Microbial occupational exposure in e-waste recycling: biological hazards, their sources, and potential toxic effects

Carla Viegas (carla.viegas@estesl.ipl.pt)
IPL: Instituto Politecnico de Lisboa https://orcid.org/0000-0002-1545-6479
Bianca Gomes
IPL: Instituto Politecnico de Lisboa
Marta Dias
IPL: Instituto Politecnico de Lisboa
Renata Cervantes
IPL: Instituto Politecnico de Lisboa
Pedro Pena
IPL: Instituto Politecnico de Lisboa
Sara Gonçalves
IPL: Instituto Politecnico de Lisboa
Marina Almeida-Silva
IPL: Instituto Politecnico de Lisboa
Elisabete Carolino
IPL: Instituto Politecnico de Lisboa
Magdalena Twaruzek
UKW: Uniwersytet Kazimierza Wielkiego w Bydgoszczy
Liliana Aranha Caetano
IPL: Instituto Politecnico de Lisboa
Susana Viegas
NOVA: Universidade Nova de Lisboa

Research Article

Keywords: Filtering respiratory protection devices, e-waste, microbial occupational exposure, cytotoxicity, Aspergillus section Fumigati

Posted Date: February 15th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3926383/v1

Abstract

E-waste management is a major environmental concern that also poses serious risks to occupational health. While e-waste workers are exposed to a variety of chemicals, little is known about occupational exposure to microbiological agents. This study aims to fill that gap. For that purpose, two e-waste facilities were assessed: one battery-recycling facility (BRF), and another facility (SRE) dedicated to e-waste sorting and storage before sending it for different processing. Filtering respiratory protection devices (FRPD, 24 samples) and settled dust (13 samples) were collected using standardized sampling approaches, and analyzed by culture-based, chemical and molecular methods. Workers also filled in a questionnaire regarding work activities and the use of FRPD. Microbial loads, fungal diversity, azole resistance, and prevalence of *Aspergillus* sections *Fumigati and Flavi* and mycotoxins (as surrogates of harmful fungal contamination) were determined. To estimate potential health effects related to exposure, the FRPD cytotoxicity was determined in human A549 lung and HepG2 hepatic cells. Microbial contaminants with pathogenicity and toxigenic potential were observed in FRPDs and settled dust. *Aspergillus* section *Fumigati* was widespread. The observed presence of *Aspergillus* section *Flavi* relates to the risk of exposure to aflatoxin B1 (a potent hepatocarcinogen). Four different mycotoxins were detected in 8% FRPD and 15% dust samples, including pathogenic fumonisin B1 and sterigmatocystin (below the limit of quantification). A low to moderate cytotoxic effect of sampled FRPD was observed, with human lung alveolar cells more sensitive than hepatic cells. No relevant azole resistance was observed, nor correlations between fungal reduced azole-susceptibility and fungal growth at 37ºC or cytotoxicity. In summary, it can be concluded that this approach using FRPD and settled dust presents a promising potential for screening occupational exposure to microbiological contaminants in e-waste management. More studies in this occupational context should be prioritized to identify critical biological hazards and to support the implementation of appropriate health risk mitigation strategies.

1 - Introduction

E-waste (electronic and electrical waste) is one of the fastest-growing waste flows in the European Union (EU) and is a major environmental concern, with less than 40% of e-waste being recycled (European Parliament 2023). The waste management sector, and more precisely the industries devoted to sorting waste are of utmost importance for the Sustainable Development Goals (SDGs) achievement, since they contribute directly and indirectly to many of the SDGs. The European Commission (EC) adopted the new circular economy action plan (CEAP) in March 2020, one of the main building blocks of the European Green Deal, Europe's new agenda for sustainable growth. The EU's transition to a circular economy will reduce pressure on natural resources and will create sustainable growth and jobs. It is also a prerequisite to achieve the EU's 2050 climate neutrality target and to halt biodiversity loss (EC 2023). To accomplish these goals, the number of units dedicated to waste sorting and the corresponding workforce is anticipated to increase in all the EU countries and partners. One of the EC's priorities is the reduction of electronic and electrical waste, although few EU countries recycle more than 50%. In 2016, Portugal reported a recycling rate of 43.5% for e-waste (European Parliament 2023).

It is well-known that e-waste workers are exposed to a variety of chemicals including metals, particulates, persistent organic compounds, and flame retardants (Gravel et al. 2023; ILO, 2021; Okeme and Arrandale 2019). However, little is known regarding microbial occupational exposure in e-waste recycling. Regarding exposure routes, workers involved in e-waste dismantling activities may be exposed to inorganic and organic contaminants through dermal contact, inhalation, and ingestion (via hand-mouth contact). Most studies assessing external chemical exposures during e-waste recycling have used air sampling and dust or soil (Akram et al. 2019).

Exposure to microbiological agents during regular waste sorting activities has been reported in different countries (Degois et al. 2017; Eriksen et al. 2022; Lavoie et al. 2006; Madsen et al. 2016; Park et al. 2011; Salamanga et al. 2022; Viegas C. et al. 2022a, 2022b; Wouters et al. 2005). In Portugal, assessments of occupational exposure in waste management determined the prevalence of microbiological hazards such as toxigenic fungi (Viegas C. et al. 2022a, 2022b, 2017; Viegas S. et al. 2015), azole-resistance (Gonçalves et al. 2021; Viegas C. et al. 2022a) and mycotoxins (Viegas C. et al. 2015; Viegas S. et al. 2014, 2018).

Since Portuguese legislation comprises the mandatory use of filtering respiratory protection devices (FRPDs) in waste sorting facilities, we have recently used FRPD sampling to assess microbial occupational exposures in this setting (Viegas C. et al. 2021a, 2021b, 2020). We found that workers in waste-sorting and belt-feeding activities are more exposed to microbial contaminants and that FRPD sampling led to similar results compared to air sampling, with higher microbial contamination and cytotoxicity levels observed in the interior layers of FRPD (compared to exhalation valves). Of utmost importance, FRPD-recovered azole-resistant *Aspergillus fumigatus* isolates were found to be harbouring the TR34/L98H mutation. Settled dust sampling has also been used to evaluate microbiological exposures in the waste sorting sector, offering significant outcomes for risk assessment and management (Salamanga et al. 2022; Scheepers et al. 2021; Viegas C. et al. 2022a). These findings provide valuable insights into the importance and methodology to follow when conducting microbial exposure assessments in the waste sorting industry, and in particular in e-waste sorting facilities.

The present study aims to address the lack of information on occupational exposure to microbial contamination in e-waste sorting facilities using novel sampling methods, such as FRPD, and their analysis by culture-based techniques and molecular methods. Additionally, it intends to determine possible deleterious health effects through FRPD cytotoxicity analysis.

2 - Materials and methods

2.1 – Study site

This study was developed between September and November of 2021 in 2 units dedicated to e-waste management: one battery recycling facility (BRF) and one dedicated to sorting and storing different e-waste before sending it to different processing facilities (SRE). Both units are located in the Lisbon district. Briefly, the BRF comprises different workstations aiming to separate the different battery components for dedicated recycling processes such as the plastics and the heavy metals components, namely: the ovens,

logistics, crucibles, and maintenance area. Additionally, a battery-breaking device and crushing and casting machines are also present on the site to support the recycling process. Regarding the SRE unit, manual sorting and storage of different electric and electronic devices it´s the main activity and comprises several storage areas to facilitate the distribution for other facilities to continue the specific recycling process.

2.2 - Samples collection

Thirteen samples of settled dust were collected (3 g from each sampling point) from the two units. In the BRF, settled dust samples were collected from nine distributed "indoor" and “outdoor” sites (furnaces, casting machine, crucibles, stacker, furnace stacker, plastic, beater, shovel, and maintenance area). In the SRE, four outdoor sites (storage 1,2,3, and storage shovel) were selected based on workers' occupancy. Regarding FRPD, 24 samples identified as FFP3 (based on EN 149 classification on filtering efficiency) were collected from the BRF and the interior layers were analyzed. A non-used FRPD was used as a control. All samples were individually packed in sterilized bags, identified, and transported in refrigerated conditions (0–4ºC).

2.3 – Questionnaire on FRPD use

The study enrolled twenty workers who completed a questionnaire about their usage of FRPDs. The questionnaire included questions about the type of FRPD, workstation, hours of use, consecutive hours of use, frequency of replacement, experience of use (in years), FRPD storage location during breaks, and whether they noticed any odours while using FRPD. Four workers in the ovens area, three workers in crucibles, and three workers in the logistics area used the FRPD. One worker used their FRPD only when managing the crushing machine, while another used it only when operating the battery-breaking machine. One worker did not provide information about the corresponding workstation. Additionally, the participants rated on a scale of 1–5, the amount of heat and sweat they felt while wearing the FRPD, and how well it adjusted their face. This study followed the guidelines of the Helsinki Declaration and Oviedo Convention and all data was stored and analyzed in accordance with the Portuguese General Data Protection Regulation (GDPR) law nº 58/2019. Workers were invited to participate voluntarily in the project, and before enrolment, all volunteers filled out a written informed consent.

2.4 – Microbial contamination

FRPDs were collected after a work shift and a 2 cm^2 section of the interior layer was extracted as previously reported (Viegas et al. 2020a). The extracted material was spread on selective culture media for fungi and bacteria and in azole-supplemented Sabouraud agar media (4.0 mg/L itraconazole (ITZ), 2.0 mg/L voriconazole (VCZ), and 0.5 mg/L posaconazole (PCZ)) as previously reported (Viegas et al. 2020b). Fungal species were identified by a mycology expert using macro and microscopic features (De Hoog, 2000).

The targeted *Aspergillus* sections *Fumigati* and *Flavi* were detected using the extracts obtained, following previously published procedures (Viegas C. et al. 2020a). Fungal DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, USA) as per the manufacturer's instructions. Fungal identification was achieved by Real-Time PCR (qPCR) using the CFX-Connect PCR System (Bio-

Rad). For each gene that was amplified, a non-template control and a positive control consisting of DNA obtained from reference strains (kindly provided by the Mycology laboratory of the National Institute of Health Dr. Ricardo Jorge) were used.

2.5 - Mycotoxins analysis

The method used to prepare samples and conduct chromatographic analysis of mycotoxins followed the procedure described in Viegas et al. (2020a). Samples of settled dust (0.10 g) and FRPD (0.05 g) were mixed with 3.0 ml and 2.5 ml respectively, of a mixture containing acetonitrile, water, and acetic acid (79/20/1; v/v/v) and shaken for 60 minutes. After centrifugation (5000 rpm for 5 minutes), the resulting samples were dried under nitrogen and dissolved in the mobile phase (A: B 7:3). Both mobile phase A (methanol/water/acetic acid, 10/89/1 (v/v/v)) and mobile phase B (methanol/water/acetic acid, 97/2/1 (v/v/v)) contained 5 mmol/L ammonium acetate. Separation was conducted on a Phenomenex Gemini C18 column (150 × 4.6 mm, 5 µm) (Torrance, CA, USA) with a flow rate of 1 ml/min and injection volume of 5 µL. The following gradient was used for mobile phase B: 0% up to 2', increasing to 50% from 2' to 5', increasing to 100% from 5' to 14', maintaining 100% up to 18', and returning to 0% by 22.5'.

Mycotoxins were detected using a Shimadzu HPLC Nexera (Tokyo, Japan) coupled with a Sciex mass spectrometry detector 5500 QTrap (Foster City, USA). The analysis was conducted in the scheduled multiple reaction monitoring (sMRM) mode for both negative and positive polarities within a single chromatographic run. The electrospray ionization (ESI)-source parameters were set as follows: curtain gas at 30 psi, collision gas at medium level, ion spray voltage set at -4500 V (negative polarity) and 5500 V (positive polarity), ion source temperature maintained at 550°C, ion source gas1 at 80 psi, and ion source gas2 at 80 psi.

2.6 - Cytotoxicity analyses

To assess the biological impact of FRPD's contaminants, we conducted an in vitro study using human lung epithelial cells (A549) and human liver carcinoma cells (HepG2). These cells were selected as they are relevant to the primary exposure routes protected by FRPD, namely, inhalation and ingestion. We exposed these cells to various concentrations of FRPD extracts and evaluated their viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which has been previously described (Hanelt et al., 1994, Viegas C. et al. 2023).

Cells were cultured using Eagle's Minimum Essential Medium (MEM) supplemented with 10,000 units of penicillin and 10 mg/mL streptomycin in 0.9% NaCl and fetal bovine serum (purchased from Sigma-Aldrich, USA). The cells were detached using 0.25% (w/v) 0.53 mM Trypsin EDTA, and then 100 µL of cell suspensions (3.0×10^5 cells/mL A549 cells; 2.0×10^5 cells/mL HepG2 cells) were serially diluted to 96-well plates. These plates were incubated with FRPD extracts for 48 hours at 5% CO2, 37°C in a humidified atmosphere. The cellular viability was determined by MTT assay at 510 nm (using the LEDETECT 96 ELISA and MikroWin2013SC software). The threshold toxicity was considered as the lowest concentration decreasing absorption to less than 50% of cellular metabolic activity (IC50).

2.7 - Statistical analysis

The Spearman's correlation coefficient was used to evaluate the relationship among contamination variables (prevalences of bacteria, fungi, azole resistance, *Aspergillus* sp., cytotoxicity) and information regarding the FRPD use. The Mann-Whitney U test was used to compare contamination variables among sampling matrices (FRPD and settled dust), and in settled dust among different units. The Simpson and Shannon indices [given by $\mathbf{ShannonIndex\ (H)} = -\sum_{\varvec{i}=1}^{\varvec{s}} \varvec{p}_{\varvec{i}} \mathbf{ln}\left(\varvec{p}_{\varvec{i}}\right)$ and $\mathbf{SimpsonIndex\ (D)} = \frac{1}{\sum_{\varvec{i}=1}^{\varvec{s}} \varvec{p}_{\varvec{i}}^{2}}$, where p_i is the proportion (n_i/n) of individuals of one particular species found (n_i) divided by the total number of individuals found (n)], were used to assess fungal species diversity. All analyses were conducted in SPSS statistical software for Windows, version 27.0. The results were considered significant at the 5% significance level.

3 – Results

3.1 – Patterns of FRPD use

The questionnaire showed that 35% of workers (n = 7) use the same FRPD for all workstations and tasks without changing it. Out of the 20 participants surveyed, 18 of them (90%) reported that they used the FRPD for seven hours, or five consecutive hours. Additionally, 8 participants (40%) mentioned they had used the FRPD for three years. One participant's experience varied depending on their seniority in the plant, with some indicating 30, 32, 33, or 36 years of experience. As for the discomfort caused by the FRPD, 10 participants (50%) reported feeling no more discomfort than usual, while two (10%) felt more discomfort than usual. A small percentage of participants (15%; 3 out of 20) reported that the FRPD caused more sweating than usual. However, all participants agreed that the FRPD was easily adjustable to fit their face.

3.2 – Microbial exposure

Total bacterial concentrations (cultivated on TSA agar) were higher in workers' FRPD from the ovens area (6.20X10^4 CFU.m^{-2}), and Gram-negative bacterial concentrations (cultivated on VRBA agar) were higher in workers' FRPD from all areas (1.05X10^4 CFU. m^{-2}). Bacterial prevalence was higher in FRPD than in settled dust (Fig. 1). Based on settled dust results, the highest total bacteria counts were found near the battery crushing machine from the RBF (TSA: 1.77 x 10^2 CFU.g^{-1}), while the Gram-negative bacteria highest counts were obtained in the SRE (VRBA: 2.56 x 10^2 CFU.g^{-1}).

The highest fungal concentrations were found in DG18 (after 5 days incubation at 27ºC), being 1.16×10^5 CFU.m^{-2} FRPD (from the battery-breaking machine area) and 2.77×10^2 CFU.g^{-1} settled dust. Fungal concentrations on MEA were 1 log lower in both FRPD and settled dust. DG18 plates incubated at 37ºC (to evaluate exposure to potentially pathogenic fungi) reached 4.00×10^3 CFU.m^{-2} FRPD and 2.60×10^1 CFU.g^{-1} settled dust (Fig. 2). The highest fungal counts, among settled dust samples from both units, were obtained in SRE (MEA: 3.9 x 10^1 CFU.g^{-1}; DG18: 2.77 x 10^2 CFU.g^{-1}).

Overall, FRPD and settled dust presented statistically significant differences regarding total bacterial (U = 0.000, p < 0.0001), fungi on MEA (U = 36.000, p < 0.0001), DG18 (U = 30,000, p < 0.0001), or SDA (U = 105,000, p = 0.038), with FRPD presenting the heaviest contamination (Table S4 – Supplementary material).

Regarding fungal variability, *Penicillium* sp. was dominant in settled dust (42.15% samples) and FRPD (74.27% samples) on DG18. *Cladosporium* sp. was dominant in FRPD (47.52% samples) on MEA. *Aspergillus* sp. was also present in settled dust (MEA: 8.77%) and in FRPD (DG18: 8.05%) (Table 1; Figure S1 – Supplementary material).

Table 1
– Fungal distribution on MEA and DG18 from FRPD and settled dust samples

	MEA			**DG18**		
Samples	Species	CFU.m^{-2}/g^{-1}	%	Species	CFU.m^{-2}/g^{-1}	%
FRPD	*Cladosporium* sp.	6.70E + 04	47.52	*Penicillium* sp.	1.66E + 05	74.27
	Penicillium sp.	6.40E + 04	45.39	*Cladosporium* sp.	3.85E + 04	17.23
	Aspergillus sp.	4.00E + 03	2.84	*Aspergillus* sp.	1.80E + 04	8.05
	Others species	6.00E + 03	4.26	Others species	1.00E + 03	0.45
	TOTAL	141E + 05	7.09	TOTAL	2.24E + 05	100.00
Settled dust	*Penicillium* sp.	2.20E + 01	19.30	*Penicillium* sp.	3.73E + 02	42.15
	Cladosporium sp.	2.00E + 01	17.54	*Cladosporium* sp.	2.35E + 02	26.55
	Aspergillus sp.	1.00E + 01	8.77	*Aspergillus* sp.	5.00E + 00	0.56
	Others species	6.20E + 01	54.39	Others species	2.72E + 02	30.73
	TOTAL	1.14E + 02	100.00	TOTAL	8.85E + 02	100.00

Based on FRPD results, the greatest fungal biodiversity on MEA was found in the ovens workstation (Shannon index (H) = 1.11, Simpson Index (D) = 9.39), followed by crucibles (Shannon index (H) = 1.61, Simpson Index (D) = 5.00), whereas on DG18 it was in all the workstations (All) (Shannon index (H) = 1.24, Simpson Index (D) = 3.05) (Table X). Regarding settled dust, the greatest fungal biodiversity was observed in the sorting plastic area both for MEA (Shannon index (H) = 0.92, Simpson Index (D) = 2.23) and DG18 (Shannon index (H) = 0.95, Simpson Index (D) = 14.42) (Tables S1 and S2– Supplementary material).

An overview of the distribution of *Aspergillus* sections can be found in Fig. 3. *Aspergillus* diversity was higher in settled dust inoculated on DG18 at 37ºC, with 5 sections identified, namely, *Nigri* (51.43%),

Fumigati (44.29%), *Nidulantes* (1.43%), *Flavi* (1.43%) and *Aspergilli* (1.43%). At 27º C, three *Aspergillus* sections were identified in DG18 (40% *Circumdati;* 40% *Nigri;* 20% *Flavi*), and two sections in MEA (80% *Nigri;* 20% *Nidulantes*). Regarding FRPD, four sections were found in MEA (50% *Nidulantes; 25% Nigri*; 12.50% *Fumigati;* 12.50% *Circumdati*), and three sections in DG18 (61.11% *Nidulantes*; 30.56% *Circumdati*; 8.33% *Aspergilli)*. In FRPD inoculated on DG18 and incubated at 37ºC, *Aspergillus* section *Fumigati* was dominant (52.00%), followed by *Nigri* (16.00%) and *Nidulantes* (12.00%) (Fig. 3).

3.3 – Screening of antifungal resistance

No fungal growth on azole-supplemented media was observed for FRPD samples. Regarding settled dust, in one sample only, *Mucor* sp. ($CFU.m^{-2}/g^{-1}$) with reduced susceptibility to 2.0 mg/L voriconazole and 0.5 mg/L posaconazole was identified in the storage unit for recycling electric and electronic devices (SRE).

3.4 – Aspergillus section *Fumigati* and section *Flavi* exposures

From the two targeted *Aspergillus* sections, *Aspergillus* section *Fumigati* was detected in 9 out of 24 FRPD samples (37.5%) from the RBF. Concerning *Aspergillus* section *Flavi* it was detected in 9 out of 24 FRPD (37.5%) and in 3 out of 9 settled dust samples from the BRF, whereas from SRE it was detected in 2 out of 4 settled dust samples (50%). In 5 FRPD both sections were detected. In 15 out of 33 samples (45.5%), section detection was obtained, while the identification was not possible to achieve by culture-based methods (Supplementary material - Table S3).

3.5 – Mycotoxin exposure

Two FRPD (used more than 5 consecutive hours and without daily replacement) were contaminated with mycotoxins: one FRPD with Fumonisin B2 below the LOQ (9 ng/g); another FRPD with fumonisin B1 (< LOQ, 12 ng/g), fumonisin B2 (9.5 ng/g), and sterigmatocystin (< LOQ, 4 ng/g). Also, two settled dust samples (from SRE) were contaminated, including one sample with mycophenolic acid (26 ng/g) and sterigmatocystin (< 6 ng/g – LOQ).

3.6 - Cytotoxicity

Low to moderate cytotoxicity was observed in 66% of the FRPD on A549 lung epithelial cells, and in 17% of the FRPD on HepG2 cells (Table 2).

Table 2
– Cytotoxicity distribution on A549 and HepG2 cells from FRPD samples collected

	A549				HepG2			
Dilution step	none	1	2	> 2	none	1	2	> 2
Cytotoxicity level	Not observed	Low	moderate	high	Not observed	low	moderate	high
N FRPD	8	14	2	0	20	4	0	0

3.7 – Statistical analysis

The following significantly positive correlations were observed among microbial exposure variables: i) total bacteria and fungi on MEA, DG18, and DG18 at 37°C; ii) fungi on MEA and fungi on DG18, DG18 at 37°C, SDA, and *Aspergillus* sp. on DG18; iii) fungi on DG18 and fungi on DG18 at 37°C, SDA and *Aspergillus* sp. on DG18; iv) fungi on SDA ($p < 0.01$) and *Aspergillus* sp. on DG18 and DG18 at 37°C. Fungal concentration on SDA was also negatively correlated ($p < 0.01$) with cytotoxicity in HepG2 cells. No correlations were detected regarding the questionnaires' answers (Table 3).

The statistically significant differences observed among the two units (BRF and SRE), by assessing the settled dust, are depicted in Fig. 4. In summary, total bacteria (VRBA, p = 0.003), fungi on DG18 (p = 0.003), fungi on DG18 at 37°C (p = 0.004), and *Aspergillus* sp. on DG18 at 37ºC (p = 0.004) were all higher at SRE.

Table 3 - Study of the relationship between bacterial and fungal contamination, azole resistance, *Aspergillus* sp., cytotoxicity, and questionnaire results. Spearman correlation coefficient results.

		Bacteria	Fungi			Azole resistance			*Aspergillus* sp.			Cytotoxicity		Questionnaire				
		VRBA	MEA	DG18	DG18 (37ºC)	SDA	ITZ	VCZ	PSZ	MEA	DG18	DG18 (37ºC)	A549	HepG2	Hours of use	Consecutive hours of use	Years of utilization	Odor during utilization	Heat_ discomfort scale	Face moisture scale
Bacteria	TSA	0.110	0.408*	0.471**	-0.124	0.166		0.001	0.016	-0.005	0.063	-0.183	-0.123	0.659	0.354	0.222	0.094	0.225	-0.118	0.400
	VRBA		0.328*	0.472**	0.473**	0.165		0.373*	0.278	0.363*	0.389*	0.298	0.071	0.375	0.164	0.238	-0.553*	-0.164	0.077	-0.175
Fungi	MEA			0.866**	0.378*	0.434**		0.237	0.204	0.251	0.536**	0.260	-0.126	-0.221	-0.146	-0.211	-0.260	-0.227	0.135	-0.261
	DG18				0.397*	0.425**		0.210	0.189	0.252	0.535**	0.268	-0.188	0.165	-0.097	-0.252	-0.381	-0.226	0.277	-0.194
	DG18 (37ºC)					0.475**		0.256	0.179	0.476*	0.572**	0.886**	0.162	-0.375	0.164	0.238	-0.239	-0.164	0.376	-0.006
Azole resistance	SDA							0.180	0.340*	0.052	0.443**	0.537**	-0.216	-0.936**	0.165	0.239	-0.040	-0.165	0.234	0.214
	ITZ																			
	VCZ								0.717**	0.522**	0.391*	0.063	0.392	0.378	0.066	0.095	-0.248	-0.066	-0.337	-0.367
	PSZ									0.391*	0.272	0.211	-0.098		0.045	0.066	-0.171	-0.045	-0.232	-0.254
Aspergillus sp.	MEA										0.455**	0.232	0.189	0.378	0.112	0.162	-0.236	-0.112	0.142	-0.085
	DG18											0.412*	0.303	-0.250	0.165	-0.154	-0.102	-0.165	0.097	-0.211
	DG18 (37ºC)												-0.180	-0.571	0.138	0.200	-0.138	-0.138	0.274	0.027
Cytotoxicity	A549													0.408			-0.209	-0.071	-0.254	-0.328
	HepG2																-0.644		-0.417	-0.605

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

Note: Correlations with values close to zero show softer colours. As the correlation values are stronger the color intensifies. Pink for positive correlations and blue for negative correlations.

4 - Discussion

Besides filling the knowledge gap regarding this specific setting (e-waste), in what concerns microbiological contamination, this study corroborates the trend found previously, regarding the utmost importance of using passive sampling methods (settled dust) and/or material collection, such as FRPD (Viegas et al. 2021a, 2020a, 2020b; Whitby et al. 2022). Previous studies recommend the use of several sampling approaches allowing overcoming the limitations of each method and contributing to a more representative scenario of microbiologic exposure (Viegas et al. 2022a; Whitby et al. 2022). In previous studies protection devices worn by workers were used to predict exposure to microbial agents and metabolites, such as mycotoxins (Viegas et al. 2021a, 2020a, b).

The use of 37ºC as incubation temperature allowed the identification of the fungal species/sections that can grow at that temperature unveiling the pathogenic potential (Andersson et al. 2023). Additionally, it was also possible to overcome the limitation in recovering fungi with clinical relevance, such as *Aspergillus* section *Fumigati* isolates that, in this kind of setting, due to fungi with higher growth rates, such as Mucorales order, *Trichoderma* sp., and *Chrysonilia sitophila* are underestimated. This is the most important drawback of using only culture based-methods to assess exposure to microbiologic agents in highly contaminated occupational environments (Cox et al. 2020; Salambanga et al. 2022; Viegas et al. 2023, 2020a,), and by using selective temperatures (37 ºC) and/or specific culture media we can better characterize the risk in the setting being assessed.

In this study, lower counts of bacteria were obtained in settled dust samples than in FRPD extracts. Not only settled dust and FRPD are different sampling/collection methods they also have quite different lab protocols to achieve the relative extracts that can lead to different microbial counts and microbial diversity, as was observed in this study. Furthermore, higher microbial counts, and more specifically bacterial counts (TSA and VRBA), and *Aspergillus* section *Fumigati* isolates, found in FRPD can be due to the interior layer favourable conditions (Cox 1989; Viegas et al. 2021a, 2021c, 2020a). In fact, during FRPD use, water vapour and sweat are released boosting material humidity, and the presence of nutritional substances that were blocked during air filtering can provide optimal conditions for microorganism growth (Donlan 2002; Jankowska et al. 2000; Majchrzycka et al. 2016; Viegas et al. 2020a). Additionally, the settled dust from this specific industry is expected to have a high chemical burden (Okeme and Arrandale 2019) and this will impact negatively the microorganism's growth (Roane et al. 2009; Gomes et al. 2020) justifying the lower counts in this matrix when comparing with FRPD and also with other studies performed in Portugal in similar settings (Viegas et al. 2022a).

In our study, the different culture media enabled a deeper characterization of the microbial counts in both units. Of note, it was possible to observe in settled dust samples that Gram-negative bacteria presented higher counts in SRE, while total bacteria counts were higher in BRF. This trend may have some seasonal influence, since all the workstations from SRE were outdoors, with expected lower temperatures and higher humidity at that time, favouring Gram-negative bacteria proliferation (Won and Ross 1966; Cox 1998). Bacteria are also more sensitive to the assessment conditions (aerosolization, collection, and culture) impacting more in their viability (Cox 1998) and, consequently, leading to quite different results among settings.

In what concerns the mycobiota, SRE presented the highest fungal counts on the settled dust samples and this can be due to the outdoor environment where the workstations are located. DG18 unveils higher fungal counts in both matrices, being also observed in different other settings (Viegas et al. 2023). DG18 has limited water content, allowing a more diversified number of species to thrive, by restricting the ones with fast growth rates (Mensah-Attipoe and Taubel 2017). Fungal species in general, and *Aspergillus* species/sections in particular, are sensitive to the occupational environment, sampling methods, and culture media (Chao et al. 2002; Viegas et al. 2021b), which restrains results' comparison among different studies. It has been previously reported that to assess occupational exposure to fungi in the waste sorting industry, FRPD should be used as collection material and DG18 as the culture media whenever *Aspergillus* section *Fumigati* is targeted (Viegas et al. 2021c). In the present study assessing fungal contamination in e-waste, *Aspergillus*' highest counts were also found in FRPD using DG18, and the *Fumigati* section was also the most prevalent in FRPD at 37ºC in the DG18. This trend was confirmed by previous studies that analyzed FRPD used by sorting waste workers (Viegas et al. 2020a, b).

The workstations presenting higher contamination in this study were the ovens for total bacteria, maybe due to the increased temperatures expected in this workstation. The FRPD used in all areas were the ones presenting higher counts of gram-negative bacteria, while for fungi the higher counts were found in the FRPD used in the battery-breaking machine. Considering fungal diversity, FRPD used by workers in ovens and the ones used by workers in all the workstations (workers without a fixed workplace) presented higher fungal diversity. Previously, it was reported that FRPD interior layers from workers with more waste contact, such as sorting the waste or feeding the belts with waste, showed an increased exposure to microbial contamination (Viegas et al. 2020a). Thus, the FRPD extracts' analyses can mimic the expected occupational exposure to microbiologic agents and help prioritize interventions for risk management (Viegas C. et al. 2021c, 2020a, b).

The settled dust samples didn´t present the same trend with the logistics workstation presenting the highest counts, although much lower than other similar settings in Portugal (Viegas et al. 2022a), maybe due to lower chemical pollution found in this workplace (Okeme and Arrandale 2019); and the plastic area the increased fungal diversity. Thus, the use of different sampling methods was useful in identifying the workstations where workers have an increased exposure to microbial contamination.

The importance of addressing fungal resistance is related to the emerging number of invasive fungal diseases worldwide. As only a few classes of antifungal drugs are available, the emergence of resistance to single and multiple drugs complicates fungal infections’ management and patients’ prognostics (Fisher et al. 2022; Global Action for Fungal Infections 2022). The recent WHO fungal priority pathogen list identifies 19 fungi with the greatest impact on public health and emerging antifungal resistance risk (WHO 2022). Although no relevant azole resistance was found in this study, *Mucor* sp. from one settled dust sample presented reduced susceptibility to voriconazole and posaconazole at tested conditions. WHO ranks Mucorales as a high priority based on criteria such as average case fatality, number of new cases annually, complications and sequelae, and antifungal resistance, among other criteria. Mucormycetes are characterized by high-level resistance to most currently available antifungal drugs, particularly to short-tailed azoles such as voriconazole, due to an evolutionarily conserved amino acid substitution of the

lanosterol 14α-demethylase (Caramalho et al. 2017; Dannaoui 2017). Standardized antifungal susceptibility testing methods and molecular detection at the species level will be further used to better characterize the antifungal susceptibility of the recovered *Mucor* isolate.

Applying culture-based methods only identifies culturable microorganisms. Furthermore, the majority of microorganisms present in a specific setting, cannot be cultured by routine laboratory assays (Cox et al. 2020; DeLong and Pace 2001), and several microorganisms, such as some pathogenic bacteria, can be in a viable but non-culturable state, but can still cause health outcomes (Li et al. 2014). Additionally, the non-viable, non-cultivable microorganisms can still be harmful to exposed workers, since several health outcomes are related to their toxigenic potential and not linked to viability or infectious potential (Cox et al. 2020). More recently, culture-based methods have gained more attractiveness due to culturomics, which combines culture-based methods with more refined tools (such as sequencing or matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)) for the identification of unidentified isolates (Cox et al. 2020). Microorganisms' viability is a crucial parameter since only viable microorganisms can lead to infection (Cox et al. 2020), justifying the culture based-methods also as the "gold standard" for occupational exposure assessments, besides clinical purposes (Kung et al. 2018). In this study, several culture media and incubation temperatures were applied together with qPCR detection of two different *Aspergillus* sections (*Fumigati* and *Flavi*). In light of previous studies (Viegas C. et al., 2020a, 2020b, 2020c, 2018; Viegas S. et al. 2015), the *Fumigati* section was suggested as a surrogate of harmful fungal contamination in waste management industry (Viegas C. et al. 2022a) and in the WHO fungal priority list to guide research, development and public health action, listed in the critical priority group due to clinical relevance, antifungal resistance, and health outcomes (WHO, 2022). Thus, it is recommended that occupational exposure to this specific section should be reduced to a minimum (Marchand et al. 2021, 2017; Viegas C. et al. 2022a). In this study, we can conclude that this section is widespread by analyzing results from culture-dependent and by qPCR, claiming attention for his dominance at 37ºC, in both matrices.

Concerning the *Flavi* section, the concern relies mainly on their ability to produce aflatoxins, although the strains belonging to this section can also cause infections. IARC Monographs reported aflatoxins carcinogenic to humans (Group 1) (IARC, 2002). In this study, section *Flavi* was detected in both matrices, although not identified by culture-based methods in FRPD. Formerly, in a study intending to assess exposure to aflatoxin B1 in workers sorting waste, through biomonitoring, all the workers participating in the study presented detectable levels of the targeted mycotoxin in blood samples (Viegas S. et al. 2014). Thus, in light of the results obtained from this section in the assessed units, the exposure to AFB1 should be considered. For all the above reasons, both culture-based and culture-independent methods should be applied to obtain a more accurate image of microbial contamination in occupational settings (Cox et al. 2020; Viegas C. et al. 2023, 2021a, 2021c, 2020a).

Fungal counts in Sabouraud presented a $p < 0.05$ level negative correlation with IC50 values in HepG2. This was an unexpected result as fungal secondary metabolites are greatly related to cytotoxicity, including in HepG2 cells (Sobral et al. 2018; Zhou et al. 2022), and fungi that were abundant in our samples, such as *Penicillium* sp. or *Aspergillus* sp. (Vu et al. 2022). However, most other studies assess the cytotoxicity of

fungal isolates or mycotoxins alone, whereas in our study we assess the effect of mixtures consisting of microbes, particles, and chemicals. As such, it is not possible to determine which sample component might present this apparent cytoprotective effect.

In the 24 FRPD samples considered in this study, two presented contamination with one of the samples presenting multiple contamination with 3 mycotoxins found in the same sample. To our knowledge, this is the first study developed where FRPD showed contamination by mycotoxins. Considering that FRPD is positioned in the workers' nose and mouth we can conclude that workers are exposed by inhalation and/or ingestion to mycotoxins, namely fumonisins B1 (FB1) and B2 (FB2) and sterigmatocystin (STE), the ones detected in our study. Human consumption of foods containing FB1 may cause esophageal cancer, and liver and kidney disease (Chen et al. 2021). FB1 is also known to have the potential to cause heart failure in humans due to damage in myocardial contractility and massive blood influx, known as Idiopathic Congestive Cardiopathy (Stoev 2015).

Although no women are working in the production area in the company engaged in this study, fumonisins are also known to be responsible for causing a high risk of bearing a child with a birth defect of the brain or spinal cord in women when exposure to high levels occurs by food consumption during early pregnancy (Missmer et al. 2006; Waes et al. 2005).

Sterigmatocystin is a known biogenic precursor of aflatoxin B1, sharing with it several structural and biological similarities (Zingales et al. 2020). This mycotoxin is hepatotoxic and nephrotoxic in animals and it has been classified as a possible human carcinogen (group 2B) by IARC (IARC, 1987).

Results regarding mycotoxins were different from a study developed in one waste-sorting industry from Portugal, where it was not found contamination by mycotoxins in the FRPD, even with a high fungal burden observed (Viegas C. et al. 2020a). The FRPD used by workers in this company (BRF) was a half mask used consecutive 5 hours and without daily replacement. This is a different usage regime than the study developed previously by Viegas C. et al. (2020a), where the FRPD was replaced daily, and this might explain the contamination found in this study.

The mycotoxin contamination found in the settled dust samples (two samples showed contamination; 15.3%) followed the same trend as a previous study developed in a waste collection centre also located in Portugal (Viegas C. et al., 2022a), where the settled dust samples collected in the waste collection trucks assessed also presented contamination by mycotoxins, however with higher number of samples being contaminated (16 samples; 53.1%). Of note, the chemical contamination can explain the fact that only settled dust samples from SRE presented mycotoxin contamination since lower chemical contamination is expected in SRE than in BRF, allowing the fungal species to thrive (Gomes et al. 2020; Roane et al. 2009;). Concerning the detected mycotoxins, the profile reported was the same observed by Viegas C. and colleagues (2022a), being the mycophenolic acid and sterigmatocystin the mycotoxins detected and also with mycophenolic acid presenting the higher concentrations.

5 - Conclusions

The study developed allowed us to recognize some specific features in this occupational setting concerning contamination/exposure to microbiologic agents:

- Microbial contamination with pathogenic and toxigenic potential was found in both analysed matrices (FRPD and settled dust);
- *Aspergillus* section *Fumigati* (surrogate of harmful fungal contamination in the waste management industry) was found widespread in the analyzed units;
- *Aspergillus* section *Flavi* results, together with previous studies concerning AFB1 exposure, suggest potential workers exposure to AFB1;
- The common and already reported chemical contamination in this setting might explain the reduced contamination (microorganisms and mycotoxins) of settled dust samples;
- The FRPD should be used in sampling protocols to assess occupational exposure to microbiologic agents since they suffer less influence from the chemical burden common in this setting;
- Exposure to mycotoxins might occur through FRPD probably because using FRPD does not imply a daily replacement.

Further studies focusing on occupational exposure to microbiologic agents in e-waste management should be prioritized in light of the results obtained in this assessment.

Declarations

Statements & Declarations

Funding

This research was funded by the Polish Minister of Education and Science, under the program "Regional Initiative of Excellence" in 2019 - 2022 (Grant No. 008/RID/2018/19) and by Instituto Politécnico de Lisboa, Lisbon, Portugal for funding the Projects IPL/2023/FoodAIIEU_ESTeSL; IPL/2023/ASPRisk_ESTeSL; IPL/2023/ARAFSawmil_ESTeSL.

H&TRC authors gratefully acknowledge the FCT/MCTES national support through the UIDB/05608/2020 and UIDP/05608/2020 and to the PhD Grants UI/BD/151431/2021 and UI/BD/153746/2022 and CE3C unit UIDB/00329/2020. We thank the companies and workers for participating in the study.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study's conception and design. Material preparation, data collection and analysis were performed by Carla Viegas, Bianca Gomes, Marta Dias, Renata Cervantes, Pedro Pena, Sara Gonçalves, Marina Almeida-Silva, Elisabete Carolino, Magdalena Twarużek, Liliana Aranha Caetano and

Susana Viegas. The first draft of the manuscript was written by Carla Viegas and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical Approval

This study complied with the Helsinki Declaration and Oviedo Convention and all data were stored and analyzed by the Portuguese General Data Protection Regulation (GDPR) law nº 58/2019.

Consent to Participate

Workers were invited to voluntarily participate in the project. Before the enrolment, all volunteers filled out a written informed consent.

Consent to Publish

Companies' owners were invited to voluntarily participate in the project and filled out a written informed consent regarding permission for results publication.

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Figures

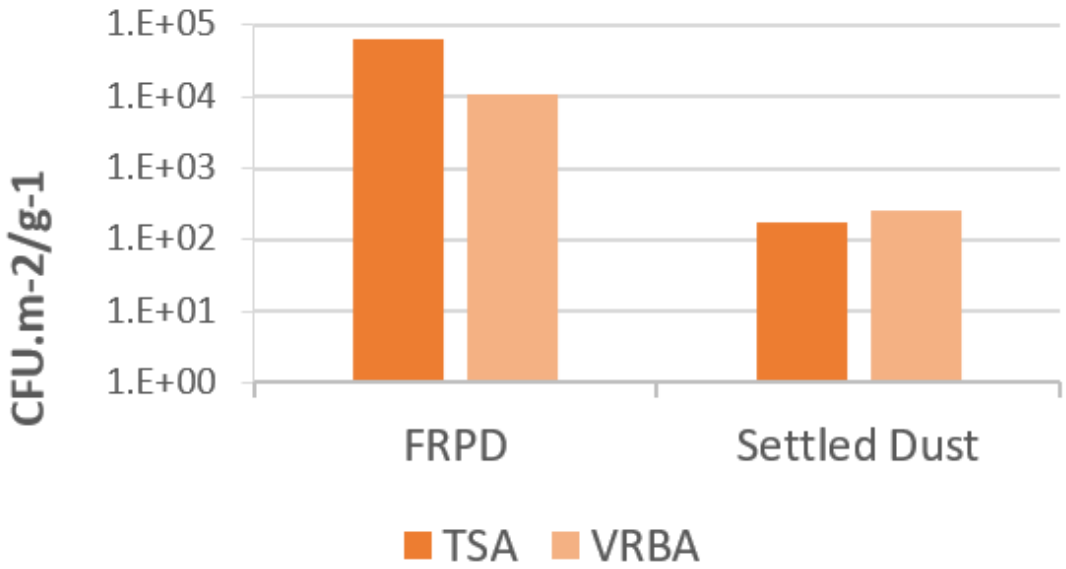

Figure 1

Highest bacteria concentration from FRPD and settled dust samples inoculated in TSA and VRBA ($CFU.m^{-2}/g^{-1}$).

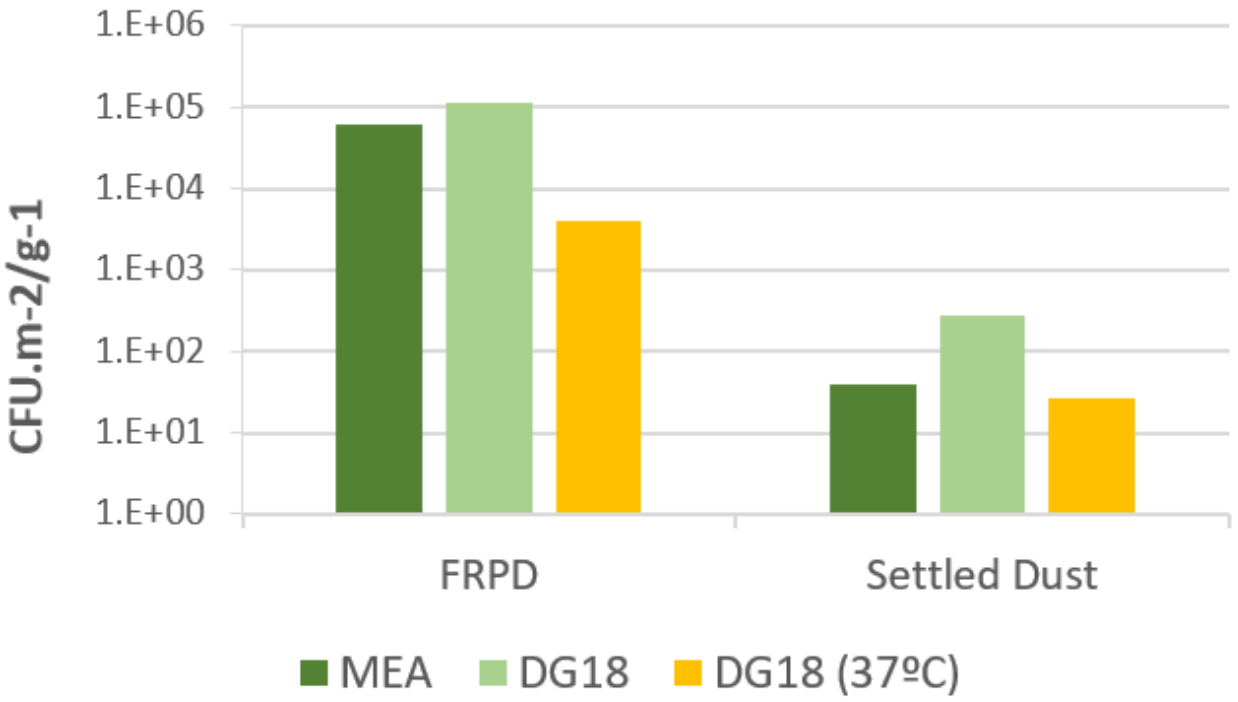

Figure 2

Highest fungal concentration from FRPD and settled dust samples inoculated in MEA and DG18 culture media ($CFU.m^{-2}/g^{-1}$)

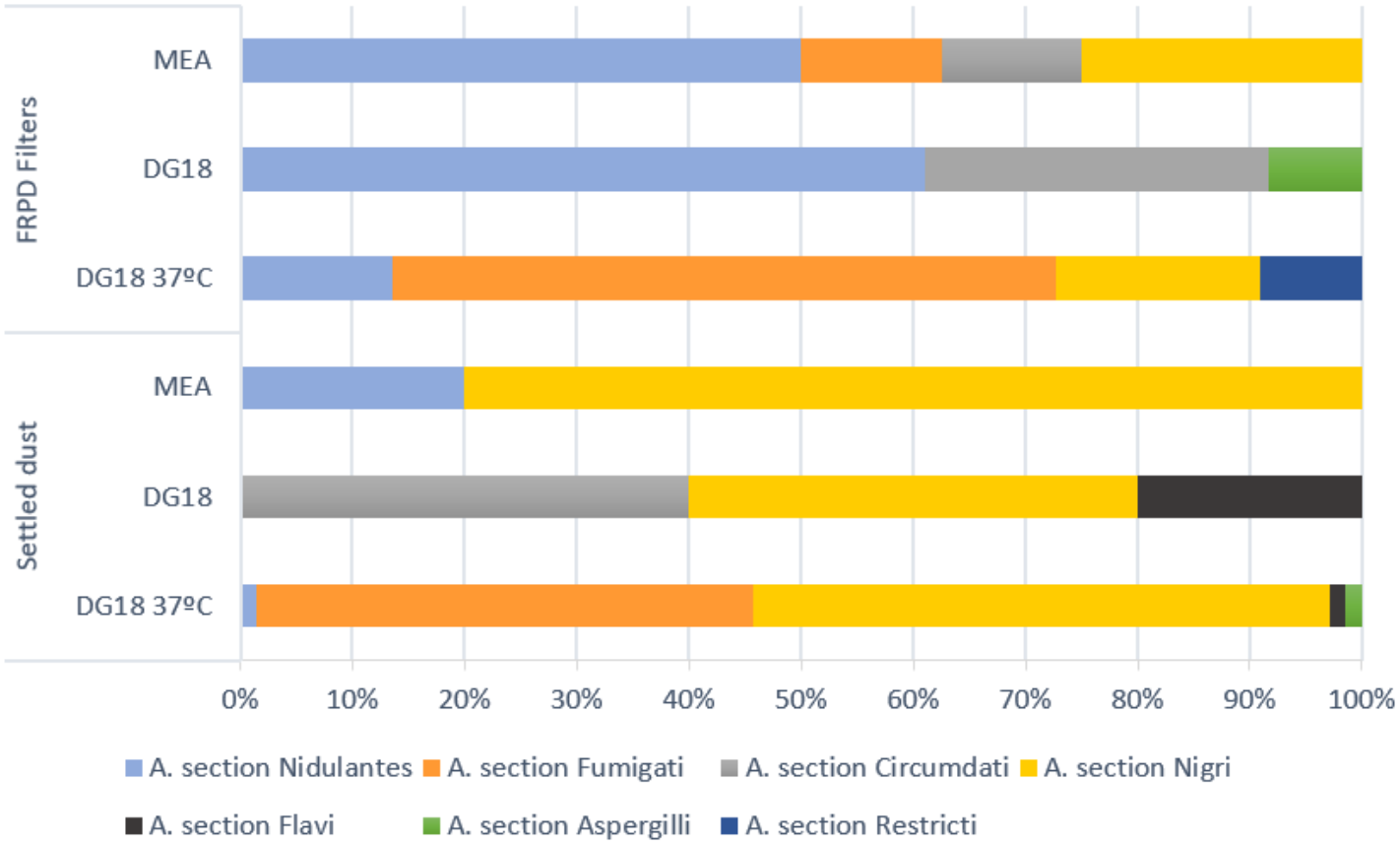

Figure 3

Aspergillus sections' distribution in MEA, DG18, and DG18 (37ºC) in FRPD and settled dust samples analyzed.

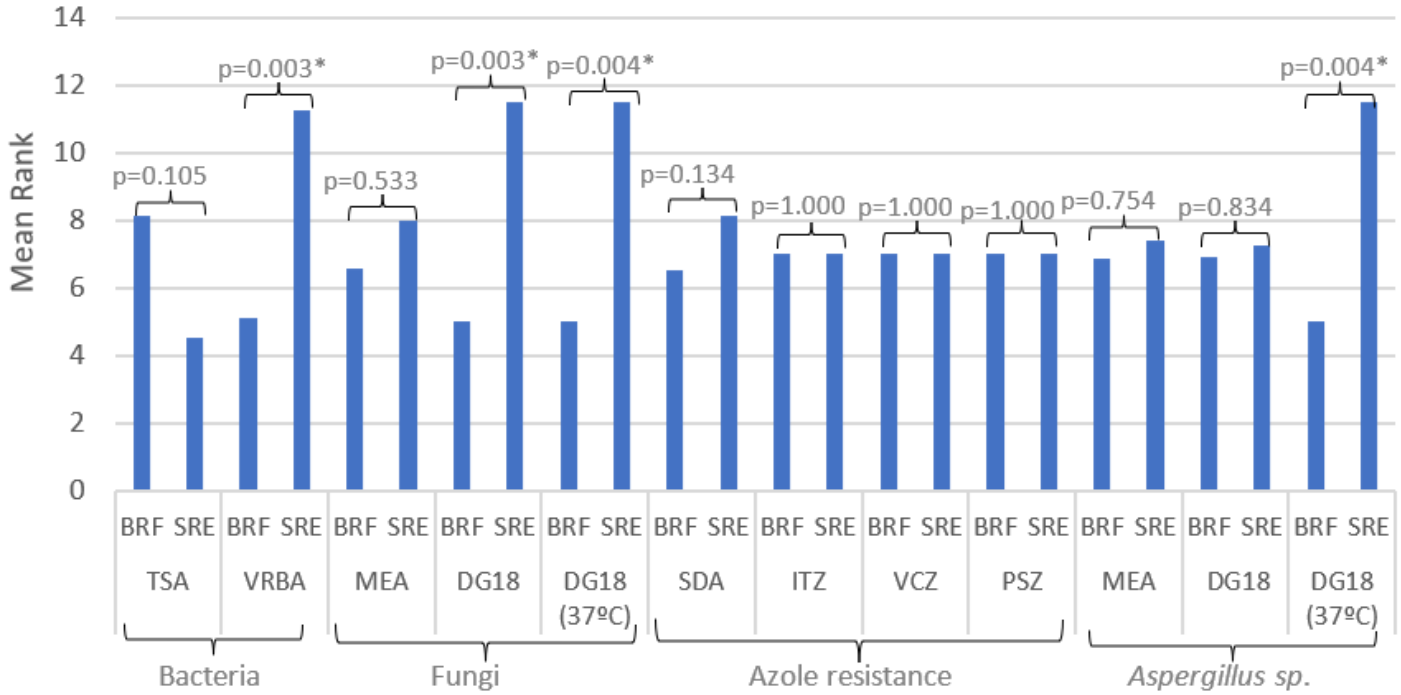

Figure 4

Comparison of bacterial and fungal contamination, azole resistance and *Aspergillus*sp. counts between BRF and SRE. Mann-Whitney test results.

Supplementary Files

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