (100%). In the warm season, *Circumdati* prevailed in the Canteen (DG18_37ºC: 50.00%), while *Nidulantes* was prominent in the Library (DG18_27ºC: 85.72%). Stage 6 in warm Classroom environments under DG18_37ºC revealed co-dominance of *Fumigati* (62.44%) and *Nidulantes* (12.52%), suggesting temperature-mediated competitive dynamics (Supplementary Tables S8).

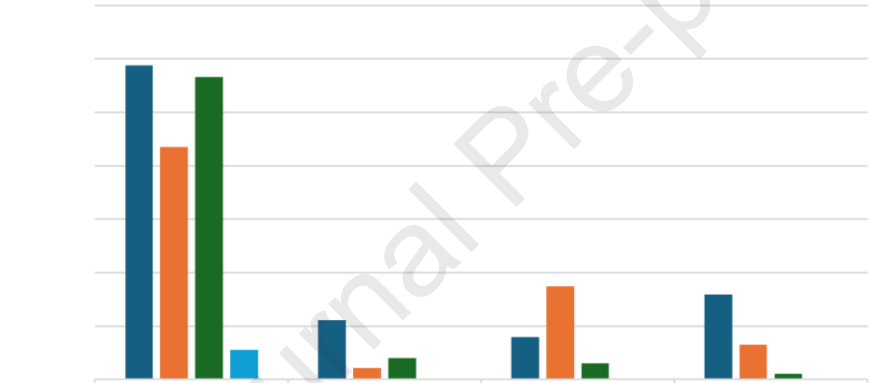
On the passive sampling, EDC in cold seasons showed *Aspergillus* sp. prevalence in the Canteen (DG18_37ºC: 100%) and Classroom (MEA: 85.07%), while *Penicillium* sp. prevailed in the Gymnasium (MEA: 51.07%). The Filters in warm seasons detected *Aspergillus* sp. as prevalent in the Canteen (DG18_27ºC: 88.46%) and Classroom (DG18_27ºC: 57.25%). EDCT cold-season Classroom samples exhibited high *Cladosporium* sp. (MEA: 77.93%), whereas MOP warm-season Toilet favoured *Aspergillus* sp. (MEA: 75.00%) (Supplementary material - Tables S9).

Section-level analysis revealed *Fumigati* prevalence in antifungal media: EDC cold-season Canteen (SDA: 100%) and Filter warm-season Classroom (DG18_37ºC:

94.89%). *Nigri* was a prominent MOP warm-season Toilet (DG18_27ºC: 77.78%). *Circumdati* and *Nidulantes* were limited to specific niches, such Filter cold-season Canteen (DG18_27ºC: 15.22%) (Supplementary material - Tables S9;S10).

3.2 Fungal growth in azole-supplemented media

From the azole resistance screening of the passive samples, EDC exhibited growth on all supplemented media and was the most contaminated matrix in ITZ (7/63) and POZ (10/63). EDCT also showed growth on all supplemented media and presented the highest level of contamination in VOZ (8/46). Filters exhibited contamination across all media. MOPS showed contamination only on SDA, with no growth observed on supplemented media. (Figure 5)



	SDA	ITZ	VOZ	POZ
EDC	58,7%	11,1%	7,9%	15,9%
EDCT	43,5%	2,2%	17,4%	6,5%
Filter	56,6%	4,0%	3,0%	1,0%
MOP	5,6%			

Figure 5 – Proportion of samples showing contamination across different non-supplemented (SDA) and supplemented media (ITZ, VOZ, POZ) by sampling method (EDC, EDCT, Filter, MOP).

3.2.1 Passive sampling fungal identification

From the azole screening, the most prevalent genera in SDA were *Aspergillus* sp. and *Penicillium* sp., with EDC Canteen samples showing 19.09% *Aspergillus* sp. and 19.09% *Penicillium* sp. ITZ media suppressed most genera except *Aspergillus* sp. (EDC Classroom: 100%) and *C. sitophila* (Filter Classroom: 50.00%). VOZ and POZ media

exhibited near-exclusive *Aspergillus* sp. growth, particularly in EDC Library (VOZ: 95.38%) and EDCT cold-season Classroom (POZ: 97.83%) (Table 2).

404 Table 2 – Passive samples fungal characterization on SDA and SDA supplemented with ITZ, VOZ, and POZ.

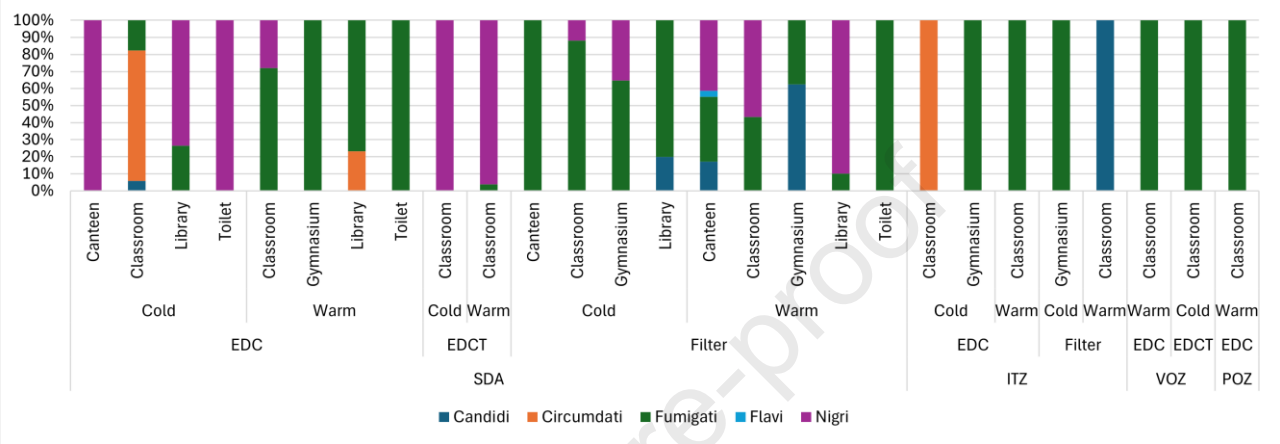
Matrix	Season	Environment	SDA		ITZ		VOZ		POZ	
			ID	%	ID	%	ID	%	ID	%
EDC	Cold	Canteen	*Aspergillus* sp.	19.08						
			Chrysosporium sp.	41.22						
			Mucor sp.	20.61						
			Penicillium sp.	19.08						
		Classroom	*Aspergillus* sp.	83.76	*Aspergillus* sp.	100.00				
			Chrysosporium sp.	6.57						
			Other species	9.67						
		Gymnasium	*Mucor* sp.	21.26	*Aspergillus* sp.	100.00			*C. sitophila*	100.00
			Penicillium sp.	78.74						
		Library	*Aspergillus* sp.	88.31						
			Mucor sp.	11.69						
		Toilet	*Aspergillus* sp.	100.00						
	Warm	Canteen	*Penicillium* sp.	100.00						
		Classroom	*Aspergillus* sp.	46.63	*Aspergillus* sp.	30.68	*Aspergillus* sp.	100.00	*Aspergillus* sp.	48.77
			Other species	3.31	*C. sitophila*	69.32			*C. sitophila*	13.30
			Mucor sp.	38.58					*Mucor* sp.	13.30
			Penicillium sp.	11.48					*Penicillium* sp.	24.63
		Gymnasium	*Aspergillus* sp.	100.00						
		Library	*Aspergillus* sp.	100.00					*Mucor* sp.	100.00
		Toilet	*Aspergillus* sp.	100.00						
EDCT	Cold	Classroom	*Aspergillus* sp.	7.69			*Aspergillus* sp.	24.98	*Paecilomyces* sp.	100.00
			Cladosporium sp.	23.06			*Paecilomyces* sp.	50.03		
			Penicillium sp.	69.25			*Penicillium* sp.	25.00		
	Warm		*Aspergillus* sp.	52.00	*Mucor* sp.	100.00	*Aureobasidium* sp.	24.99		
			Cladosporium sp.	16.00			*Cladosporium* sp.	24.99		

Matrix	Season	Environment	SDA		ITZ		VOZ		POZ	
			ID	%	ID	%	ID	%	ID	%
			Mucor sp.	6.00			*Mucor* sp.	24.99		
			Paecilomyces sp.	12.00			*Penicillium* sp.	25.02		
EDCT	**Warm**	Classroom	*Penicillium* sp.	14.00						
		Canteen	*Aspergillus* sp.	100.00						
			Alternaria sp.	6.25	*C. sitophila*	50.00				
			Aspergillus sp.	70.83	*Penicillium* sp.	50.00				
	Cold		*Other species*	2.08						
		Classroom	*Penicillium* sp.	20.83						
		Gymnasium	*Aspergillus* sp.	100.00	*Aspergillus* sp.	100.00				
		Library	*Aspergillus* sp.	100.00						
			Aspergillus sp.	96.67			*Mucor* sp.	100.00		
Filter		Canteen	*Other species*	3.33						
			Other species	2.94	*Aspergillus* sp.	100.00				
			Aspergillus sp.	88.24						
		Classroom	*Other species*	8.82						
	Warm		*Aspergillus* sp.	88.89			*Penicillium* sp.	100.00		
		Gymnasium	*Rhizopus* sp.	11.11						
			Aspergillus sp.	83.33	*Mucor* sp.	100.00	*C. sitophila*	100.00		
			Mucor sp.	8.33						
		Library	*Penicillium* sp.	8.33						
		Toilet	*Aspergillus* sp.	100.00						
MOP	**Warm**	Classroom	*Cladosporium* sp.	100.00						

405

406 *Fumigati* section demonstrated broad resistance, dominating SDA (EDC Classroom: 55.32%), ITZ
407 (EDC Classroom: 100%), and VOZ (EDC Classroom: 100%). *Nigri* prevailed in POZ (EDCT
408 Classroom: 100%) and SDA (Filter Gymnasium: 56.00%). *Circumdati* showed niche-specific
409 prevalence in EDC Library (POZ: 88.73%), while *Nidulantes* was only from Filter cold-
410 season Canteen (DG18_27ºC: 18.18%) (Figure 6).

411



412 Figure 6 - *Aspergillus* section identified from Passive samples on SDA, and SDA supplemented
413 with ITZ, VOZ, and POZ.

414

415 **3.3 Molecular detection of *Aspergillus* sections**

416 Out of the 226 passive samples analysed by qPCR, fungal DNA from three *Aspergillus* sections,
417 *Fumigati*, *Flavi* and *Nidulantes*, was successfully detected. Positive signals with quantifiable Cq
418 values were found across multiple matrices, including mops, filters, and EDCTs, and in all 11
419 schools surveyed. *Fumigati* was the most frequently detected section, present in all three sample
420 types. *Flavi* was identified in mops, filters, and EDCTs, while *Nidulantes* was detected only in
421 filters and EDCTs. A direct comparison with culture-based methods revealed a significantly higher
422 detection rate by qPCR. Of the total qPCR-positive samples, the vast majority were not recovered
423 by classical microbiology techniques: out of 31 samples positive for *Fumigati*, only 3 were
424 confirmed by microscopy; all 17 samples positive for *Flavi* and all 10 positives for *Nidulantes*
425 were detected exclusively by qPCR (Supplementary material – Table S11).

426

427 **3.4 Mycotoxins contamination**

From the 207 environmental samples analysed across multiple matrices, mycotoxins were detected in a small number of samples (4 out of 207; 1.93 %). Two EDC samples (3.2 %; 2 out of 63) collected from classroom environments showed contamination with mycophenolic acid. Additionally, two settled dust filters (2.3 %; 2 out of 88) revealed the presence of ochratoxin A, one from a classroom and one from the same school's canteen, while sterigmatocystin was detected in a single settled dust filter from the same canteen where ochratoxin A was found (1.1 %; 1 out of 88). None of the other analysed samples showed detectable levels of mycotoxins.

3.5 Particulate matter, temperature and humidity measurements

Supplementary Figure S3 represents the PM2.5 and PM10 mass concentrations compared with the legal thresholds defined by Portuguese Ordinance 138-G/2021, establishes the requirements for indoor air quality (IAQ) in commercial and service buildings in Portugal (DRP, n.d.). $PM_{2.5}$ concentrations demonstrated seasonal variation. During the warm season, 42 school spaces exceeded 25 µg.m-3, with 18 surpassing 50 µg.m-3. The highest warm season concentrations were found in the library of rural school S6 (87.3 µg.m-3) and the canteen of urban school S7 (97.9 µg.m-3). In contrast, the cold season had fewer exceedances, with 19 spaces above 25 µg.m-3 and 9 above 50 µg.m-3. The highest cold season levels were observed in the toilet of rural school S8 (140 µg.m-3) and the library of urban school S10 (82 µg.m-3). On PM_{10} in the warm season, 30 spaces exceeded 50 µg.m-3 and 16 exceeded 100 µg.m-3, with a peak concentration of 389 µg.m-3 in the gym of urban school S5. During the cold season, only 16 spaces exceeded 50 µg.m-3, and 4 exceeded 100 µg.m-3. The highest cold season concentrations were detected in the toilet of rural school S8 (270 µg.m-3) and the library of urban school S9 (241 µg.m-3). The detailed particle number concentrations (from PM0.3 to PM10) and environmental conditions are presented in Supplementary Figure S4. Analysis of the data reveals distinct patterns, with urban schools generally exhibiting higher concentrations of the finer particle fractions (PM0.3, PM0.5, and PM1) compared to rural schools, particularly in settings like canteens and gyms. Furthermore, a clear seasonal influence is observed, where particle levels across most size fractions are significantly elevated during the cold season for both rural and urban locations.

Regarding temperatures and humidity levels, rural areas recorded higher temperatures but lower relative humidity levels. In comparison, urban areas were notably cooler yet displayed higher

458 relative humidity. This pattern suggests an inverse relationship between temperature and moisture
459 content across the sites, where the warmer rural air corresponds with drier conditions, and the
460 cooler urban air corresponds with more humid conditions. (Supplementary material – Table S12).

461 **3.6 Comparisons and correlation analysis**

462 In the warm season, the following correlations were detected (Table 3):

463 a) Rural environment: i) in MAS100, greater fungal contamination in MEA related to greater con-
464 tamination in DG18 medium (r_S=0.470, p=0.015); ii) in filters, greater fungal contamination in
465 MEA related to higher values in SDA (r_S=0.672, p=0.001); iii) in EDCT, greater fungal con-
466 tamination in DG18 related to higher values in SDA (r_S=0.785, p=0.036); iv) in EDC, higher
467 fungal contamination in MEA related to higher fungal contamination in DG18 (r_S= 0.761,
468 p=0.001) and in DG18 at 37ºC (r_S=0.664, p=0.005) and with higher values in SDA (r_S=0.713,
469 p=0.002) and in supplemented media ITZ (r_S=0.548, p=0.028) and VCZ (r_S=0.524, p=0.037);
470 b) Urban environment: i) in MAS100, higher fungal contamination in MEA related to higher fun-
471 gal contamination in DG18 (r_S=0.729, p<0.001); ii) in filters, higher fungal contamination in
472 MEA related to higher fungal contamination in DG18 (r_S=0.647, p=<0.001), to higher values
473 in SDA (r_S=0.524, p=0.007), higher fungal contamination in DG18 related to higher fungal
474 contamination in DG18 at 37ºC (r_S=0.679, p<0.001) and to higher counts in SDA (r_S=0.473,
475 p=0.017), and higher fungal contamination in DG18 at 37ºC related to higher values in SDA
476 (r_S=0.500, p=0.011); iii) in EDCT, higher fungal contamination in DG18 related to higher fun-
477 gal contamination in DG18 at 37ºC (r_S=0.609, p=0.021); iv) in EDC, higher fungal contamina-
478 tion in MEA related to higher fungal contamination in DG18 (r_S=0.471, p=0.048).

479 In the cold season, the following correlations were detected (Table 4):

480 a) Rural environment: i) in MAS100, higher fungal contamination in MEA related to higher fungal
481 contamination in DG18 (r_S=0.874, p=<0.001); ii) in EDCT, higher fungal contamination in
482 MEA related to higher fungal contamination in DG18 (r_S=0.809, p=0.005) and to higher values
483 in SDA (r_S=0.669, p=0.034).
484 b) Urban environment: i) in MAS100, higher fungal contamination in MEA related to higher fun-
485 gal contamination in DG18 (r_S=0.447, p=0.003); ii) in filters, higher fungal contamination in
486 DG18 related to higher fungal contamination in DG18 at 37ºC (r_S=0.444, p=0.018); iii) in
487 EDCT, higher values of azole resistance in VCZ related to higher values in PSZ (r_S=0.645,

p=0.044); iv) in EDC, higher fungal contamination in DG18 related to higher fungal contamination in DG18 at 37ºC (r_S=0.582, p=0.029), to higher azole resistance values in ITZ (r_S=0.661, p=0.010) and to higher values in PSZ (r_S=0.764, p=0.001), higher values in SDA related to higher values in PSZ (r_S=0.558, p=0.038) and higher values in ITZ related to higher values in PSZ (r_S=0.793, p=0.001).

Table 3 - Study of the relationship between fungal contamination (in different culture media) and resistance to azoles (in different culture media). Spearman correlation coefficient results.

Season	Context	Matrices		Media	Fungi			Azole screening		
					DG18	DG18 (37ºC)	SDA (CTL)	ITZ	VCZ	PSZ
Warm	Rural	MAS100	Fungi	MEA	0.470*					
		MOP	Fungi	DG18		-0.417				
		Filter	Fungi	MEA	0.436	0.171	0.672**			0.221
				DG18		0.328	0.300			0.140
				DG18 (37ºC)			0.032			0.170
			Azole resistance	SDA						0.341
		EDCT	Fungi	MEA	0.468	0.233	0.396		-0.520	
				DG18		0.643	0.785*		0.000	
				DG18 (37ºC)			0.558		0.228	
			Azole resistance	SDA					-0.212	
		EDC	Fungi	MEA	0.761**	0.664**	0.713**	0.548*	0.524*	0.258
				DG18		0.740**	0.570*	0.619*	0.627**	0.348
				DG18 (37ºC)			0.760**	0.330	0.466	0.239
			Azole resistance	SDA				0.446	0.610*	0.352
				ITZ					0.826**	0.491
				VCZ						0.684**
	Urban	MAS100	Fungi	MEA	0.729**					
		MOP	Fungi	MEA	0.061	0.745	-0.441			
				DG18		-0.258	-0.535			
				DG18 (37ºC)			-0.338			
		Filter	Fungi	MEA	0.647**	0.365	0.524**	-0.099	0.302	
				DG18		0.679**	0.473*	0.323	0.263	

				DG18 (37ºC)			0.500*	0.210	0.067		
			Azole resistance	SDA				0.059	0.243		
				ITZ					0.308		
		EDCT	Fungi	MEA	0.431	0.423	-0.111	-0.229	-0.072	-0.229	
				DG18		0.609*	0.239	-0.383	-0.350	0.418	
				DG18 (37ºC)			0.299	-0.258	-0.300	0.405	
			Azole resistance	SDA				0.441	-0.075	0.361	
				ITZ					-0.145	-0.077	
				VCZ						-0.145	
		EDC	Fungi	MEA	0.471*	0.116	0.164	0.258	0.258	0.258	
				DG18		0.412	0.136	0.305	0.305	0.305	
				DG18 (37ºC)			0.141	0.406	0.406	0.406	
			Azole resistance	SDA				0.291	0.291	0.291	
Cold	Rural	MAS100	Fungi	MEA	0.874**						
		MOP	Fungi	MEA	0.000	-0.333					
				DG18		0.816					
		Filter	Fungi	MEA	0.224	0.401	0.052	-0.147			
				DG18		0.440	0.119	0.000			
				DG18 (37ºC)			0.009	0.108			
			Azole resistance	SDA				-0.362			
		EDCT	Fungi	MEA	0.809**	-0.059	0.669*		-0.059	-0.412	
				DG18		-0.175	0.507		-0.175	-0.524	
				DG18 (37ºC)			-0.261			-0.111	
			Azole resistance	SDA					-0.261	-0.261	
				VCZ						-0.111	
		EDC	Fungi	MEA	-0.042	0.366	-0.525				
				DG18		-0.198	0.109				
				DG18 (37ºC)			0.211				
	Urban	MAS100	Fungi	MEA	0.447**						
		Filter	Fungi	MEA	0.333	0.088	0.238				
				DG18		0.444*	-0.031				
				DG18 (37ºC)			-0.026				
		EDCT	Fungi	MEA	0.296	0.117	-0.316		-0.079	-0.058	
				DG18		0.395	-0.060		0.011	0.412	
				DG18 (37ºC)			-0.047		0.112	-0.166	
			Azole resistance	SDA					0.049	0.063	
				VCZ						0.645*	
		EDC	Fungi	MEA	0.259	0.257	0.183	0.514		0.499	
				DG18		0.582*	0.161	0.661*		0.764**	

		DG18 (37ºC)	0.081	0.335	0.472
	Azole	SDA		0.298	0.558*
	resistance	ITZ			0.793**

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

496

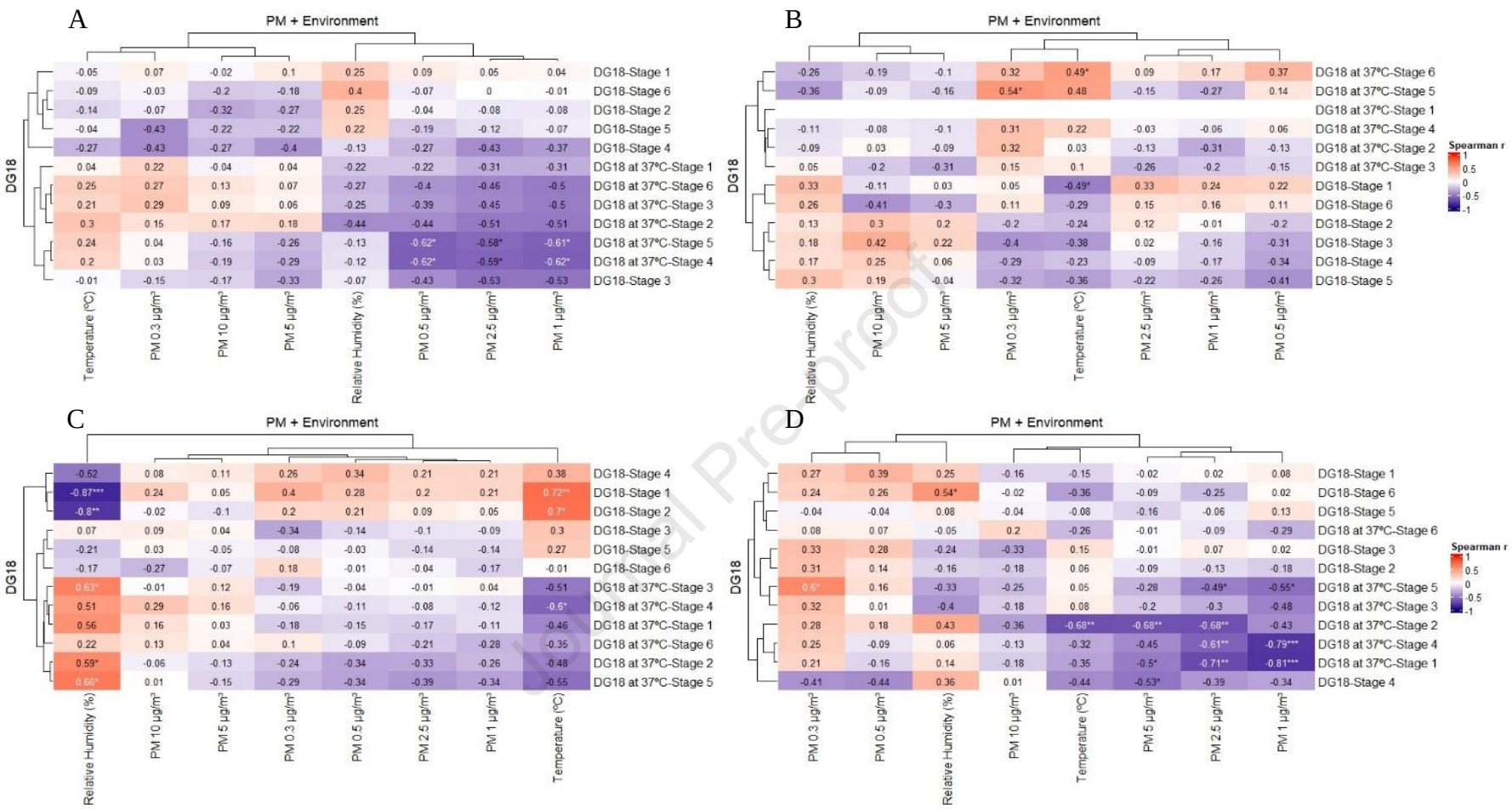
497 *Comparison analysis*

498 Statistically significant differences in fungal contamination were detected between rural and urban
499 contexts: for MEA (U=13414.5, p=0.049) and DG18 at 37ºC (U=4690.0, p=0.028), contamination
500 was higher in the urban environment; however, for DG18 at 37ºC in stage 1 (U=731.5, p=0.047)
501 and stage 4 (U=641.0, p=0.017), it was higher in the rural environment (Supplementary Table
502 S13).
503 Significant seasonal differences were found within each context. In the rural setting, the warm
504 season had greater contamination in DG18 – stage 1 (U=106.0, p=0.048) and in DG18 at 37ºC
505 across stages 1 to 5 ($p<0.05$). Similarly, in the urban context, the warm season showed greater
506 contamination in DG18 (U=4208.0, p=0.044), DG18 at 37ºC (U=1341.0, p=0.043), and specific
507 stages of DG18 at 37ºC: stage 1 (U=216.0, p=0.010), stage 3 (U=176.5, p=0.002), and stage 4
508 (U=187.5, p=0.015) (Supplementary Table S14).
509 Within the rural context, significant differences between sampling sites were also detected: DG18,
510 stage 3 was more contaminated in the canteen (χ^2=8.966, p=0.011), DG18 at 37ºC in the
511 gymnasium (χ^2=11.614, p=0.020), and azole resistance to ITZ was highest in the classroom
512 (χ^2=11.614, p=0.020) (Supplementary Table S15).
513 In the cold season, significant correlations in rural areas were only between fungal contamination
514 in DG18 at 37ºC (stages 4 and 5) and lower concentrations of PM0.5 (rS=-0.62, $p<0.05$ for both)
515 and PM1.0 (rS=-0.62, $p<0.05$; rS=-0.61, $p<0.05$) (Figure 7A). In urban areas, cold season
516 correlations included: higher contamination in DG18 at 37ºC stage 5 with higher PM0.3 (rS=0.54,
517 $p<0.05$); higher contamination in DG18 at 37ºC stage 6 with higher temperature (rS=0.49, $p<0.05$);
518 and higher contamination in DG18 stage 1 with lower temperature (rS=-0.49, $p<0.05$) (Figure 7B).
519 During the warm season in rural areas, correlations detected were: higher contamination in DG18
520 stage 1 with higher temperature (rS=0.72, $p<0.01$) and lower relative humidity (rS=-0.87,
521 $p<0.001$); higher contamination in DG18 stage 2 with higher temperature (rS=0.70, $p<0.05$) and
522 lower relative humidity (rS=-0.80, $p<0.01$); higher contamination in DG18 at 37ºC stage 3 with
523 higher relative humidity (rS=0.63, $p<0.05$); higher contamination in DG18 at 37ºC stage 4 with

lower temperature (rS=-0.60, p<0.05); and higher contamination in DG18 at 37ºC stages 2 and 5 with higher temperature (rS=0.59, p<0.05; rS=0.66, p<0.05) (Figure 7C).

In urban areas during the warm season, correlations included: higher contamination in DG18 stage 6 with higher relative humidity (rS=0.54, p<0.05); higher contamination in DG18 at 37ºC stage 5 with lower PM1.0 (rS=-0.55, p<0.05) and PM2.5 (rS=-0.49, p<0.05) and higher PM0.3 (rS=0.60, p<0.05); higher contamination in DG18 at 37ºC stage 2 with lower PM2.5 (rS=-0.68, p<0.01), PM5.0 (rS=-0.68, p<0.01), and temperature (rS=-0.68, p<0.01); higher contamination in DG18 at 37ºC stage 4 with lower PM1.0 (rS=-0.79, p<0.01) and PM2.5 (rS=-0.61, p<0.01); and higher contamination in DG18 at 37ºC stage 1 with lower PM1.0 (rS=-0.81, p<0.001) and PM2.5 (rS=-0.71, p<0.01) (Figure 7D).

534



535 Figure 7 - Correlation matrix and dendrogram for studying the relationship between fungal contamination and particulate matter and
536 environmental conditions. Results of Spearman's correlation coefficient. A. Cold season, rural areas. B. Cold season, urban areas. C.
537 Warm season, rural areas. D. Warm season, urban areas.
538

4 - Discussion

4.1 Compliance assessment

The present findings reveal limitations in the current Portuguese regulatory framework for microbial contamination in school environments. Existing regulations rely primarily on active air sampling and on a quantitative indoor/outdoor (I/O ≤ 1) criterion, without school-specific fungal exposure thresholds (DRP, n.d.; C. Viegas et al., 2019). However, the results show that even when compliance with this criterion was achieved, potentially pathogenic and toxigenic fungi were detected, indicating that quantitative thresholds alone may not fully reflect indoor fungal risk. Higher indoor fungal loads on DG18 during the warm season, particularly in moisture-prone areas such as bathrooms, highlight the influence of microenvironmental conditions on fungal contamination and the selective proliferation of xerophilic species) (C. Viegas et al., 2024). These localized contamination hotspots are not adequately captured by generalized standards or by active air sampling alone. In addition, passive sampling revealed persistent fungal reservoirs in dust and surfaces, even when airborne concentrations appeared compliant. These findings support the need for a more comprehensive regulatory approach for schools, integrating qualitative assessment, multi-method sampling strategies, and consideration of microenvironment-specific risks to better protect children's health (Cervantes et al., 2025; C. Viegas et al., 2019).

4.2 Particulate matter

A substantial proportion of the monitored school spaces did not comply with the particulate matter limits established by national legislation for commercial and service buildings, which sets 8-hour mean concentration limits of 25 $\mu g.m^{-3}$ for $PM_{2.5}$ and 50 $\mu g.m^{-3}$ for PM_{10} (DRP, n.d.; *WHO Global Air Quality Guidelines*, n.d.). Exceedances were particularly frequent during the warm season, with several indoor environments presenting concentrations well above guideline values, including canteens and gymnasiums. Similar patterns have been previously reported in Portuguese schools, where $PM_{2.5}$ and PM_{10} concentrations in classrooms frequently exceed national and international recommendations (Felgueiras et al., 2024; Madureira et al., 2015, 2016). Both chronic and short-term exposure to elevated levels of fine and coarse particulate matter have been consistently associated with adverse respiratory outcomes in children, including increased prevalence of wheezing (Liu et al., 2018). The elevated concentrations of ultrafine particle fractions ($PM_{0.3}$, $PM_{0.5}$ and PM_1), particularly in urban schools, suggest a relevant contribution from outdoor

combustion-related sources, such as traffic infiltration (Jeong et al., 2019). These findings highlight the need for targeted mitigation strategies, combining improved ventilation and filtration in high-risk indoor spaces with operational measures addressing identified indoor sources, in order to reduce children's exposure to particulate matter in school environments (Branco et al., 2019, 2024).

4.3 Complementarity of Active and Passive Sampling Methods

Active air sampling provides a snapshot of culturable airborne fungi at a specific moment, reflecting short-term inhalation exposure (Cervantes et al., 2022; Collins et al., 2023; King & McFarland, 2012), whereas passive sampling captures the accumulation and resuspension of settled dust over time (Cervantes et al., 2022; Whitby et al., 2022). Seasonal patterns observed with active methods, such as higher airborne loads in canteens and classrooms during the warm season, contrast with the consistently high contamination detected by passive samplers in filters and in moisture, and activity-prone areas such as bathrooms and gymnasiums, indicating the presence of substantial fungal reservoirs. This contrast is not contradictory but complementary. Passive methods act as long-term integrators of fungal presence, revealing contamination that may be missed during short active sampling periods, while personal EDCT sampling further reflects individual exposure (Cervantes et al., 2025; Noss et al., 2008; Whitby, 2022). In the present assessment, active air sampling provided the quantitative indoor/outdoor ratios required under Portuguese Ordinance 138-G/2021; however, qualitative analysis demonstrated non-compliance through the identification of fungal species explicitly listed in the legislation, including *Aspergillus* sections *Nidulantes*, *Flavi*, *Circumdati*, and *Fumigati* (Table 2). Passive sampling was indispensable to corroborate this hidden risk profile, identifying potentially pathogenic and toxigenic fungi that were under-represented in air samples. (Supplementary material - Tables S9; S10). Differences in fungal profiles obtained by active and passive methods further reflect the influence of spore characteristics on sampling efficiency. Active samplers preferentially collect small, dry, and readily aerosolized spores, explaining the predominance of *Cladosporium*, *Penicillium* and *Aspergillus* in air samples (Madsen et al., 2016; Mbareche et al., 2018). In contrast, passive methods act as a safety net for larger, heavier, gelatinous, or clustered propagules that settle rapidly and are often under-represented in short-term air sampling, which accounts for the dominance of *Aspergillus* sections *Fumigati* and *Nigri* and the detection of genera such as *Mucor* and *Paecilomyces* in passive samples (Dias et al., 2024; Minahan et al., 2024). The stage-

dependent distribution observed with the Andersen six-stage cascade impactor further highlights the relevance of particle size in health risk assessment. The predominance of fungal contamination at stages 4 and 5, corresponding to particle sizes of approximately 2.1 µm and 1.1 µm respectively, indicates that a substantial fraction of airborne fungal propagules is within the respirable range, capable of reaching the lower airways and alveolar regions upon inhalation, which is of particular concern in a children's school environment. This validates the principle that combined strategies are necessary to obtain a complete and accurate risk assessment.

The combined use of MEA and DG18 influenced both fungal counts and community composition, particularly affecting the recovery of *Aspergillus* sections (Charres et al., 2025; Pinheiro et al., 2014; C. Viegas, Dias, et al., 2020). Differences between media, especially in passive samples, reflect the selection of distinct ecological groups within the fungal community, while incubation at 37 °C, despite presenting lower counts, enables the identification of thermotolerant species of relevance for health risk assessment in school environments (Noss et al., 2008; C. Viegas, Dias, et al., 2022; C. Viegas et al., 2024). Higher viable fungal loads indoors compared with outdoors during the cold season are consistent with reduced ventilation, closed building envelopes, and increased occupancy, which promote the accumulation of fungal aerosols through human shedding and resuspension from settled dust (Heo et al., 2017; J. Qian et al., 2012). As ventilation increases during warmer months, these patterns shift, and peaks observed in high-activity spaces such as gymnasiums highlight occupant movement and mechanical disturbance as key drivers of aerosolization and particle resuspension (Heo et al., 2017; H. Qian & Zheng, 2018; J. Qian et al., 2012). These dynamics help explain the spatial hotspots identified in the study, particularly in moisture-rich environments such as bathrooms, areas with organic residues like canteens, and activity-intensive rooms such as gymnasiums, where conditions favour sporulation, nutrient availability, and disturbance-driven release (Andersson et al., 2023; Flannigan et al., 2016; C. Viegas et al., 2024). The performance of sampling with passive methods, particularly filters, in detecting these localized hotspots underscores their role as time-integrated collectors of settled dust and debris, capturing fungi associated with reservoirs that may be missed by short-term active air sampling (Nazaroff, 2016; C. Viegas, Dias, et al., 2022). Culture-based methods remain essential for identifying metabolically active, allergenic, or pathogenic fungi, including those selectively recovered on MEA or DG18, which shape the fraction of the community that can be isolated and inform health-relevant exposure assessments (Pitkäranta et al., 2011; Samson et al.,

2019). These findings support the integration of qualitative, species-level identification and resistance screening into routine school monitoring programs. A combined strategy using active air sampling to accurately assess microbial contamination, including antifungal resistance profile, providing a more meaningful risk assessment to inform targeted mitigation measures in school environments (Frankel et al., 2012; Minahan et al., 2024).

4.4 Exploratory Associations between Particulate Matter and Fungal Contamination

The associations between particulate matter and fungal contamination assessed on DG18 were generally limited and highly context-dependent, consistent with studies showing weak or variable correlations between fungal bioaerosols and particle concentrations in indoor environments (Hargreaves, n.d.). During the cold season, correlations were scarce overall, with rural schools predominantly exhibiting negative relationships between fine particle fractions and fungal contamination, whereas urban schools showed more varied patterns involving ultrafine particles and environmental variability (Shammi et al., 2021). During the warm season, a greater number of associations emerged, particularly in rural contexts, highlighting the influence of meteorological and microclimatic conditions on airborne fungi (Canha et al., 2015). In urban environments, correlations appeared more linked to particulate matter size fractions, suggesting that particle size may shape fungal distribution patterns under complex urban atmospheric conditions (Marcovecchio & Perrino, 2021; Shammi et al., 2021). Overall, these findings indicate that the interaction between particulate matter and culturable fungal contamination is complex and likely secondary to broader environmental drivers such as seasonality, humidity, and local context (Marcovecchio & Perrino, 2021).

4.5 Azole Resistance Screening in Passive Matrices

Aligned with the One Health framework advanced by major European health and regulatory agencies (ECDC, EFSA, EMA, EEA, and WHO Europe) (74,75), which identify antimicrobial resistance, including antifungal resistance, as a priority requiring integrated environmental monitoring (WHO, n.d.-b), the screening of passive samples using azole supplemented media highlights that azole-resistant fungi are present in indoor school environments, with contamination patterns strongly influenced by microenvironment and matrix type. Certain spaces, such as bathrooms, gymnasiums, classrooms, and libraries, emerge as potential hotspots, emphasizing the

importance of monitoring not only air but also long-term fungal reservoirs in dust and surfaces (C. Viegas et al., 2010). The predominance of *Aspergillus* species, particularly sections with known resistance potential, reinforces the need for targeted surveillance strategies in schools to better assess exposure risks. Looking forward, and as a new approach in school settings a more detailed investigation will be conducted using isolates recovered from these samples, following EUCAST standards, with sequencing and mutation analyses to characterize azole resistance mechanisms in *Aspergillus fumigatus* (EUCAST, n.d.). These analyses will provide a deeper understanding of the prevalence, diversity, and potential health implications of resistant strains in indoor school environments.

4.6 Molecular Methods

The presented results demonstrate a clear disparity in sensitivity between molecular and culture-based methods. The qPCR analysis revealed a widespread presence of *Aspergillus* sections that was largely undetected by classical techniques. This underscores a primary advantage of molecular tools in environmental monitoring: their ability to detect non-viable, non-culturable, or stressed fungal propagules that still contribute to environmental exposure and may pose a health risk. This capability makes qPCR an essential tool for comprehensive exposure assessment. However, this methodological comparison also highlights the fundamental complementarity of both approaches. While qPCR offers superior sensitivity and specificity for target DNA, it cannot distinguish between viable and non-viable cells or provide isolates for further characterization (e.g.,antifungal resistance testing, precise species-level identification, or mycotoxin profiling). Furthermore, a positive qPCR signal is not directly equivalent to an exposure dose or an indicator of infectivity, as DNA may persist from non-viable or fragmented propagules. In this regard, culture-based methods, though less sensitive, are essential for confirming fungal viability, and to recover the isolates, which are required for functional characterization, including antifungal susceptibility testing and the detection of azole resistance, as well as for identifying unexpected or non-target microorganisms. Molecular methods provide a robust, sensitive measure of total target burden for risk assessment, while classical microbiology remains vital for confirming biological relevance, isolating strains, and completing the taxonomic picture. This combined approach yields the most complete understanding of environmental fungal contamination.

4.7 Mycotoxins

Although the overall frequency of mycotoxin detection was low in this study, the identification of ochratoxin A and sterigmatocystin, both classified by the International Agency for Research on Cancer (IARC) as *possibly carcinogenic to humans* (Group 2B), and mycophenolic acid in dust samples from school environments is noteworthy given their documented toxicological profiles (IARC, n.d.; WHO, n.d.-a). Ochratoxin A, produced by several *Aspergillus* and *Penicillium* species, has demonstrated nephrotoxic and genotoxic effects in experimental models and has been associated with renal pathology in epidemiological contexts (García-Esparza et al., 2025). Sterigmatocystin, a precursor of aflatoxin-like compounds, also shows genotoxic and carcinogenic potential in animal studies (IARC, n.d.). Mycophenolic acid exhibits bioactive immunomodulatory properties that may have relevance under chronic exposure (To et al., 2016). The low detection rate is unlikely to reflect a methodological limitation, as EDCs were deployed for 30 days per location, providing an extended integration period for mycotoxin accumulation; rather, it likely mirrors genuinely low mycotoxin levels in the sampled environments. Although the detected concentrations and prevalence do not indicate a dramatic contamination scenario, these findings support the need for continued environmental surveillance in indoor settings, particularly as climate change is projected to alter fungal ecology and increase mycotoxin exposure risk (EEA, n.d.) .

Overall, the present findings highlight the complexity of indoor environmental exposure in school settings, where compliance with quantitative thresholds does not necessarily reflect biological risk. The combined evidence from particulate matter measurements, active and passive fungal sampling, molecular detection, and mycotoxin screening demonstrates that exposure is shaped by seasonality, microenvironmental conditions, building use, and methodological approach. From a practical perspective, these results support targeted interventions in specific school microenvironments, particularly moisture-prone and high-activity areas such as bathrooms, gymnasiums and canteens. Increasing cleaning frequency in these spaces, improving ventilation efficiency during periods of reduced air exchange, and adapting mitigation measures to room function and occupancy may help reduce both airborne exposure and long-term fungal reservoirs. Importantly, the findings reinforce the need to move beyond single-method compliance assessments toward integrated monitoring strategies that combine active air sampling, passive dust collection, molecular tools and culture-based approaches. Such strategies provide a more biologically meaningful evaluation of exposure, enabling the detection of toxigenic species and

resistance indicators. Given its consistent detection across sampled environments, its well-established clinical relevance, and its designation as a critical priority pathogen by the World Health Organization, *Aspergillus fumigatus* may represent a suitable sentinel organism for environmental fungal risk and antifungal resistance surveillance in school settings (Salambanga et al., 2022). It should be acknowledged that PM measurements were obtained using a direct-reading optical particle counter, which may be subject to inherent uncertainties, including potential overestimation under elevated relative humidity conditions. Although the instrument was calibrated and humidity was simultaneously recorded, no gravimetric validation was performed; however, given that PM served as a contextual parameter rather than a primary outcome, this limitation is unlikely to affect the main conclusions of the study. These approaches ultimately support the development of school-specific guidelines to better protect children's health.

Conclusions

This study implemented a multifaceted environmental assessment campaign in primary school settings, integrating particulate matter fraction with active and passive fungal sampling, multi-media culture approaches, incubation at different temperatures, molecular detection, mycotoxin screening, and resistance indicators. The findings demonstrate that exploiting the complementarity of sampling and analytical methods is essential to avoid the loss of critical information and to achieve a more accurate characterization of indoor microbial contamination, while also highlighting the limitations and inadequacies of the current legal framework in place. Particulate matter characterization provided important contextual information for interpreting microbial contamination patterns. Airborne particles act as carriers for fungal spores, fragments, and metabolites, influencing their persistence, deposition, and resuspension dynamics. The integration of PM and microbiological data therefore contributes to a more mechanistic understanding of indoor fungal contamination and potential health risks. The combined methodological framework revealed marked spatial and seasonal heterogeneity, highlighting the influence of microenvironmental conditions, building use, and ventilation dynamics on both airborne contamination and long-term fungal reservoirs. Importantly, culturomics approaches and incubation temperatures at 37 °C proved particularly valuable for detecting thermotolerant species with potential pathogenic relevance. The detection of fungal growth on azole-supplemented media further indicates the presence of species with suggested resistance profiles, reinforcing the relevance of incorporating antifungal resistance considerations into routine assessments of school

environments. Overall, the results support the adoption of school-specific indoor air quality assessment strategies based on integrated, multi-method monitoring approaches capable of capturing biological, toxicological, and resistance-related dimensions of contamination. Aspergillus fumigatus may serve as a sentinel organism for fungal risk and antifungal resistance surveillance in school environments.

Based on the present findings, the following considerations are proposed for school environmental monitoring and regulatory frameworks:

• Recognition of primary schools as a distinct environmental exposure context

Adoption of integrated active and passive sampling methods

• Use of multiple culture media and incubation conditions, including selective media and incubation at 37 °C

• Systematic integration of molecular and culture-based methods

• Incorporation of antifungal resistance screening, particularly using azole-supplemented media

• Development of school-specific regulatory criteria

• Consideration of *Aspergillus fumigatus* as a sentinel/surrogate organism for fungal risk surveillance

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Highlights

• High fungal exposure influenced by building conditions, ventilation, and seasonality.

• Quantitative cutoffs are not suitable for fungal contamination assessment.

• Active + passive sampling methods reveal complementary information.

• Pathogenic, thermotolerant, toxigenic fungi were widespread in all sampling sites.

• Azole resistant *Aspergillus* sections were detected across all school environments.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: