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Cytotoxic effect of filtering respiratory protective devices from the waste sorting industry: is *in vitro* toxicology useful for risk characterization?

Carla Viegas [a,b,c,*,1], Magdalena Twarużek [d,1], Marta Dias [a], Beatriz Almeida [a], Elisabete Carolino [a], Ewelina Soszczyńska [d], Iwona Ałtyn [d], Susana Viegas [a,b,c], Liliana Aranha Caetano [a,e]

[a] H&TRC- Health & Technology Research Center, ESTeSL- Escola Superior de Tecnologia da Saúde, Instituto Politécnico de Lisboa, Portugal
[b] NOVA National School of Public Health, Public Health Research Centre, Universidade NOVA de Lisboa, Portugal
[c] Comprehensive Health Research Center (CHRC), Portugal
[d] Kazimierz Wielki University, Faculty of Biological Sciences, Department of Physiology and Toxicology, Chodkiewicza 30, 85–064, Bydgoszcz, Poland
[e] Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

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ABSTRACT

The use of Filtering Respiratory Protective Devices (FRPD) is mandatory in Portugal to protect workers from the waste industry of harmful exposures. Deleterious health effects of exposure to bioburden via inhalation and/or ingestion include respiratory symptoms and nephrotoxicity. Between January and February 2019, 118 FRPD samples were collected in one waste sorting industry and characterized regarding microbial contamination and cytotoxicity, defined as cell metabolic activity, through the MTT colorimetric assay (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide). Cytotoxic effect was classified according to percentage of extinction values with respect to the control group, as follows: absent (≥90); low (80%–90%, +); medium (60%–79%, ++); and high (below 60%, +++). For 113 samples the MTT assay revealed a cytotoxic effect in A549 cells, of which 81 presented high cytotoxicity. In SK cells, a cytotoxic effect was observed in 56 samples, of which five displayed a high cytotoxic effect. Several moderate ($p < 0.05$) to strong ($p < 0.01$) correlations were found between higher bacterial and fungal counts both in interior layers (fungi and bacteria) and in exhalation valves (fungi) of FRPD samples and reduced cell metabolic activity of SK cells. On the basis of the obtained results for the cytotoxic effect of FRPD samples on two different cells lines, it was determined that A549 cells exhibited a cytotoxic effect for a higher number of FRPD, whereas the SK cells model correlated better with the other assessed parameters, namely, bacterial and fungal counts and conditions of FRPD use. Although the results are not conclusive on the most appropriate cell line to assess FRPD cytotoxicity, they reinforce the importance of *in vitro* toxicology in exposure assessments to determine the cytotoxicity of mixtures of contaminants, for better risk characterization and selection of appropriate risk management measures.

1. Introduction

Waste sorting is one of the most critical working environments since waste is frequently contaminated by organic matter that functions as a nutrient substrate to microorganisms. In many occupational environments of waste industry, workers are exposed during long periods to high concentrations of microbial contamination. Several studies have lately stressed health risks related with those environments (Marth et al., 1997; Eker et al., 2012).

Microbial exposures are the leading inducers of several respiratory health symptoms, such as asthma, decline in lung function, bronchial hyper-responsiveness, chronic bronchitis, wheeze, and cough (Schenker et al., 1998; Linaker and Smedley, 2002; Sigsgaard and Schlünssen, 2004; Cleave et al., 2010; Basinas et al., 2012; Reynolds et al., 2013). The well-known occurrence of saprophytic fungi and nephrotoxic fungal toxins in waste settings (Viegas et al., 2014, 2018), associated with ineffective protective measures of workers, can also prompt renal toxicity related to exposure to mycotoxins (Bennett and Klich, 2003;

* Corresponding author. ESTeSL – Escola Superior de Tecnologia da Saúde de Lisboa, Av. D. João II, Lote 4.69.01 | 1990-096, Lisbon, Portugal.
E-mail address: carla.viegas@estesl.ipl.pt (C. Viegas).
[1] These authors contributed equally to this work.

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Hope and Hope (2012).

Due to the recognized increased risk of microbiologic agents' exposure and the need of the employer to protect workers from the exposure risks, the use of Filtering Respiratory Protective Devices (FRPD) is mandatory in the Portuguese waste industry. Previous studies stated the suitability of using FRPD as passive sampling methods, since these devices assessment mimic the workers exposure to fungi and bacteria (Viegas et al., 2019a,b).

To our knowledge, nothing is known about the conditions of use of FRPD. Some hygienic measures taken by workers (such as where the FRPD is kept when not in use) or even the FRPD frequency replacement can impact on the FRPD microbial contamination. During the FRPD use, the exhalation of humid air by workers and sweat production increases the moisture content in the filter material (Jachowicz et al., 2019) leading to increase of the microbial contamination. Thus, information collected from workers about smell during FRPD use or if the workers felt more heat moisture than normal can be useful.

Bioaerosols in the waste sorting industry consist of complex mixtures of organic and inorganic dust suspended in the air, including bacteria and endotoxins, fungi and mycotoxins, as well as particles of plant and animal origin. These factors can cause irritative, toxic and allergic reactions in workers after exposure through inhalation, resulting in several respiratory diseases (Douwes et al., 2003; Corrao et al., 2012; Rim and Lim, 2014). The evaluation of toxicity and health effects of simultaneous exposure to complex mixtures of biological agents and particles present in the air of specific occupational environments, without prior knowledge of the composition of the mixture or its properties, can be performed through *in vitro* biological testing using relevant cell cultures (Viegas et al., 2017).

The relation between the presence of bioburden (previously characterized by culture-based and molecular methods) in FRPD used in the waste sorting industry and the potential cytotoxic effects was investigated in this study through the MTT assay using two different cell lines. To our knowledge, this is the first study that will allow to characterize the risk resulting from the FRPD conditions of use and identifying the most suitable risk management measures.

2. Materials and methods

2.1. Waste sorting industry information

This study evaluates the cytotoxic effect displayed by 118 FRPD samples collected from one waste sorting unit in Lisbon, Portugal, and is part of an enlarged exploratory study with financial support aiming to characterize bioburden retained by FRPD through culture based-methods and molecular tools (Viegas et al., 2020). The assessed waste sorting unit processes both sorted and non-sorted waste of different types, with a capacity of 105 thousand tons of waste per year, as follows: 50 thousand tons/yr of paper and board; 30 thousand tons/yr of glass; 25 thousand tons/yr of packaging. Both manual and mechanical processes are used in waste sorting, namely, ballistic and optical separation, packaging and dispatch, as regularly used in the recycling industry.

2.2. FRPD sampling and bioburden evaluation

One hundred and eighteen FRDP samples were collected randomly in the Winter season (on a weekday, between January and February 2019) at workstations where workers spend more time with FRPD and with a greater risk of exposure to microbiologic agents (Viegas et al., 2015, 2020) (Table 1). FRPD samples were individually packed in hygienic wrapping (to protect against contamination) and kept refrigerated until 4 days prior analyses. The conditions of use for each FRPD were determined through a questionnaire on where the FRPD was kept when not in use, whether the FRPD smelled during use, whether workers felt more moisture on the face than normal, whether the FRPD was well adjusted, and the number of hours used before replacement, and used for statistical analyses. The bioburden retained by FRPD after use was evaluated by culture-based methods and molecular detection. Bioburden densities (colony-forming units, CFU. m^{-2}) were calculated and fungal species were identified microscopically as previously described (Viegas et al., 2020).

Table 1
FRPD number collected by each workstation.

FRPD Sample Information		
Workstations	Tasks	FFR number
FMW	Feeding machines with waste	33
SW	Sorting waste	54
MI	Machines inspection	12
MSVO	Machines and special vehicles operator	13
Not specified (without information)	–	8

2.3. Cytotoxicity evaluation

The washed extracts of the interior layer of collected FRPD samples were used for cytotoxicity evaluation through the MTT assay in two distinct cell lines: primary swine kidney (SK) monolayer cells and human A549 adenocarcinoma cells. The MTT assay has often been used to measure cytotoxicity in different cell lines, including cell lines of animal and human origin (Hanelt et al., 1994; Lewis et al., 2004; Fornelli et al., 2004; Viegas et al., 2017). The assay measures the conversion of the tetrazolium salt, 3- [4,5, dimethylthiazol-2-yl] -2-5 diphenyltetrazolium (MTT) to MTT formazan in cells' mitochondria, thus providing an indication of cell respiration competence and metabolic activity. The A549 cell line is broadly used in lung cell biology (Swain et al., 2010) and was used as a model for alveolar cells. The SK cells are a valid alternative to primary human cells for renal *in vitro* toxicology, due to high similarity in renal physiology between the two species (Heussner and Bingle, 2015).

First, cells were cultured in tissue culture flasks (TPP) with Minimum Essential Medium (MEM) with Earle's Salts supplemented with penicillin and streptomycin and fetal bovine serum (all reagents from Sigma-Aldrich, USA) at 5% CO2, 37 °C, and humid atmosphere. Grown cells were then harvested with trypsin/EDTA (1:10,v:v) in phosphate buffered saline (PBS) and prepared at densities of $2{,}5 \times 10^5$ cells/ml in 100 μl culture medium. Next, cells were incubated with a series of dilutions prepared with the FRPD samples. Cytotoxicity, defined in terms of cell metabolic activity, was determined by spectrophotometric analysis of the reduction of the yellow MTT tetrazolium salt to insoluble formazan in an ELISA microplate reader (ELISA LEDETECT 96, biomed Dr. Wieser GmbH; MikroWin, 2013SC software) at the wavelength of 510 nm (Hanelt et al., 1994). Cytotoxic effect was classified according to percentage of extinction values with respect to the control group, as follows: absent (≥90); (low (80%–90%, +); medium (60%–79%, ++); and high (below 60%, +++). The threshold toxicity level (assessed by the dilution method) was defined as the lowest concentration of the FRPD extract capable of causing a drop in absorption to <50% of cell division activity.

2.4. Statistical analysis

The data analysis was performed and descriptive statistics was applied, using either frequency, median or graphical representations in accordance with the nature of the data. The normality of the data was tested using the Kolmogorov-Smirnov test (n's > 50) or through the Shapiro-Wilk test (n's ≤ 50). In the comparison of the workstations regarding the fungal and bacterial contamination and the cytotoxicity (A549 and SK cells), the Kruskal-Wallis test was used, since the assumption of normality was not verified. Whenever statistically

significant differences were detected, the Kruskal-Wallis comparison test was used. To compare the fungal and bacterial contamination and cytotoxicity between interior layer and exhalation valves, the Wilcoxon test was used, since the assumption of normality was not verified. For the study of the relationship between the fungal and bacterial contamination, the cytotoxicity and the characteristics of the FRPD mentioned by the workers, Spearman's correlation coefficient was used, since the assumption of normality was not verified. Statistical software SPSS V23 was applied for statistical analysis. The results were considered significant at a 5% significance level.

3. Results

3.1. FRPD conditions of use

The number of hours per day of FRPD use varied between 1 h and 24 h and the number of consecutive hours of use varied between 50 min and 18 h. The majority (89.3%) of the workers reported replacing the FRPD daily. Most workers reported that they kept the FRPD in their pockets during breaks (79.3%) (Table 2).

3.2. FRPD bioburden characterization

A previous study characterized the FRPD bioburden both in interior layers and in exhalation valves (Table 3) by using the obtained extracts (Viegas et al., 2020). Overall, the higher frequencies were presented by *Chrysonilia sitophila* on interior layers (55.1% MEA; 59.6% DG18), *Aspergillus* sp. on MEA on exhalation valves (44.1%), and *C. sitophila* on DG18 (36.3%). Gram– bacteria were detected with higher frequency in both matrixes (53.2% Interior Layers; 55.4% Exhalation Valves - VRBA) than total bacteria (48.8% Interior layer; 44.6% Exhalation valves - TSA) (Table 3).

DNA from fungal biomass (by dd-PCR) was detected in all samples, ranging from quantification limit (2 copies/μl) to more than 1600 copies (Viegas et al., 2020). *Aspergillus* section *Fumigati* was detected on FRPD interior layers (40 out of 118) and on the exhalation valves (2 out of 118) (Viegas et al., 2020). The FRPD from workers with more waste contact (FMW and SW) showed an increased exposure to bioburden (Viegas et al., 2020).

3.3. Cytotoxicity evaluation

Among the analyzed FRPD samples (n = 118), a cytotoxic effect was observed in 113 cases in A549 cells (95.8%), and in 56 cases in SK cells (47.5%) (Table 4). Distribution of IC50 values per workstation is presented on Table 5.

3.4. Correlation analysis

Correlation analysis was performed with the already reported bioburden characterization (Viegas et al., 2020). This was done to understand if the bioburden influence the cytotoxicity results obtained in this study.

In the interior layer of the FRPD the following correlations were detected: i) bacterial counts on TSA with moisture on the face (r_S = 0.256, p = 0.011), with % of metabolic active A549 (r_S = 0.229, p = 0.013) and SK (r_S = −0.191, p = 0.039) cells. These results reveal that greater bacterial counts on TSA in the interior layer of the FRPD is related to greater moisture on the face and with reduced cell metabolism in SK; ii) bacterial counts on VRBA with usage time per day (r_S = −0.199, p = 0.038), with moisture in the face (r_S = 0.212, p = 0.036), with the FRPD fit (r_S = 0.202, p = 0.046) and with % of metabolic active A549 cells (r_S = 0.382, p < 0.0001). These results show that greater bacterial counts on VRBA in the interior layer is related to shorter usage time per day, greater moisture in the face and greater FRPD fit; iii) fungal contamination on MEA with number of daily hours of FRPD use (r_S = 0.212, p = 0.025), with number of consecutive hours of FRPD use (r_S = 0.287, p = 0.002), with usage time per day (r_S = −0.258, p = 0.007), with moisture in the face (r_S = 0.385, p < 0.0001), with % of metabolic active A549 (r_S = 0.423, p < 0.0001) and SK (r_S = −0.370, p < 0.0001) cells. This means that greater fungal contamination on MEA in the interior layer is related with greater number of daily hours and consecutive hours use, shorter usage time per day, greater moisture in the face, greater FRPD fit, and reduced cell metabolism in SK; iv) Fungal contamination on DG18 with number of daily hours of use (r_S = 0.197, p = 0.038), with number of consecutive hours of use (r_S = 0.266, p =

Table 2
FRPD conditions of use.

		n (%)	Minimum – Maximum	Mean ± Standard Deviation	Median (Interquartil range)
Hours of daily use (h)			1.00–24.00	7.59 ± 3.65	7 (7–7)
Consecutive hours of use (h)			0.50–18.00	6.622 ± 2.73	6 (7–5)
Daily replacement	No	12 (10.7%)			
	Yes	100 (89.3%)			
Usage time (day)			0.625–5.00	1.13 ± 0.68	1 (1–1)
Place where is kept	Pocket	88 (79.3%)			
	Folded in the bench	19 (17.1%)			
	Inside the packaging	2 (1.8%)			
	Shelf	2 (1.8%)			
Smell during use	No	90 (82.6%)			
	Yes	3 (2.8%)			
	Sometimes	16 (14.7%)			
More heat than normal	No more than normal	76 (77.6%)			
	Very little more than normal	5 (5.1%)			
	Little more than normal	15 (15.3%)			
	Much more than normal	2 (2.0%)			
	Excessively more than normal	0 (.9%)			
Moisture on the face	Did not create	44 (44.9%)			
	Very little	16 (16.3%)			
	Few	37 (37.8%)			
	Lots of	1 (1.0%)			
	Overly	0 (0.0%)			
Well adjusted	Very little	0 (0.0%)			
	Little	0 (0.0%)			
	Not much or little	2 (2.0%)			
	Adjusts	75 (76.5%)			
	It fits a lot	21 (21.4%)			

Table 3
Bioburden distribution on FRPD analyzed.

Interior Layer						Exhalation Valves					
Fungal distribution											
MEA			DG18			MEA			DG18		
Species	**CFU/m²**	**%**	**Species**	**CFU/m²**	**%**	**Species**	**CFU/m²**	**%**	**Species**	**CFU/m²**	**%**
C. sitophila	22.5×10^5	55.14	*C.sitophila*	27.5×10^5	59.62	*Aspergillus* sp.	12.2×10^5	44.06	*C.sitophila*	10.0×10^5	72.33
Penicillium sp.	7.95×10^5	19.48	*Penicillium* sp.	16.1×10^5	34.92	*C.sitophila*	10.0×10^5	36.02	*Aspergillus* sp.	2.39×10^5	17.27
Other species	$7{,}57 \times 10^5$	18.55	*Aspergillus* sp.	2.41×10^5	5.22	*Chrysosporium* sp.	5.00×10^5	18.01	*Penicillium* sp.	1.38×10^5	9.93
Aspergillus sp.	2.79×10^5	6.84	*Other species*	0.11×10^5	0.24	*Other species*	0.53×10^5	1.91	*Other species*	0.06×10^5	0.47
Total	40.8×10^5	100	Total	46.2×10^5	100	Total	27.8×10^5	100	Total	13.8×10^5	100
Bacterial distribution											
TSA			**VRBA**			**TSA**			**VRBA**		
Mean (SD) CFU.m^{-2}			Mean (SD) CFU.m^{-2}			Mean (SD) CFU.m^{-2}			Mean (SD) CFU.m^{-2}		
1.8×10^5 (1.3×10^5)			2.1×10^5 (3.0×10^5)			1.2×10^5 (1.3×10^5)			1.5×10^5 (2.7×10^5)		

Adopted from Viegas et al. (2020).

Table 4
Level of cytotoxicity of FRPD samples collected.

Cytotoxicity Group	A549		SK	
	No. of samples	IC50	No. of samples	IC50
absence of cytotoxicity ≥90%	5	n.d.	62	n.d.
low cytotoxic effect (+) 80 to <90%	3	n.d.	31	n.d.
medium cytotoxic effect (++) 60 to <80%	29	n.d.	20	n.d.
high cytotoxic effect (+++) <60%	81	62 out of 81 (76.5%) samples with extinction values < 50% 58 of samples IC50 – 5 mm²/ml 3 of samples IC50 – 2,5 mm²/ml 1 of samples IC50 – 1,25 mm²/ml	5	5 out of 5 (100%) samples with extinction values < 50% 5 of samples IC50 – 5 mm²/ml

n.d., IC50 not determined for extinction values ≥ 50%.

0.005), with usage time per day ($r_S = -0.225$, $p = 0.019$), with moisture in the face ($r_S = 0.373$, $p < 0.0001$), with % of metabolic active A549 ($r_S = 0.258$, $p = 0.005$) and SK ($r_S = -0.284$, $p = 0.002$) cells. These results reveal that greater fungal contamination on DG18 in interior layer is related with greater number of daily hours and consecutive hours of use, shorter usage time per day, greater moisture in the face, greater FRPD fit, and reduced cell metabolism in SK; v) Fungal biomass concentration in interior layer with usage time per day ($r_S = -0.191$, $p = 0.049$), which reveals that higher fungal biomass concentration is related to interior layer and shorter usage time per day; vi) *Aspergillus* section *Fumigati* counts with more heat than normal ($r_S = 0.321$, $p = 0.019$), which indicates that higher *Aspergillus* section *Fumigati* counts in interior layer is related with greater heat produced by the use of the FRPD (Table 6). In the exhalation valves the following correlations were detected: i) bacterial counts in VRBA with usage time per day ($r_S = -0.367$, $p < 0.0001$), with FRPD fit ($r_S = 0.212$, $p = 0.036$) and with % of metabolic active A549 cells ($r_S = 0.271$, $p = 0.036$), which reveals that bacterial counts in VRBA is related with smaller usage time per day and greater FRPD fit; ii) Fungal contamination on MEA with consecutive hours of use ($r_S = 0.261$, $p = 0.006$), with moisture in the face ($r_S = 0.382$, $p < 0.0001$), with % of metabolic active A549 ($r_S = 0.463$, $p < 0.0001$) and SK ($r_S = -0.315$, $p = 0.001$) cells. These results indicate that greater fungal contamination in MEA is related with greater consecutive hours of use and moisture in the face and lower cell metabolism in SK; iii) Fungal contamination on DG18 with consecutive hours of use ($r_S = 0.211$, $p = 0.026$), with moisture in the face ($r_S = 0.283$, $p = 0.005$), with % of metabolic active A549 ($r_S = 0.232$, $p = 0.011$) and SK ($r_S = -0.209$, $p = 0.023$) cells. These results point out that greater fungal contamination in DG18 is related with higher consecutive hours of use and moisture in the face and lower cell metabolism in SK; iv) Fungal biomass concentration with % of metabolic active SK cells ($r_S = -0.210$, $p = 0.024$), which reveals that greater fungal biomass concentration is related lower cell metabolism in SK (Table 6).

4. Discussion

A good *in vitro* model should be as close as possible to *in vivo*

Table 5
FRPD samples' IC50 values per workstation.

Workstation	IC50 (mm²/ml)									
	A549					SK				
	n.d.	10	5	2.5	1.25	n.d.	10	5	2.5	1.25
FMW (n = 40) n (%)	17 (42.5%)	0	23 (57.5%)	0	0	38 (95.0%)	0	2 (5.0%)	0	0
MI (n = 4) n (%)	2 (50.0%)	0	*2 (50.0%)*	0	0	4 (100.0%)	0	0	0	0
MSVO (n = 13) n (%)	7 (53.8%)	0	5 (38.5%)	1 (7.7%)	0	4 (100.0%)	0	0	0	0
SW (n = 53) n (%)	27 (50.9%)	0	23 (43.4%)	2 (3.8%)	1 (1.9%)	51 (96.2%)	0	2 (3.8%)	0	0
n. s. (n = 8) n (%)	3 (37.5%)	0	5 (62.5%)	0	0	7 (87.5%)	0	1 (12.5%)	0	0

n.d., IC50 not determined for extinction values ≥ 50%; FMW, Feeding machines with waste; SW, Sorting waste; MI, Machines inspection; MSVO, Machines and special vehicles operator; n.s., not specified (without information).

Table 6
Study of the relationship between bacterial and fungal contamination, FRPD conditions of use reported by workers, and cell metabolic activity (Spearman's correlation coefficient).

			Interior layer						Exhalation valves						% of cell metabolic activity	
			Bacterial contamination		Fungal contamination		Fungal biomass	Aspergillus section Fumigati	Bacterial contamination		Fungal contamination		Fungal biomass	Aspergillus section Fumigati		
			TSA	VRBA	MEA	DG18			TSA	VRBA	MEA	DG18			A549	SK
Interior Layer	Bacterial contamination	TSA		0.150	0.307**	0.292**	0.020	−0.098	0.141	0.138	0.337**	0.340**	0.161	0.284	0.229*	−0.191*
		VRBA			0.189*	0.084	0.015	0.032	0.077	0.527**	0.168	0.017	0.248**	0.182	0.382**	−0.094
	Fungal contamination	MEA				0.806**	0.293**	0.266*	−0.115	0.150	0.546**	0.563**	0.269**	0.136	0.423**	−0.370**
		DG18					0.444**	0.336**	−0.047	0.110	0.412**	0.500**	0.215*	0.168	0.258**	−0.284**
	Fungal biomass							0.589**	0.074	0.040	−0.012	0.185*	0.209*	0.155	−0.116	0.094
	Aspergillus section Fumigati								−0.096	−0.111	0.020	0.072	0.139	0.140	0.219	0.050
Exhalation valves	Bacterial contamination	TSA								−0.035	−0.042	−0.062	0.052	−0.244	−0.020	0.046
		VRBA									0.145	0.042	0.261**	0.241	0.271**	0.012
	Fungal contamination	MEA										0.589**	0.260**	0.163	0.463**	−0.315**
		DG18											0.282**	0.301	0.232*	−0.209*
	Fungal biomass													0.226	0.165	−0.210*
	Aspergillus section Fumigati														0.071	0.256
Characteristics and habits of use of the masks	Hours of daily use		0.105	0.055	0.,212*	0.,197*	0.185	0.180	0.009	−0.010	0.154	0.169	0.097	−0.025	0.080	0.024
	Consecutive hours of use		0.156	0.121	0.,287**	0.266**	0.,022	0.025	0.021	−0.046	0.261**	0.211*	0.055	0.114	0.123	−0.112
	Usage time (day)		−0.012	−0.199*	−0.258**	−0.225*	−0.191*	0.022	0.052	−0.367**	−0.143	−0.128	−0.173	0.087	−0.268**	−0.043
	More heat than normal		0.048	0.075	0.046	0.086	0.129	0.321*	0.117	0.022	0.161	0.159	0.165	0.291	0.236**	−0.098
	Moisture in the face		0.256*	0.212*	0.385**	0.373**	−0.017	0.129	0.047	0.162	0.382**	0.283**	0.089	0.041	0.404**	−0.163
	FRPD fit		−0.099	0.202*	0.069	0.,128	0.146	0.024	−0.032	0.212*	−0.098	−0.009	−0.032	0.201	−0.075	−0.084
% of cell metabolic activity	A549															−0.113

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

behavior, in order to reflect most likely the *in vivo* situation. The two cell lines were used since both lungs and kidney are target organs of contaminants present in the FRPD: lung cells contact directly with contaminants after exposure by inhalation, whereas kidney cells might contact with both the original molecules and/or their metabolites. In previous toxicity studies conducted with composite environmental samples, the use of relevant cell cultures also provided useful data for the analysis of negative health effects of co-exposure to multiple stressors (Viegas et al., 2017).

The present study showed that the percentage of cell metabolic activity decreased in a more pronounced way in A549 cells, with swine kidney cells revealing a lower sensibility to contaminants present at FRPD samples. Considering that lung cells are exposed directly to the contaminants, this model might be more related with the real exposure scenario. The cell-to-cell variability in cytotoxic response may be due to the different metabolic activity of the target cells. The most common explanations for the varying cytotoxic response of different cell types to contaminants are related to differences in: target enzymes or other cell constituents that can interact with the contaminant; metabolic activation of a non-toxic precursor molecule in a metabolite with non-specific toxicity; efficiency of detoxification mechanisms by reducing cells' susceptibility to the toxic agent (Shier et al. 1991).

While no statistically significant correlations between bioburden and decreased percentage of metabolic active cells were found in A549 cells, the statistical analysis of SK cells revealed moderate ($p < 0.05$) to strong ($p < 0.01$) correlations between higher bacterial and fungal counts and reduced percentage of cell metabolic activity in interior layers (TSA, MEA, DG18) and exhalation valves (MEA, DG18) of FRPD samples, and a moderate correlation between fungal biomass in FRPD exhalation valves and percentage of SK cell metabolic activity.

Swine kidney cells were used in this study as model for renal toxicity. The highest cytotoxicity levels in swine kidney cells were obtained with 8 FRPD samples with confirmed presence of *Aspergillus* (the most prevalent) and *Penicillium* at the interior layer (7 out of 8 FRPD) and at the exhalation valve (7 out of 8 FRPD) (Viegas et al., 2020). Most of these 8 FRPD also presented some of the highest levels (>6000 CFU m^{-2}) of total fungal burden in MEA and/or DG18 (7 out of 8 FRPD, considering interior layer and exhalation valve), ranging from 6 to 8 h of use per day while being kept at workers' pocket during breaks in most cases.

It should be highlighted that workers behavior regarding the FRPD use impacts on microbial contamination. Indeed, besides most of the workers reported that they kept the FRPD in their pockets during breaks, some also refer in the shelf without any cover or folded in the bench increasing FRPD contamination. Additionally, if after the breaks hygienic measures, such as washing the hands are not taken, can lead to a higher number of FRPD contamination sources.

Aspergillus sections *Fumigati* and *Nigri*, the most prevalent on interior layers and exhalation valves (Viegas et al., 2020), are able to cause occupational diseases and their cytotoxicity has been previously measured (Bunger et al., 2004). Schulz et al. previously used the MTT test to determine cytotoxicity for *A.* section *Nigri* and *A.* section *Fumigati* spore extracts describing lower IC50 levels for *A.* section *Nigri* than for *A.* section *Fumigati* (Schulz et al., 2004). These species are also well known producers of mycotoxins: *A.* section *Fumigati* produces gliotoxin, fumagillin, helvolic acid (fumigacin), fumitremorgin A and Asp-hemolysin, and *A.* section *Nigri* produces ochratoxin A, malformin, fumonisins and toxic oxalates. In a previous biomonitoring study, performed in workers from the same unit, it was already reported occupational exposure to ochratoxin A and aflatoxin B1 even with the reported use of FRPD by most of the workers (Viegas et al. 2014, 2018).

It is known that renal toxicity often relates to exposure to mycotoxins Bennett and Klich (2003); Hope and Hope (2012). Mycotoxins are metabolites of filamentous fungi that can be toxic for humans even in low concentrations which are mainly produced by five genera of filamentous fungi, namely *Aspergillus*, *Penicilium*, *Fusarium*, *Alternaria*, and *Claviceps* (Steyn, 1995). Saprophytic fungi and nephrotoxic fungal toxins are natural contaminants of feeds and foods and their presence in waste settings has been well documented (Viegas et al., 2014, 2018).

A previous study from Gniadek et al. (2017) also identified cytotoxic strains of fungi from *A.* sections *Circumdati*, *Nigri* or *Flavi* from the hospital environment in SK cells, which may pose an additional risk for immunocompromised individuals. Furthermore, it was also reported an association between cytotoxicity and microbial content (Happo et al., 2014) and the influence of the viable component of the microorganisms on cytotoxic potential (Croston et al., 2016), reinforcing the need to use culture based-methods to assess occupational exposure to bioburden (Madsen et al., 2020; Viegas et al., 2020).

Moisture, as expected, influences the microbial contamination in the interior layer and exhalation valve and this can be potentiated by the consecutive hours of use. Indeed, it is the moisture content of materials that allows microbial growth, because it determines the water available for the germination of spores (Valentín, 2007). Also, higher *Aspergillus* section *Fumigati* counts linked with heat can be explained by the stress resistance of this *Aspergillus* section that may be relevant to its ability to adapt to the stress of growth in the human host (Bhabhra and Askew, 2005).

The human A549 adenocarcinoma cell line was used in this study as a model for alveolar cells. On the basis of the obtained cytotoxicity assessment results of the extracts from the FRPD samples collected, conclusions can be drawn on the potential risk for health as the result of the toxic influence after a significant inhalation of a large number of fungal spores. The higher sensitivity of A549 cells observed in our study is of concern, as inhalation is an important exposure route in the waste sorting plant which the FRPD is intended to protect. Moreover, the environment should be treated as a potential source of exposure to fungi and may predispose individuals suffering from asthma to fungal colonization of airways (Fairs et al., 2013).

The available literature is sparse in what concerns to the toxicity of composite mixtures of bioaerosols in the environment, mostly focusing on the toxicity of specific bacterial or fungal species (e.g., MRSA or *Aspergillus* section *Fumigati*) found in the specific environments such as hospital wards, or dealing with the fungal genera most associated with dampness in dwellings (*Penicillium* sp., *Stachybotrys* sp., *Chaetomium* sp. or *Aspergillus* sp.). The exposure to bioburden and consequent health risks, however, cross all occupational settings. The waste management industry is a setting of concern as it presents high-load contamination by saprophytic fungi and bacteria, due to decomposition of organic matter, while it is a sector where a low educational level and health risks awareness of workers might be associated with low adherence to protection measures, such as the use of individual protection equipment, as is the case of FRPD.

No significant differences were found between workstations. The cytotoxicity of the samples might be related to the type and load of microorganisms, microbial components (such as beta-glucan from fungi or lipopolysaccharide from bacteria) and microbial metabolites (endotoxins, mycotoxins), while it can also relate to particulate matter or other contaminants occurring on FRPD samples. Besides organic contaminants, inorganic components (not assessed in this study) such as metals, persistent organic pollutants, bisphenols and phthalates, that originate from the residues sorted in the waste sorting unit, might also be present and have cytotoxic effect, particularly in lung cells. Viegas et al. (2017) have previously discussed the limitations of occupational exposure limits, which are mainly dedicated to single exposures, thus, not assessing the real context of multiple exposures in occupational settings.

Through the assessment of the cytotoxicity of composite real environmental samples, it may be assumed that the exposure to bioburden or toxins produced by bacteria or fungi on affected FRPD (or on other individual protection equipment) may be a significant factor predisposing for infections and toxicity in sensitive workers, besides causing other common health effects (allergy, irritant, nephrotoxicity).

Summing up, the high cytotoxicity of FRPD samples collected in the

waste sorting environment, especially in lung cells, as observed in this study, may represent an additional risk of exposure for workers in this occupational setting, which is greater for susceptible and/or immunocompromised workers in particular.

Therefore, there is a need to consider suitable risk management measures, such as higher frequency in FRPD substitution during the day and awareness of workers for the need of constantly use the individual protection equipment and the proper storage of FRPD when not in use.

5. Conclusions

The cytotoxicity observed in A549 cells suggests the inhalation route as a critical exposure route in the waste sorting industry, since metabolic activity of these cells was impaired by contaminants present in the collected FRPD samples. Suitable and adjustable risk management measures targeting to protect workers from exposure by inhalation route should be a priority for investment.

The statistical analysis showed that higher bacterial and fungal counts on FRPD are associated to higher cytotoxicity levels in SK cells. The integrated study of cytotoxicity and microbial contamination levels revealed that the highest cytotoxicity levels (SK cells) were found in FRPD where *Aspergillus* sp. and *Penicillium* sp. were the most prevalent fungal species, although no conclusion can be drawn on which species is the most cytotoxic. More studies on this topic, including the co-assessment of particulate matter and chemical contaminants, are needed to explain the importance of cytotoxicity in composite environmental samples. The use of *in vitro* toxicology studies is useful to address cytotoxicity of contaminant mixtures as present in FRPD. *In vitro* models coupled with culture based-methods is a relevant approach to obtained useful information on the possible health effects of co-exposure to multiple stressors. This approach is of upmost importance in exposure assessments aiming the risk characterization and the selection of the most appropriate risk management measures. Additionally, training and education programs for workers should be developed to have their engagement on selecting the risk management measures, such as protection devices, to guarantee their proper use.

Authors credits

Carla Viegas, Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Roles/Writing - original draft; Writing - review & editing., Magdalena Twarużek, Investigation; Formal analyses; Methodology; Resources; Supervision; Validation; Writing - review & editing., Marta Dias, Formal analysis; Methodology, Beatriz Almeida, Formal analysis; Methodology, Elisabete Carolino, Formal analysis; Methodology; Writing - review & editing., Ewelina Soszczyńska, Formal analysis; Methodology, Iwona Ałtyn, Formal analysis; Methodology, Susana Viegas, Methodology; Writing - review & editing., Liliana Aranha Caetano, Formal analysis; Methodology; Supervision; Validation; Writing - review & editing.

Declaration of competing Interest

No conflict of interest exists.

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