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Assessment of Children's Potential Exposure to Bioburden in Indoor Environments

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Abstract: The exposure to particles and bioaerosols has been associated with the increase in health effects in children. The objective of this study was to assess the indoor exposure to bioburden in the indoor microenvironments more frequented by children. Air particulate matter (PM) and settled dust were sampled in 33 dwellings and four schools with a medium volume sampler and with a passive method using electrostatic dust collectors (EDC), respectively. Settled dust collected by EDC was analyzed by culture-based methods (including azole resistance profile) and using qPCR. Results showed that the PM2.5 and PM10 concentrations in classrooms (31.15 μg/m^3 and 57.83 μg/m^3, respectively) were higher than in homes (15.26 μg/m^3 and 18.95 μg/m^3, respectively) and highly exceeded the limit values established by the Portuguese legislation for indoor air quality. The fungal species most commonly found in bedrooms was *Penicillium* sp. (91.79%), whereas, in living rooms, it was *Rhizopus* sp. (37.95%). *Aspergillus* sections with toxigenic potential were found in bedrooms and living rooms and were able to grow on VOR. Although not correlated with PM, EDC provided information regarding the bioburden. Future studies, applying EDC coupled with PM assessment, should be implemented to allow for a long-term integrated sample of organic dust.

Keywords: indoor air quality; microenvironments; schools; dwellings; bioburden; electrostatic dust collector

1. Introduction

Children are more susceptible to air pollutants compared to adults since they breathe more air relative to their body weight, their immune system is still in development and they have a lower ability to deal with the toxicity due to their undeveloped airways [1,2]. Children spend more than 85% of their time in indoor environments, mainly at home and school [3] and therefore it is essential to assess the indoor air quality (IAQ) in these microenvironments to estimate their integrated exposure to air pollutants.

Pollutants such as particulate matter (PM) are linked to an increase in morbidity and mortality [4,5]. PM is a complex mixture of small-diameter particles with different physical and chemical characteristics. PM is classified according to their diameter (e.g., PM2.5 and PM10, which are particles with an aerodynamic diameter smaller than 2.5 and 10 μm, respectively), because this physical characteristic highly affects the penetration into the respiratory tract [6,7]. PM2.5 or fine particles reach the lower respiratory tract, while the PM2.5–10 or coarse particles can reach the upper respiratory tract. In addition, the health impact of the PM depends on its composition, which is highly determined by the emission sources.

Bioaerosols are usually defined as PM with biological origins such as microorganisms, pollen and plant fibers. The exposure to biological agents can lead to a wide range of adverse health effects, including allergies, infection diseases, breathing problems and cancer [4].

Previous studies reported a wide range of environmental factors that influence bioburden (covering bacteria and fungi) indoors, such as the occupancy of the spaces [8,9], building layout, ventilation [10] and cleaning procedures including the type of products applied [4]. Furthermore, poor maintenance of heating, ventilation and air conditioning systems can also enhance the hazardous effects of many biological and nonbiological pollutants [11]. Due to the influence of these multiple environmental variables, sampling bioburden should be performed by passive methods, together with more conventional air sampling [12–15]. Indeed, passive methods allow defining the contamination of a larger period of time (ranging from weeks to several months), whereas air samples can only replicate the load from a shorter period of time (mostly minutes) [16].

The electrostatic dust collector (EDC) is a passive collection device easy-to-use that comprises an electrostatic polypropylene cloth [17]. The use of this device is gradually increasing since it is low-cost and effective for the collection of dust [16,18,19], and it has already been applied for the bioburden assessment in several indoor environments [16,19–27].

The emergence worldwide of drug-resistant human pathogenic fungal species, such as *Candida* sp. and *Aspergillus fumigatus*, and the increasing reports of therapeutic failure against fungal infections caused by environmental resistant strains [28–30], has revealed the need of surveillance of fungal resistance in the indoor and outdoor environments, which is mostly described for *Aspergillus* section *Fumigati* [31–36].

In this study, the exposure to PM and bioburden in the indoor microenvironments frequented by children was assessed by particle measurement and by the use of EDCs. This work also explored the suitability of EDCs for identifying critical control points of indoor exposure to PM, and for characterizing the bioburden present indoors. The fungal burden was also characterized through molecular detection of the species with toxigenic potential and also via analysis of antifungal resistance profile.

2. Materials and Methods

2.1. Location of the Studied Schools and Dwellings

This work was developed in the framework of the LIFE Index Air. Available online: (http://www.lifeindexair.net/) (accessed on 14-09-2020) and was conducted in 33 dwellings (D1–D33) and 4 schools (S1–S4) located in the city of Lisbon, Portugal from September 2017 to October 2018. Figure 1 shows the location of the studied schools and homes.

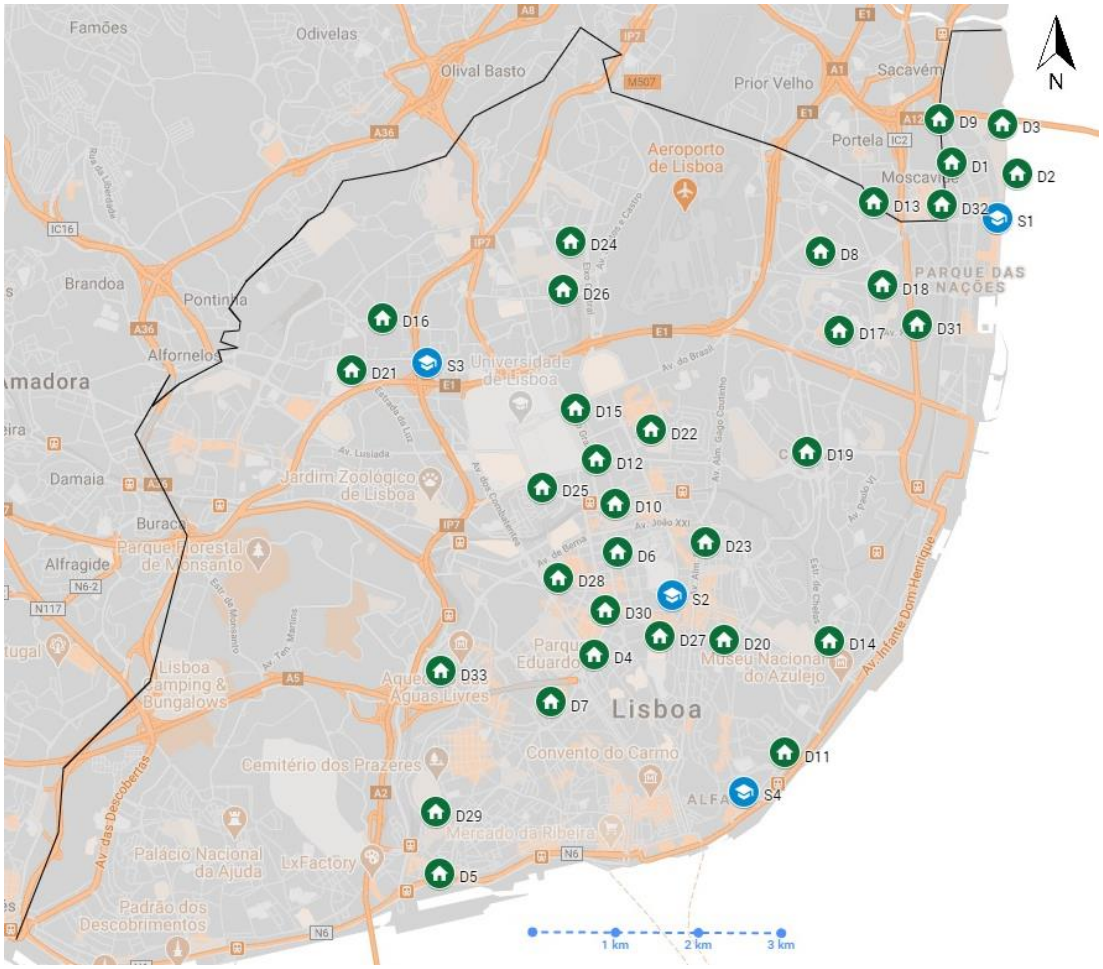

Figure 1. Location of the studied schools (blue) and dwellings (green) in Lisbon, Portugal.

2.2. Air Particulate Matter and Settled Dust Sampling

PM2.5 and PM2.5–10 was sampled with a medium volume sampler (MVS6, Leckel, Sven Leckel, Germany), which was installed in the living room of the dwellings and in a classroom of the schools, as described by Faria et al. (2020). Filters were analyzed by gravimetry before and after sampling with a microbalance (Sartorius R160P, Greifensee, Switzerland) and PM mass concentration was determined by dividing the filter loads by the volume of filtered air. All microenvironments were monitored for 5 days during the occupied period, summing a total of 330 sampled filters.

Dust was collected through a passive method using an electrostatic dust collector (EDC), which comprises an electrostatic polypropylene cloth [17]. Dust was collected from 30 to 44 days in an EDC with a surface exposure area of 0.00636 m^2. In the dwellings, the EDCs were exposed in the living room (a total of 33) and in the children's bedroom (a total of 31) and in schools, the EDCs were placed in the classrooms (a total of 4). The EDC was then used for the bioburden assessment.

2.3. Electrostatic Dust Cloth Extraction and Bioburden Characterization

In order to determine the mass of the collected dust, each EDC was weighted after sampling and subtracted to the mean of 10 EDCs weighted before sampling. Settled dust collected by the EDC was analyzed by culture-based methods and using real-time PCR (qPCR), targeting 4 different *Aspergillus* sections (*Flavi, Fumigati, Circumdati* and *Nidulantes*). The target fungi were selected based on the classification as indicators of harmful fungal contamination [37].

EDC samples were subject to extraction and bioburden characterized by culture-based methods as previously described [16,19,22,26,27]. EDC were washed and 0.15 mL seeded onto 2% malt extract

agar (MEA) with 0.05 g/L chloramphenicol media; dichloran glycerol (DG18) agar-based media; tryptic soy agar (TSA) with 0.2% nystatin for total bacteria assessment; violet red bile agar (VRBA) for Gram-negative bacteria.

Samples were also spread (0.15 mL) onto Sabouraud dextrose agar (SDA) media supplemented with 4 mg/L itraconazole (ITR), 1 mg/L voriconazole (VOR) or 0.5 mg/L posaconazole (POS, protocol adapted from the EUCAST 2017 guidelines) [38] for the screening of antifungal resistance [19].

Incubation of MEA, DG18 and azole screening plates at 27 °C for 5 to 7 days and TSA and VRBA plates at 30 and 35 °C for 7 days, respectively, was performed.

Molecular identification of the different fungal species/strains was achieved by qPCR using the CFX-Connect PCR System (Bio-Rad, Hercules, CA, USA) on EDC collected (bedrooms n = 31; living rooms n = 33; classrooms = 4). Reactions included 1× iQ Supermix (Bio-Rad), 0.5 µM of each primer (Table 1), and 0.375 µM of TaqMan probe in a total volume of 20 µL. Amplification followed a three-step PCR: 50 cycles with denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 30 s (Table 1). Nontemplate control was used in every PCR reaction. For each gene that was amplified, a nontemplate control and positive control were used, consisting of DNA obtained from a reference that belonged to the culture collection of the Reference Unit for Parasitic and Fungal Infections, Department of Infectious Diseases of the National Institute of Health, from Dr. Ricardo Jorge. These strains have been sequenced for ITS B-tubulin and Calmodulin.

Table 1. Sequence of primers and TaqMan probes used for real-time PCR.

***Aspergillus* Sections Targeted**	**Sequences**	**Reference**
Flavi (Strains with toxigenic potential)		
Forward Primer	5′-GTCCAAGCAACAGGCCAAGT-3′	
Reverse Primer	5′-TCGTGCATGTTGGTGATGGT-3′	[39]
Probe	5′-TGTCTTGATCGGCGCCCG-3′	
Fumigati		
Forward Primer	5′-CGCGTCCGGTCCTCG-3′	
Reverse Primer	5′-TTAGAAAAATAAAGTTGGGTGTCGG-3′	[40]
Probe	5′-TGTCACCTGCTCTGTAGGCCCG-3′	
Circumdati		
Forward Primer	5′-CGGGTCTAATGCAGCTCCAA-3′	
Reverse Primer	5′-CGGGCACCAATCCTTTCA-3′	[41]
Probe	5′-CGTCAATAAGCGCTTTT-3′	
Nidulantes		
Forward Primer	5′–CGGCGGGGAGCCCT-3′	
Reverse Primer	5′–CCATTGTTGAAAGTTTTGACTGATcTTA-3′	
Probe	5′–AGACTGCATCACTCTCAGGCATGAAGTTCAG-3′	[42]

2.4. Statistical Analysis

The statistical software SPSS V24.0 for Windows® was used for data analysis. The results were considered significant at a 5% significance level. The frequency analysis (n, %) was applied for the qualitative data, and the minimum, maximum, median and interquartile range were calculated for the quantitative data. The median and the interquartile range were used, since outliers were detected and the mean and standard deviation were influenced by these values. The Shapiro-Wilk test was applied to test data normality, and Spearman's correlation coefficient to study the relationship between two quantitative variables. Kruskal–Wallis test was used to compare EDC weight, fungal counts on MEA and DG18 and bacteria counts on TSA and VRB among the different sampling locations, since the assumption of normality was not verified. When statistically significant differences were detected, the Kruskal–Wallis multiple comparisons test was the analyses selected. For the comparison of the concentration of the particles between the two sampling locations (classroom and living room) the Mann–Whitney test was used, since the assumption of normality was not verified.

3. Results

3.1. Particulate Matter Assessment

The PM2.5 and PM10 average concentrations in the classrooms were 31.15 and 57.83 μg/m^3, respectively, with a range between 19.47 and 52.91 μg/m^3 for PM2.5 and between 32.72 and 109.02 μg/m^3 for PM10. Table 2 shows that in dwellings, the concentrations ranged between 6.05 and 67.96 μg/m^3 for PM2.5 and between 9.14 and 72.95 μg/m^3 for PM10, with an average concentration of 15.26 μg/m^3 and 18.95 μg/m^3, respectively. The PM2.5 concentrations exceeded the 8-hr limit value established by the Portuguese legislation for indoor air quality (Portaria 353-A/2013, 25 μg/m^3) in 50% of the schools and in 12% of the dwellings and the PM10 limit value (50 μg/m^3) was exceeded in 50% of the schools and in 3% of the dwellings.

Regarding the settled dust collected by the EDC, the schools presented an average level of 1.42 g/m^2/d with a range between 1.28 and 1.57 g/m^2/d and the dwellings registered an average of 3.36 g/m^2/d with a range between 1.27 and 11.16 g/m^2/d. In dwellings, the living room presented an average amount of 3.6 g/m^2/d and the bedroom of 3.11 g/m^2/d (Table 2).

Table 2. Settled dust (g/m^2/d) and PM2.5 and PM10 concentrations (μg/m^3) measured in dwellings and schools.

		Settled Dust (g/m^2/d)	PM2.5 (μg/m^3)	PM10 (μg/m^3)
Schools	Average	1.42	31.15	57.83
	Range (min–max)	1.28–1.57	19.47–52.91	32.72–109.02
Dwellings	Average	3.36	-	-
	Range (min–max)	1.27–11.16	-	-
Living Rooms	Average	3.60	15.26	18.95
	Range (min–max)	1.28–11.16	6.05–67.96	9.14–72.95
Bedrooms	Average	3.11	-	-
	Range (min–max)	1.27–10.74	-	-

3.2. Bacterial Contamination Assessment

From the 31 samples collected in the bedrooms, the total bacteria contamination ranged from below the detection limit to 1.42×10^3 CFU/m^2/d, with the Gram-negative bacteria contamination, ranging from below the detection limit to 3.15×10^1 CFU/m^2/d.

Total bacteria contamination in the 33 EDC collected in living rooms ranged from below the detection limit to 3.42×10^3 CFU/m^2/d, with the Gram-negative bacteria contamination, ranging from below the detection limit to 4.60×10^1 CFU/m^2/d.

In the 4 EDC samples collected in the classrooms, the total bacteria contamination ranged from below the detection limit to 6.2×10^1 CFU/m^2/d, while there was no contamination by Gram-negative bacteria (Table 3).

Table 3. Bacteria contamination (CFU/m^2/d) in each studied location.

Location			Total Bacteria	Gram-Negative Bacteria
	Average	N	CFU/m^2/d	CFU/m^2/d
Bedrooms	Range (min–max)	31	*–1.42×10^3	*–3.15×10^1
Living Rooms	Range (min–max)	33	*–3.42×10^3	*–4.60×10^1
Classrooms	Range (min–max)	4	*–6.2×10^1	-

N—Number of samples collected. *–Below the detection limit.

3.3. Fungal Contamination Assessment

A total of 31 EDC were collected from bedrooms. The fungal contamination in these samples ranged from lower the detection limit to 2.00×10^3 CFU/m^2/d (D30) in MEA, and from lower the detection limit to 2.81×10^3 CFU/m^2/d (D32) in DG18. The most commonly found fungal species in MEA was *Penicillium* sp. (2.00×10^3 CFU/m^2/d; 89.43%), followed by *Cladosporium* sp. (1.59×10^2 CFU/m^2/d; 7.10%) and *Chrysosporium* sp. (2.56×10^1 CFU/m^2/d; 1.14%; Table 4). In DG18, the most prevalent species were *Cladosporium* sp. (2.81×10^3 CFU/m^2/d; 90.44%), *Penicillium* sp. (2.07×10^2 CFU/m^2/d; 6.67%) and *Aspergillus* sp. (1.05×10^2 CFU/m^2/d; 1.23%; Table 4). Four different *Aspergillus* sections were identified in the EDC samples from the bedrooms, two found in MEA (*Nigri* and *Fumigati*; 1.05×101 CFU/m^2/d), and two in DG18 (*Candidi* and *Circumdati*; 3.81×101 CFU/m^2/d; Figure 2).

In the 33 EDC collected from the living rooms, the fungal contamination ranged from lower the detection limit to 5.24×10^3 CFU/m^2/d (D3, D6 and D28) in MEA, and from lower the detection limit to 2.62×10^3 CFU/m^2/d (D32). In MEA, the most common was *Rhizopus* sp. (5.24×103 CFU/m^2/d; 38.11%), followed by *Chrysonilia* sp. (5.24×10^3 CFU/m^2/d; 38.11%) and *Chrysosporium* sp. (2.64×10^3 CFU/m^2/d; 19.19%); in DG18, *Chrysonilia* sp. (2.62×10^3 CFU/m^2/d; 76.55%), followed by *Penicillium* sp. (3.54×10^2 CFU/m^2/d; 10.33%) and *Cladosporium* sp. (1.7×10^2 CFU/m^2/d; 4.96%) were the most prevalent (Table 4). A total of eight *Aspergillus* sections were identified in the samples from the living room. Five different sections were found in MEA, including *Aspergillus* section *Fumigati* (6.18×101 CFU/m^2/d), *Flavi* and *Nigri* (2.62×10^1 CFU/m^2/d; Figure 2). In DG18, six *Aspergillus* sections were identified, with the most prevalent being *Nidulantes* (7.89×10^1 CFU/m^2/d), followed by *Fumigati* (3.67×10^1 CFU/m^2/d) and *Clavati* (1.57×10^1 CFU/m^2/d; Figure 2).

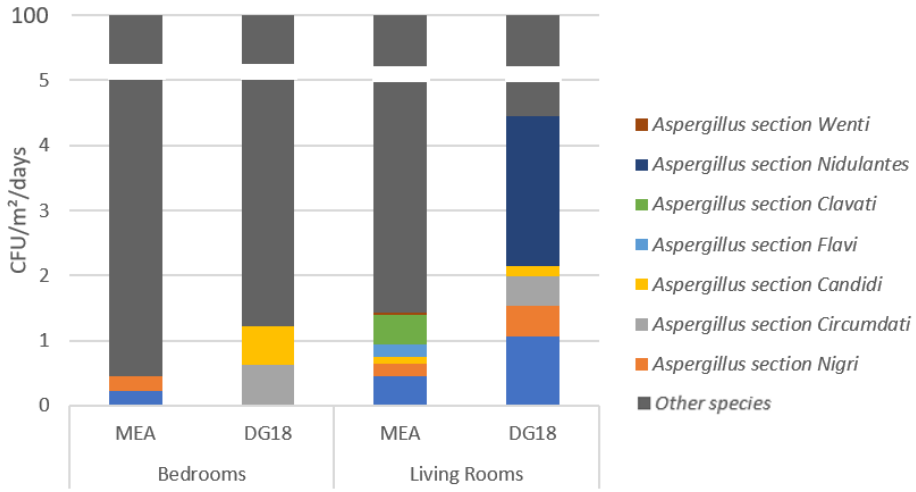

Figure 2. *Aspergillus* sections identified in the electrostatic dust collectors (EDC) samples from the bedrooms and the living rooms.

Four EDC were recovered from classrooms. The fungal contamination in the MEA samples ranged from the lower detection limit (S1) to 1.76×10^1 CFU/m^2/d (in the three remaining samples), and in DG18 from the lower detection limit (S1 and S3) to 1.02×10^1 CFU/m^2/d (in S4). Three different fungal species were identified in the MEA samples: *Penicillium* sp. (1.76×10^1 CFU/m^2/d; 64.21%), *Chrysonilia* sp. and *Cladosporium* sp. (4.91×10^1 CFU/m^2/d; 17.90%; Table 4). Four fungal species were found in DG18: *Chrysosporium* sp. (1.02×10^1 CFU/m^2/d; 40.79%), *Aspergillus* section *Nidulantes*, *Chrysonilia* sp. and *Cladosporium* sp. (1.02×10^1 CFU/m^2/d; 19.74%; Table 4).

Table 4. Fungal species found in each studied location.

Location	Genus/Species	MEA			DG18		
		N	CFU/m^2/d	%	N	CFU/m^2/d	%
Bedrooms	*Alternaria* sp.	2	1.05×10^1	0.47	1	1.05×10^1	0.34
	Aureobasidium sp.	1	5.24×10^0	0.23	1	5.24×10^0	0.17
	Chrysosporium sp.	3	2.56×10^1	1.14	2	9.49×10^0	0.31
	Cladosporium sp.	8	1.59×10^2	7.10	14	2.81×10^3	90.44
	Geotrichum sp.	1	4.14×10^0	0.18	1	5.24×10^0	0.17
	Penicillium sp.	17	2.00×10^3	89.43	12	2.07×10^2	6.67
	Aspergillus sp.	2	1.05×10^1	0.47	2	3.81×10^1	1.23
	Fusarium sp.	2	2.18×10^1	0.97	0	*	*
	Crysonilia sitophila	0	*	*	2	2.10×10^1	0.68
Living rooms	*Alternaria* sp.	1	5.24×10^0	0.04	0	*	*
	Aspergillus sp.	2	1.33×10^2	0.97	2	1.68×10^2	4.91
	Aureobasidium sp.	1	4.91×10^0	0.04	0	*	*
	Chrysonilia sp.	2	5.24×10^3	38.11	1	2.62×10^3	76.55
	Chrysosporium sp.	4	2.64×10^3	19.19	8	6.68×10^1	1.95
	Cladosporium sp.	13	2.22×10^2	1.61	12	1.7×10^2	4.96
	Fusarium sp.	0	*	*	1	2.46×10^1	0.72
	Geotrichum sp.	0	*	*	2	1.48×10^1	0.43
	Penicillium sp.	14	2.65×10^2	1.93	16	3.54×10^2	10.33
	Rhizopus sp.	2	5.24×10^3	38.11	0	*	*
	Ulocladium sp.	0	*	*	1	5.24×10^0	0.15
Classrooms	*Penicillium* sp.	2	1.76×10^1	64.21	0	*	*
	Chrysonilia sp.	1	4.91×10^0	17.90	1	4.91×10^0	19.74
	Cladosporium sp.	1	4.91×10^0	17.90	1	4.91×10^0	19.74
	Aspergillus sp.	0	*	*	1	4.91×10^0	19.74
	Chrysosporium sp.	0	*	*	1	1.02×10^1	40.79

N—Number of isolates observed. *—Lower the detection limit.

3.4. Azole-Resistance Screening

Seventeen different fungal species were detected on azole-resistance screening in 61 EDC samples, of which 11 were able to grow in at least one azole among the tested conditions. Noteworthy, *Aspergillus* sections *Candidi* and *Nigri* were able to grow on VOR in two distinct samples. Reduced susceptibility to multiazoles (i.e., fungal ability to grow in more than one azole) was observed in 14 EDC samples, for five different fungal species, including *Penicillium* sp. (VOR+POS in three samples), *Chrysosporium* sp. (VOR+POS in one sample, ITR+VOR in one sample) or *Cladosporium* sp. (ITR+VOR in two samples, VOR+POS in three samples, ITR+VOR+POS in one sample; Table 5). Similar to the results obtained with MEA in dwellings (Table 4), some of the most frequent fungal species were *C. sitophila* (83.05% SAB, 11.17% POS, 1.68 VOR), *Cladosporium* sp. (40.44% ITR, 38.33% VOR, 37.03% POS, 13.22% SAB) and *Penicillium* sp. (45.60% VOR, 27.21% ITR, 21.65% POS, 2.29% SAB; Table 5).

Table 5. Fungal species found on azole-screening media.

Species/Sections/Complexes	SAB			ITR			VOR			POS		
	N	CFU/m^2/d	%	N	CFU/m^2/d	%	N	CFU/m^2/d	%	N	CFU/m^2/d	%
Alternaria sp.	8	4.91×10^1	0.22	0	*	0	1	5.24×10^1	0.94	0	*	0
Aspergillus section *Aspergilli*	1	5.24×10^0	0.02	0	*	0	0	*	0	0	*	0
Aspergillus section *Candidi*	0	*	0	0	*	0	1	5.24×10^0	0.94	0	*	0
Aspergillus section *Fumigati*	2	9.38×10^0	0.04	0	*	0	0	*	0	0	*	0
Aspergillus section *Nigri*	14	6.91×10^1	0.31	0	*	0	2	9.38×10^0	1.68	0	*	0
Aspergillus section *Nidulantes*	21	9.02×10^1	0.41	0	*	0	0	*	0	0	*	0
Aureobasidium sp.	0	*	0	2	1.05×10^1	16.18	5	2.40×10^1	4.30	1	4.14×10^1	4.41
Crysonilia sitophila	3000	1.84×10^4	83.05	0	*	0	2	9.38×10^0	1.68	2	1.05×10^1	11.17
Chrysosporium sp.	24	5.24×10^1	0.24	1	5.24×10^0	8.09	3	1.54×10^1	2.76	3	2.41×10^1	25.73
Cladosporium sp.	561	2.92×10^3	13.22	6	2.62×10^1	40.44	55	2.14×10^2	38.33	7	3.47×10^1	37.03
Fusarium incarnatum-equiseti species complex	2	1.05×10^1	0.05	0	*	0	0	*	0	0	*	0
Fusarium oxysporum species complex	0	*	0	1	5.24×10^0	8.09	0	*	0	0	*	0
Geotrichum sp.	0	*	0	0	*	0	2	1.05×10^1	1.88	0	*	0
Litchemia sp.	2	1.05×10^1	0.05	0	*	0	0	*	0	0	*	0
Penicillium sp.	162	5.07×10^2	2.29	3	1.76×10^1	27.21	53	2.54×10^2	45.60	5	2.03×10^1	21.65
Syncephalastrum racemosum	1	4.91×10^0	0.02	0	*	0	0	*	0	0	*	0
Paecilomyces sp.	1	1.57×10^1	0.07	0	*	0	0	*	0	0	*	0
Ulocladium sp.	0	*	0	0	*	0	2	1.05×10^1	1.88	0	*	0

*—Lower the detection limit.

3.5. Molecular Assessment

None of the *Aspergillus* sections targeted (*Circumdati, Flavi, Fumigati* and *Nidulantes*) on the EDC were amplified by RT-PCR.

3.6. Correlation Analysis

Regarding the EDC weight, significant correlations, with moderate or low intensity, were detected with particles PM2.5 (rS = −0.395, $p = 0.015$), particles PM10 (rS = −0.486, $p = 0.002$), bacterial contamination on TSA (rS = −0.252, $p = 0.042$) and with *Aspergillus* prevalence on MEA (Rs = 0.555, $p = 0.049$). These results show that higher EDC weights are related to lower concentrations of particles (PM2.5 and PM10), lower bacterial contamination on TSA and higher *Aspergillus* prevalence on MEA (Table 6).

Considering the concentration of PM, only a significant positive correlation was detected, with a strong intensity, between the PM2.5 and PM10 (rS = 0.957, $p < 0.0001$), which means that higher concentrations of particles PM2.5 are related to higher concentrations of PM10 (Table 6).

Regarding fungal contamination on MEA, significant positive and moderate correlations were detected with (i) fungal contamination on DG18 (rS = 0.457, $p < 0.0001$), (ii) fungal presence on VOR (rS = 0.281, $p = 0.020$) and (iii) fungal detection on POS (rS = 0.280, $p = 0.021$), indicating that higher fungal contamination on MEA is related with higher fungal contamination on DG18 and with fungal counts on VOR and on POS (Table 6).

Regarding the fungal contamination on DG18, significant correlations of weak intensity and positive direction were detected with the fungal presence on VOR (rS = 0.262, $p = 0.031$) and on POS (rS = 0.276, $p = 0.023$), and with *Aspergillus* prevalence on DG18 (rS = 0.459, $p = 0.042$), revealing that higher fungal contamination on DG18 is related with the higher fungal counts on VOR and POS and *Aspergillus* prevalence on DG18 (Table 6).

Finally, a significant correlation, of weak intensity and in a positive direction, between fungal presence on VOR and POS (rS = 0.250, $p = 0.039$), which indicates that higher fungal counts on VOR are related with higher fungal counts on POS (Table 6).

Table 6. Study of the relationship between the weight of EDCs, particulate matter (PM2.5 and PM10), bacterial (TSA and VRBA) and fungal (MEA and DG18) contamination, fungi in azole-screening media (ITR, VOR and POS) and *Aspergillus* prevalence (MEA and DG18).

Variables		Particles (μg/m³)		Bacteria (CFU/m²/d)		Fungi (CFU/m²/d)		Fungi in Azole-Screening Media			*Aspergillus* Prevalence (CFU/m²/d)	
		PM2.5	**PM10**	**TSA**	**RB**	**MEA**	**DG18**	**ITR**	**VOR**	**POS**	**MEA**	**DG18**
	EDC Weight (g/m²/d)	−0.395 *	−0.486 **	−0.252 *	0.118	0.038	0.210	0.030	0.083	0.000	0.555 *	0.253
Particles (μg/m³)	PM2.5		0.957 **	0.240	0.123	−0.084	−0.013	0.164	−0.084	−0.014	−0.386	−0.392
	PM10			0.220	0.114	−0.066	0.023	0.153	−0.064	0.027	−0.426	−0.447
Bacteria (CFU/m²/d)	TSA				0.146	0.005	0.058	0.053	0.034	0.119	−0.379	−0.203
	RB					0.069	0.141	0.055	0.033	−0.009	0.019	−0.106
Fungi (CFU/m²/d)	MEA						0.457 **	−0.097	0.281 *	0.280 *	0.420	0.196
	DG18							−0.019	0.262 *	0.276 *	0.107	0.459 *
Fungi in azole-screening media	ITR								0.078	0.026	−0.324	0.029
	VOR									0.250 *	0.335	0.287
	POS										−0.032	−0.155
Aspergillus prevalence (CFU/m²/d)	MEA											0.355

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).

3.7. Comparison between Sampling Locations

From the comparison between sampling locations, only significant differences were detected in: (i) the EDC weight (χ_(K-W)^2 (2) = 6.74, $p = 0.046$), showing that in the classroom the EDC had less weight; (ii) the concentration of PM2.5 (U = 15.000, $p = 0.013$) and PM10 (U = 8.000, $p = 0.005$) particles presented the classroom as the sampling location with the highest concentrations (Table 7).

Table 7. Comparison of EDC weight, particulate matter concentration, fungal and bacterial contamination, fungal presence in azole-screening media and *Aspergillus* prevalence between sampling locations (Kruskal–Wallis test or Mann–Whitney test).

Variables		Ranks			Test Statistics		
		Location	N	Mean Rank	χ^2 Kruskal–Wallis or Mann–Whitney U	Df	*p*
	EDC Weight (g)	Classroom	4	11.75	6.174 *	2	0.046 ***
		Living room	33	37.68			
		Bedroom	31	34.05			
		Total	68				
Particles	PM2.5 (μg/m^3)	Classroom	4	31.75	15.000 **		0.013 ***
		Living room	33	17.45			
		Total	37				
	PM10 (μg/m^3)	Classroom	4	33.50	8.000 **		0.005 ***
		Living room	33	17.24			
		Total	37				
Bacterial contamination	TSA (CFU/m^2/d)	Classroom	4	25.88	0.774 *	2	0.679
		Living room	32	33.25			
		Bedroom	30	34.78			
		Total	66				
	RB (CFU/m$^{2/d}$)	Classroom	4	31.00	0.491 *	2	0.782
		Living room	33	34.08			
		Bedroom	31	33.22			
		Total	68				
Fungal contamination	MEA (CFU/m^2/d)	Classroom	4	25.38	3.228 *	2	0.199
		Living room	33	38.65			
		Bedroom	31	31.26			
		Total	68				
	DG18 (CFU/m^2/d)	Classroom	4	21.75	3.306 *	2	0.192
		Living room	33	38.18			
		Bedroom	31	32.23			
		Total	68				
Fungal presence in Azoles	ITR	Classroom	4	46.00	5.049 *	2	0.080
		Living room	33	35.12			
		Bedroom	31	32.35			
		Total	68				
	VOR	Classroom	4	26.50	5.273 *	2	0.072
		Living room	33	39.77			
		Bedroom	31	29.92			
		Total	68				
	POS	Classroom	4	38.00	5.920 *	2	0.052
		Living room	33	38.18			
		Bedroom	31	30.13			
		Total	68				
Aspergillus prevalence	MEA	Classroom	4	4.50	3.338 *	2	0.188
		Living room	5	8.60			
		Bedroom	4	7.50			
		Total	13				
	DG18	Classroom	4	5.88	4.530	2	0.104
		Living room	8	13.25			
		Bedroom	8	10.06			
		Total	20				

* Kruskal–Wallis test. ** Mann–Whitney test. *** Statistically significant differences at a 5% significance level.

4. Discussion

To contribute to the assessment of children's exposure to particles and bioburden, EDC was exposed for an extended period to collect dust in two home locations and at schools [27] (Figure 1). Although with some downsides, that rely mainly on the fact that bioaerosols are highly dynamic, thus difficult to collect in a representative way [43], settled dust is considered to be a long-term integrated sample of particles that have been airborne. As such this method is more reliable to sample bioaerosols [44]. Indeed, settled dust evidences a composite view of bioaerosols in the indoor environment that is being assessed [19,22,27]. Therefore, EDC permits consistent estimation of exposure, since a single EDC analysis is equal to the sum of several air-impaction measurements [45]. Furthermore, EDC allows for an exclusive identification of some fungal species and higher fungal diversity, when compared to air samples obtained by impaction or even with other passive methods [27]. The coupling of this sampling method with particle measurement allowed a more complete analysis of children's exposure in their daily lives.

Indoor particle exposure constitutes a significant percentage of overall exposure, as children spend the majority of the time indoors [3]. In our study, both fractions (PM2.5 and PM10) had higher concentrations in schools than in dwellings, which is related to children's activity during classes, resuspension of PM and inadequate ventilation [3]. Studies carried out in European cities showed similar concentrations in schools [46,47] and in dwellings [48–50].

The settled dust presented a different pattern, characterized by higher levels in the dwellings. This difference between the PM and the collected dust by the EDC behavior has already been found in other studies, which indicated that settled dust is less influenced by the short-term variability of the indoor activities and ventilation [51,52]. Particle deposition depends on the size of the particles, their sedimentation processes (diffusion in the case of very small particles or gravity in the case of larger particles) [52], the amount of furniture in the spaces [53], the type of ventilation and air turbulence [54].

The importance of using different culture media was validated and followed the same tendency as previously reported in studies performed in different indoor environments [16,19,26,27,41,55]. Regarding bacteria detection, no contamination by Gram-negative bacteria was detected in classrooms, which can partially be explained by less tolerance to the environmental conditions of these species [56]. In what refers to fungal contamination, it was possible to detect different species in both culture media applied (MEA and DG18), with higher diversity of *Aspergillus* sections on living rooms as observed on DG18. Indeed, the exclusive identification by DG18 of *Aspergillus* sections *Circumdati* and *Nidulantes*, both with toxigenic potential [57], on living rooms should be highlighted. Another concern regarding the toxigenic potential of the fungal species was the detection of *Aspergillus* section *Flavi* on the living rooms and of *Aspergillus* section *Fumigati* present in both sampling locations. Additionally, *Aspergillus* sections *Circumdati*, *Flavi*, *Fumigati* and *Nidulantes* identification should be emphasized since all the four *Aspergillus* sections are considered as indicators of harmful fungal contamination and, although our study has not detected these toxigenic species, their analysis should be performed in order to better contribute to the implementation of corrective measures [37]. Indeed, these species can produce mycotoxins that can become airborne on conidia or smaller fragments suggesting a potential inhalation or ingestion by indoor occupants [58]. Mycotoxins are known to have a wide array of adverse health effects or being carcinogenic to humans [59].

Culture-based methods were able to provide positive results within *Aspergillus* genera, whereas the *Aspergillus* sections were not detected with molecular tools. Despite these observations, molecular tools are generally a suitable solution to overcome the nonviable/nonculturable limits of the commonly used culture-based methods as they might also provide a more exhaustive diversity profile (e.g., high throughput sequencing), unlike culture methods that might reveal less abundant taxa in an environment. However, culture-independent molecular methods often only identify most of the organisms until taxonomic levels [60,61] and this level of identification is insufficient for exposure assessment. Furthermore, it has already been reported that the viability of microorganisms can affect their

inflammatory and/or cytotoxic potential and only viable microorganisms can cause infections, justifying the preference of culture-based methods [62–64].

As fungal resistance to available azole drugs is an emergent global health problem [65], especially with *Aspergillus fumigatus* [29,66,67], an exploratory screening of the frequency of fungal reduced susceptibility to azoles in dwellings and schools was conducted in this study. Some nonpathogenic species exhibited reduced susceptibility to one or more azoles, including *Aspergillus* sections *Nigri* and *Candidi*. In order to confirm the resistance phenotype of these species, further susceptibility tests and/or molecular detection of resistance mutations must be performed. So far, azole-resistant isolates with identical genetic profiles were found to be globally distributed and sourced from both clinical and environmental locations, thus, reinforcing azole resistance as an international public health concern [67]. In Portugal, some resistant *Aspergillus* sp. have already been found in the environment (data not published), but never in this context. If the resistance phenotype is confirmed, it will be a novelty as it has never been described in these environments.

The statistical analysis revealed some positive correlations that suggest (more evident on MEA than on DG18) that fungal reduced susceptibility to azole drugs, such as voriconazole and posaconazole, might be developed when higher fungal contamination is present in those environments. Moreover, it seems that reduced susceptibility to voriconazole and posaconazole are also related among these two azoles. This can be important (if azole resistance is confirmed) to understand the development of resistance, since voriconazole and posaconazole, though belonging to the same azole class, differ in their molecular structure: voriconazole is a short-tailed triazole (similar to triazole fungicides used in agriculture), whereas posaconazole (such as itraconazole) is a long-tailed triazole [68]. Understanding how fungal mutations affect drug affinity is necessary for the design of improved azoles that might overcome fungal resistance [69].

5. Conclusions

The indoor exposure to PM and bioburden at children's dwellings and schools was assessed by particle measurement and by using EDC. Results showed that the PM concentrations in classrooms highly exceeded the limit values established by the Portuguese legislation for indoor air quality. Although not correlated with PM, EDC provided information regarding the bioburden present indoors unveiling the presence of fungal species with toxigenic potential and nonpathogenic species exhibited reduced susceptibility to one or more azoles, including *Aspergillus* sections *Nigri* and *Candidi.*

Future studies at a larger scale, applying the same sampling approach—EDC coupled with particulate matter assessment—should be implemented to allow for a long-term integrated sample of organic dust.

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