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Cytotoxicity of filtering respiratory protective devices from the waste sorting industry: A comparative study between interior layer and exhalation valve

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ABSTRACT

Filtering respiratory protection devices (FRPD) are mandatory for workers to wear in the Portuguese waste-sorting industry. Previous results regarding microbial contamination found on FRPD interior layer raised the question of whether microbial contamination from the exhalation valve would also have cytotoxicity effects. Since the FRPD exhalation valves are very close to workers' nose and mouth, they represent a source of exposure to bioburden by inhalation. This study aimed to evaluate the cytotoxicity of the microbial contamination present in the FRPD exhalation valves. For this purpose, the cytotoxicity effects were determined through the MTT assay in two different cell lines (human A549 epithelial lung cells, and swine kidney cells) and compared with previous results obtained with FRPD interior layers. The contamination present in the FRPD exhalation valves presented some cytotoxicity on epithelial lung cells, suggesting the inhalation route as a potential route of exposure through the use of FRPD in the waste-sorting industry. Half-maximal (50%) inhibitory concentration (IC50) values were lower for FRPD interior layer than exhalation valves in lung cells, with overall cytotoxicity lower in exhalation valves when compared to interior layer ($z = -4.455$, $p = 0.000$). Higher bacterial counts in TSA were correlated with lower IC50 values, thus, higher cytotoxicity effect in lung cells. No statistically significant differences were detected among different workplaces.

1. Introduction

Waste-sorting plant workers are exposed to a significant health risk associated with the presence of high concentrations of bioburden (comprising fungi and bacteria) in this environment (Kozajda and Szadkowska-Stanczyk, 2009; Madsen et al., 2020). This might be demonstrated by increasing written evidence reporting associations between working in waste-sorting plants and the occurrence of health problems, including several respiratory diseases (Douwes et al., 2003; Malta-Vacas et al., 2012; Corrão et al., 2012; Rim and Lim, 2014).

One of the major features of the waste sorting environment is the fact that workers are exposed to a complex mixture of contaminants, of chemical and biological origin, which, although difficult to fully characterize, is certainly responsible for biological responses that can result in health effects (Viegas et al., 2017; Ladeira et al., 2020). Several studies in the last decades underline the importance of understanding the mechanisms of adverse effects of airborne fungi on human health, ranging from irritating and cytotoxic effects of fungal metabolites (mycotoxins or volatile organic compounds) to immunosuppression and inflammatory response to β-glucans from the fungal cell wall (Smith et al., 1992; Hanelt et al., 1994; Maličev et al., 2007; Pieckova, 2012). More recent studies (Viegas et al., 2017; Ladeira et al., 2020) have warned of the need for more data on the effects of combined and multiple exposure to complex mixtures for public health and, particularly, in

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occupational settings.

As in other European countries, employers are obliged by Portuguese legislation to assess occupational risks (Viegas et al. 2016; Directive 89/391/EEC). The assessment of biological risks in specific environments can be carried out by evaluating toxicity and health effects resulting from occupational exposure to complex mixtures. Since bioburden analysis cannot alone predict harmful effects to the human health, *in vitro* toxicological studies, using relevant and established cell lines, can be developed to characterize biological responses to composite samples (e.g., air or inhalable fractions, or respiratory protection devices) and provide important data on the biological risks of occupational exposure, even in the absence of information on the composition of mixtures (Viegas et al., 2017, 2020bd).

In the Portuguese waste-sorting industry, it is mandatory for workers to wear filtering respiratory protection devices (FRPD). During the use of FRPD by workers, the exhalation of humid air through the exhalation valves (Majchrzycka et al., 2016; Jachowicz et al., 2019), together with the organic matter retained by filter material, can synergistically boost microbial contamination (Majchrzycka et al., 2016; Jachowicz et al., 2019; Viegas et al., 2020abcd). The purpose of FRPD exhalation valves (EV) is to reduce heat build-up in humid conditions, with low resistance filter technology helping workers breathe easily (Viegas et al. 2020b). Since the EV is very close to the workers' nose and mouth, it will be important to characterize microbial contamination for the assessment of workers' exposure to bioburden by inhalation (Viegas et al. 2020b).

Previous studies in the waste sorting industry related high levels of microbial contaminants in FRPD (Viegas et al. 2020b) with cytotoxicity effects (Viegas et al. 2020d) of the FRPD interior layer (IL) in two different cell lines (human A549 epithelial lung cells, and swine kidney (SK) cells) through the MTT assay. Those results raised the question of whether the microbial contamination found on FRPD exhalation valve (EV) would also exhibit cytotoxicity effects.

Based on these assumptions, in this study we use the MTT assay to determine the adverse effects of microbial contamination of FRPD exhalation valves on cell viability. This approach aimed to conclude about the efficacy of FRPD protection in the waste sorting industry, and to define a suitable sampling strategy for the characterization of biological risks in this occupational environment. The cytotoxicity effect of microbial contaminants from FRPD exhalation valves (EV) was measured in two different cell lines through the MTT assay, and compared with previous results of FRPD interior layers (IL). The relations between microbial contamination in EV (evaluated by classic microbiology and molecular detection) and cytotoxicity (IC50) were determined.

2. Materials and methods

2.1. Sampling strategy and FRPD characterization

Four workstations of one waste sorting industry in Lisbon, Portugal, were selected for the collection of FRPD (n = 118) used by workers in those areas and assessment of the cytotoxicity of FRPD exhalation valves (EV) (n = 118) (Fig. 1). The waste-sorting industry and the workplaces where the FRPD were collected have been previously fully described (Viegas et al. 2015, 2020b,d). The waste sorting industry receives inorganic waste of different types: Ferrous Metal Packaging; Non - Ferrous Metal Packaging; Paper/Card; Glass Packaging; Plastic Packaging. The sorting is performed with the combination of manual and mechanical processes of separation, with the use of ballistic and optical separation, packaging and dispatch (Viegas et al. 2015, 2020b,d).

Fig. 1. Exhalation valve and FRPD.

Samples were collected randomly in the Winter season (between January and February 2019) on a weekday, at workstations where workers spend more time wearing FRPD, and with increased risk of exposure to microbiologic agents (due to the nature of activities there developed) (Viegas et al., 2015; 2020abcd)), as follows: Feeding machines with waste (FMW) (N = 33), Sorting waste (SW) (N = 54), Machines inspection (MI) (N = 12), Machines and special vehicles operator (MSVO) (N = 13), and eight more FRPD from non-identified workstation (Viegas et al. 2020b,d).

The FRPD samples were individually packed in sterilized bags and kept refrigerated (4 to 8 °C) until 4 days before analyses. The EV were removed from FRPD with a sterilized clamp in aseptic conditions and both matrixes (IL and EV) were extracted with 10 mL of 0.1% Tween™ 80 saline solution (NaCl 0.9%) for 30 min at 250 rpm on an orbital laboratory shaker (Edmund Bühler SM-30, Hechingen, Germany). Four different culture media were used in order to enhance the selectivity for bacterial and fungal growth. The extracts of a control sample (FRPD without prior use) were submitted to the same assays performed. All samples (FRPD IL and EV) were characterized regarding bioburden densities (bacteria and fungi colony-forming units, $CFU.m^{-2}$) and fungal species identification (morphological characterization and molecular detection) as retained by FRPD after use and already reported (Viegas et al. 2020b,d).

2.2. Cytotoxicity evaluation

The cytotoxicity studies were conducted in the Faculty of Biological Sciences of the Kazimierz Wielki University, Bydgoszcz, Poland.

The first step of the cytotoxicity assessment procedure involved a series of test dilutions prepared and transferred onto A549 and SK cells. The choice of an appropriate cell line is critical when conducting studies to elucidate induced toxicity in humans. The human A549 epithelial lung cell line is largely used in lung cell biology (Swain et al. 2010) and was applied as a model for alveolar cells, while the swine kidney (SK) cells are a valid alternative to primary human cells for renal *in vitro* toxicology, due to high similarity in renal physiology (Heussner and Dietrich, 2013).

The cells were maintained in MEM (Minimum Essential Medium Eagle) supplemented with antibiotics (10,000 units penicillin and 10 mg streptomycin per mL in 0.9% NaCl) (Sigma-Aldrich, USA)) and foetal bovine serum (Sigma-Aldrich, USA). Cells were detached from the bottom of the culture vessel with 0.25% (w / v) Trypsin 0.53 mM EDTA. The released cells were suspended in the culture medium then their number was counted using the Scepter™ 2.0 Cell Counter (Merck). The cells prepared in this way were applied 100 µl each to a 96-well plate.

The evaluation of cytotoxicity was conducted by incubating the FRPD EV extracts with the A549 or SK cells (densities of $2{,}5 \times 10^5$ cells/ml) for 48 h in the supplemented MEM medium at 5% CO2, 37 °C, and humid atmosphere. The same IL and EV extracts used for the microbiological contamination characterization (including the control sample) were used for the cytotoxicity evaluation.

The cytotoxicity level was defined as cell metabolic activity, through spectrophotometric analysis of the reduction of tetrazolium salt, 3- [4,5, dimethylthiazol-2-yl] -2-5 diphenyltetrazolium (MTT) tetrazolium salt to formazan in the mitochondria at the wavelength of 510 nm (Hanelt et al. 1994) in an ELISA microplate reader (ELISA LEDETECT 96, biomed Dr. Wieser GmbH; MikroWin 2013SC software). The MTT assay has commonly been used to measure cytotoxicity in different cell lines, comprising cell lines of animal and human origin (Fornelli et al., 2004; Viegas et al., 2017) and provides an indication of cell respiration competence and metabolic activity. Prinsloo and colleagues have

already used the MTT assay to determine the effect of microbial contaminants in cell viability (Prinsloo et al. 2013).

The threshold toxicity level was defined as the lowest concentration of the FRPD EV extract causing a dropping in absorption to < 50% of cell division activity.

2.3. 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). For the study of the relationship between the fungal and bacterial contamination, the cytotoxicity, Spearman's correlation coefficient was used, since the assumption of normality was not verified. To compare the cytotoxicity between interior layer and exhalation valves, the Wilcoxon test was used, since the assumption of normality was not verified. In the comparison of the workstations regarding 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. Statistical software SPSS V26 was applied for statistical analysis. The results were considered significant at a 5% significance level.

3. Results

3.1. Preliminary FRPDs' IL and EV bioburden characterization

This research was conducted by means of the MTT assay on the group of exhalation valves (EV) from 118 FRPD collected in four different workstations from one Portuguese waste sorting industry. The same EV extracts were analysed and results reported previously (Viegas et al. 2020b,d). In fact, the previous bioburden assessment of both FRPDs' EV and IL (Viegas et al. 2020b,d) has revealed high frequencies of fungal contamination, with *Chrysonilia sitophila* accounting for 55.1% (MEA) to 59.6% (DG18) on IL, and *Aspergillus* sp. with 44.1% (MEA) and *C. sitophila* with 36.3% (DG18) on EV. The most dominant species of *Aspergillus* genus was *Aspergillus* section *Fumigati* both in IL and EV. Regarding bacteria, Gram– bacteria was detected with higher frequency in both matrixes (53.2% IL; 55.4% EV - VRBA) than total bacteria (48.8% IL; 44.6% EV - TSA) (Table 1).

Having characterized the bioburden in FRPD's IL and EV through culture-based methods, fungal biomass (through dd-PCR) was also determined in that previous study (Viegas et al. 2020b). Fungal DNA was detected in all FRPDs, ranging from quantification limit (2 copies / µl) to more than 1600 copies (Viegas et al. 2020b). *Aspergillus* section *Fumigati* was detected with great variability among FRPDs' IL (33%; 40 out of 118 FRPD) and EV (1.6%; 2 out of 118 FRPD) (Viegas et al. 2020b, d). The FRPD (IL and EV) of the FMW and SW workstations (with greater contact with waste) had the highest levels of microbial contamination, suggesting greater exposure to microbial risk for workers in these areas (Viegas et al. 2020b,d).

3.2. Cytotoxicity of FRPDs' exhalation valves (and comparison with FRPDs' interior layers)

Among the analysed FRPD exhalation valves (n = 118), 38 EV (32.2%) presented a half-maximal (50%) inhibitory concentration (IC50) value of 10 mm^2/ml in A549 cells (95.8%), against 6 EV (5.1%) in SK cells. The results obtained in the A549 cells reveals that IC50 values are lower in IL than in EV. Distribution of IC50 values per workstation is presented on Table 2. It must be taken in consideration that the use of antibiotics in cell culture medium, to protect susceptible mammalian cells from bacterial infection, might have compromised to some extent the bacterial contamination of the FRPD EV extracts, hence, the measured cytotoxicity may be underestimated. On the other hand, the interpretation of results should also consider that *in vitro* responsiveness is higher than the observed *in vivo* (with additional protection of the immune system).

3.3. Correlation and comparisons analysis

Correlation analysis was achieved with the bioburden characterization (Viegas et al. 2020b) and the cytotoxicity grading by means of the MTT assay (IC_{50} (mm^2/ml) of 118 IL (Viegas et al. 2020d) and 118 EV (present research). This was done to understand the differences on cytotoxicity effects between IL and EV from FRPD, and to study the relation of the observed cytotoxicity with the bioburden on IL and EV. The detailed overview of the results with the 118 IL and 118 EV are shown in the Table 3.

Regarding bacterial contamination (in TSA and VRBA), fungal contamination (in MEA and DG18), fungal biomass, *Penicillium/Aspergillus* and *Fumigati* section of the IL, no correlation with IC50 values was found, either in the IL either in the EV. In EV, only a correlation was detected between bacterial counts in TSA and IC50 values in A549 ($r_S = -0.319$, $p = 0.047$), which indicates that greater bacterial contamination in TSA is related to lower IC50 values, thus, higher cytotoxicity in A549 (Table 3).

On the basis of the comparison of IC50 values for IL and EV in A549 cells, it was found that the EV showed significantly higher IC50 values, thus, lower cytotoxicity ($z = -4.455$, $p = 0.000$) than IL (Table 4). No comparison analysis was possible with SK cells.

Concerning the EV, statistically significant differences were only detected regarding fungal biomass ($\chi^2_{K\text{-}W}(3) = 13.649$, $p = 0.003$), having been verified that the SW and FMW workplaces presented higher

Table 1
Bioburden distribution on IL and EV analysed.

IL						EV					
Fungal contamination											
MEA			DG18			MEA			DG18		
Species	CFU/m^2	%	Species	CFU/m^2	%	Species	CFU/m^2	%	Species	CFU/m^2	%
C. sitophila	22.5x10^5	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 × 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 contamination											
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. (2020b,d)

Table 2
Distribution of IC50 values per workstation.

Workstation	IC_{50} (mm^2/ml)															
	Interior Layer[1]								Exhalation valves							
	A549				SK				A549				SK			
	10	5	2.5	1.25	10	5	2.5	1.25	10	5	2.5	1.25	10	5	2.5	1.25
FMW (n = 40)	0	23	0	0	0	2	0	0	13	0	0	0	5	0	0	0
MI (n = 4)	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MSVO (n = 13)	0	5	1	0	0	0	0	0	3	0	0	0	0	0	0	0
SW (n = 53)	0	23	2	1	0	2	0	0	18	1	0	0	1	1	0	0
n.s. (n = 8)	0	5	0	0	0	1	0	0	4	0	0	0	0	0	0	0

[1] Results from Viegas et al. (202b,d).

Table 3
Study of the relationship between bacterial counts (TSA and VRBA), fungal counts (MEA and DG18), Fungal biomass, *Penicillium/Aspergillus*, *Fumigati* section with IC50 values (in A549 and SK cells) in the IL and in the EV.

			Interior Layer		Exhalation Valves	
			IC_{50} (mm^2/ml)			
			A549	SK	A549	SK
Interior Layer	Bacterial counts	TSA	−0.058	–	0.074	−0.509
		VRBA	0.201	–	−0.032	0.569
	Fungal counts	MEA	0.101	–	−0.144	0.103
		DG18	0.067	–	−0.216	−0.612
	Fungal biomass (uL)		−0.084	–	−0.216	−0.204
	Penicillium / Aspergillus (uL)		−0.014	–	−0.231	−0.204
	Fumigati section (uL)		0.105	–	0.020	−0.354
Exhalation Valves	Bacterial counts	TSA	−0.061	–	−0.319*	0.535
		VRBA	0.104	–	−0.187	−0.450
	Fungal counts	MEA	0.125	–	−0.150	−0.212
		DG18	0.028	–	−0.154	0.000
	Fungal biomass (uL)		0.001	–	0.195	−0.612
	Penicillium / Aspergillus (uL)		0.128	–	0.042	−0.655
	Fumigati section (uL)		0.239	–	–	0.000
Interior Layer	IC_{50} (mm^2/ml)	A549		–	−0.082	–
		SK			–	–
Exhalation Valves		A549				–

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

Table 4
Comparison of the interior layer and exhalation valves in relation cytotoxicity (in A549 and SK): Wilcoxon test results.

			N	Ranks		Test Statistics [d]	
				Mean Rank	Sum of Ranks	z	p
IC_{50} (mm^2/ml)	A549 in the Exhalation Valves - A549 in the Interior Layer	Negative Ranks	0[a]	0.00	0.00	−4.455[e]	0.000*
		Positive Ranks	22[b]	11.50	253.00		
		Ties	1[c]				
		Total	23				
	SK in the Exhalation valves – SK in the Interior Layer [f]						

a. A549 in the Exhalation Valves < A549 in the Interior Layer. b. A549 in the Exhalation in the Valves > A549 in the Interior Layer. c. A549 in the Exhalation Valves = A549 in the Interior Layer. d. Wilcoxon Signed Ranks Test. z. Based on positive ranks. e. Based on negative ranks. f. It was not possible to make this comparison, given the very low number of FRPD with simultaneous information of Interior layer and exhalation valves. *Statistically significant differences at the 5% significance level.

values (Fig. 2(A)). With regard to IC50 values, no statistically significant differences were detected between the different workplaces, either in the IL or in the EV (Fig. 2 (B)).

4. Discussion

Since 2008, European countries have committed to reduce waste production and to maximize recycling and re-use, aiming to ensure the full implementation of waste policy goals in all member states (Council of the European Union 2008). The waste sorting industry is one of the key points to achieve the EU's milestones and also the Sustainable Development Goals (SDGs) proposed by the World Health Organization. The increased waste recovery promoted by circular economy leads to a large workforce involved in waste management. Thus, while improving waste management helps to reduce health and environmental problems, workers' exposure to contaminants during waste sorting can have a negative impact on workers' health. It is therefore of the utmost importance to ensure safe working conditions. In view of the EU's commitment, exposure assessment in this occupational setting is crucial for adequate risk characterization and management.

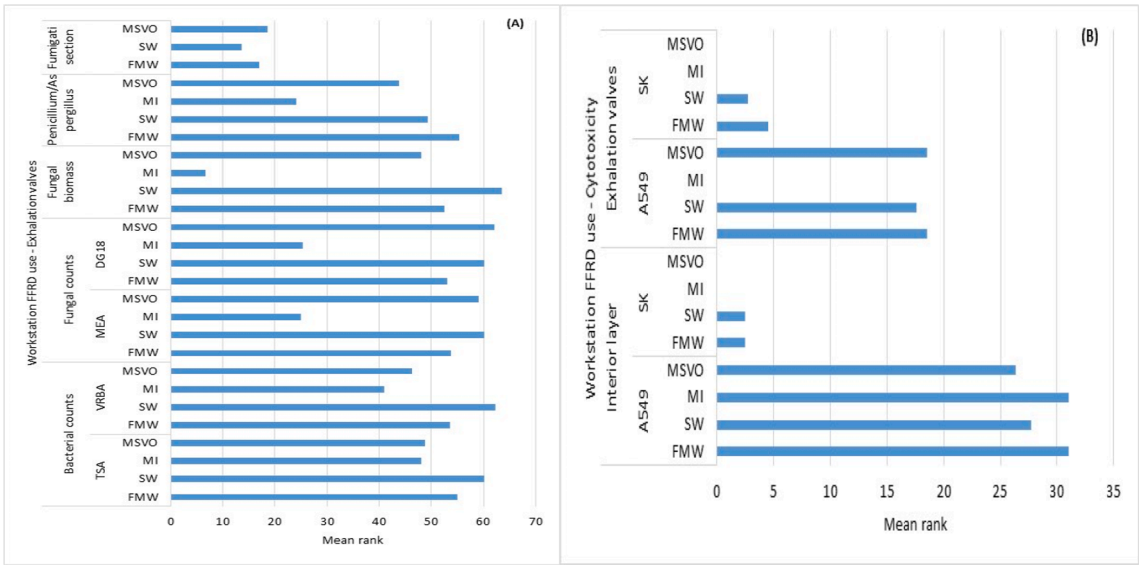

Fig. 2. Comparison of bacterial contamination (TSA and VRBA), fungal contamination (MEA and DG18), Fungal biomass, *Penicillium/Aspergillus* and *Fumigati* section in the EV and IC50 values in the IL and EV (the mean ranks are represented - Kruskal-Wallis test results).

Previous studies performed in the same waste–sorting industry where the FRPD were collected, reported similar results for fungal species distribution. In the same unit and relying on active (air sampling) and passive methods (surface swabs) the prevalent fungal genus found on the environment were quite similar with the ones found in the FRPD (Viegas et al. 2015) and the use of FRPD as a passive sampling method was suggested (Viegas et al. 2020b), driven also by the fact the FRPD (IL and EV) of the workstations with greater contact with waste had the highest levels of microbial contamination (Viegas et al. 2020b, d).

The differences in cytotoxicity between EV and IL were statistically significant ($z = -4.455$, $p = 0.000$) on lung cells (being lower in EV). The MTT assay, as applied in this study, was successful in discriminating between IL and EV of FRPD. The test results clearly indicated that the EV caused less adverse effects on the lung cells' viability than IL. These differences might be a consequence of lower accumulation of contaminants in the exhalation valve, since EV is closer to the workers' nose and mouth, thus, contaminants can be inhaled and be less accumulated than in FRPD interior layer. As such, exhalation valves are suitable matrices to screen for microbial contaminants of FRPD and assessment of occupational exposure to bioburden, since they can be a contamination source for exposure by inhalation (Viegas et al. 2020b).

Lung epithelial cells have proved to be an important first line of defence of the host's innate immune system, both as a physical barrier and as producing inflammatory factors, and can even kill microbes directly (Krishnan et al., 2010; Osherov, 2012; Parker and Prince, 2011). Changes of gene expression in lung epithelial cells related to exposure to fungal infections, namely, by *A. fumigatus,* have also been described (Osherov, 2012; Chen et al., 2015). The human cell line of type II origin, A549 cells, can be exposed to contaminants *in vitro* in order to evaluate the effects of exposure through inhalation to those contaminants on the lung epithelium (Seagrave and Nikula, 2000). Among the adverse effects that exposure to air pollutants can cause, impairing lung function, is cell death. Although other adverse effects can occur, independently or in a related way (e.g. alterations in gene expression with cytokine production) (Chen et al. 2015), in this study the assessed parameter was cell viability by measuring cellular metabolic activity by the MTT reduction assay. A meaningful link between the *in vitro* results and their implication for human health still needs to be established. A contributing factor that requires further elucidation is the effect chemical contaminants and particles, potentially present in FRPD, would have on cell viability.

Regarding SK cells, they are routinely used to assess mycotoxin cytotoxicity (Hanelt et al. 1994) and nephrotoxic effects of xenobiotics in humans (Gunness et al. 2010) and in this study they were used as model for renal toxicity. It is known that renal toxicity often relates with exposure to mycotoxins (Hope and Hope (2012). In a previous study (Viegas et al. 2020a), *Aspergillus* section *Nidulantes* exerted strong cytotoxic effects on SK, probably due to sterigmatocystin, a hepatotoxic and carcinogenic mycotoxin produced by this *Aspergillus* section whose toxic effects include kidney and liver damage (Gniadek et al. 2017; Viegas et al. 2020a). The fact that no mycotoxins were recovered from FRPD IL and EV in this setting (Viegas et al. 2020b) explains the low cytotoxicity effect of FRPD in SK cells observed in this study.

As abovementioned, the cytotoxicity effect of EV samples might be related with the presence of chemicals (Viegas et al., 2017; Ladeira et al., 2020), particulate matter (such as micro- and nanoplastics; not assessed in this study) (Xu et al. 2019) and chemicals and natural toxins that they carried or due to natural toxins that are airborne (Gniadek et al., 2017; Viegas et al., 2020bcd). Nevertheless, we postulate that the observe cytotoxicity effects are most likely linked to microbial contamination than chemical pollutants, as high levels of chemical contaminants would cause acute toxicity among workers, whereas low chemical concentrations rarely have an effect on cell viability (Prinsloo et al. 2013). Of note, a statistically significant (at the 0.05 level (2-tailed) correlation was determined between bacterial counts in TSA and IC50 values in EV (on A549 cells), indicating that greater bacterial contamination (TSA) is related to higher cytotoxicity (on A549 cells). No significant correlations were observed, however, between fungal contamination and cytotoxicity. These results were surprising, as in a previous study evaluating the air-conditioning filters from 18 forklifts operating in the waste sorting, the cytotoxicity effect was correlated with fungal contamination (*Alternaria* species), while no correlation was found with bacterial contamination (Viegas et al. 2020a). Thus, the identification, besides quantification, of the microbiota in occupational exposure assessments should be more often considered in order to better predict health risks for workers in specific settings.

More studies on this issue, including the co-assessment of chemical contaminants, are needed to explain the importance of cytotoxicity assessment in composite environmental samples (Viegas et al. 2017, 2020b,c). The results from this study also show that the use of relevant cell lines provides useful information on biological responses triggered by co-exposure to multiple stressors, including microbial contaminants (Segura et al., 2009; Viegas et al., 2017). Further *in vitro* toxicology studies with additional cell lines and other biomarkers are important to

better estimate health risks. In summary, *in vitro* toxicology combined with an accurate assessment of exposure to microbial and chemical contamination is a relevant approach to obtain useful information on the potential health effects of co-exposure to multiple stressors.

5. Conclusions

This study demonstrates how filtering respiratory protection devices (FRPD) can be used in biological effect studies using relevant cells lines (A549 as a model for the human alveolar epithelia, and swine kidney as renal cells) with the MTT assay to evaluate microbial contamination. The MTT assay characteristics (rapid, efficient and cost-effective) make this cell-based assay attractive to demonstrate whether filtering respiratory protection devices used in a variety of settings are adequate for human protection.

The cytotoxicity effect observed for FRPD EV in epithelial lung cells suggests inhalation as a potential route of exposure through the use of FRPD in the waste-sorting industry. Based on the differences found in the cytotoxicity effects between EV and IL, and the lack of correlation between them, we propose that both should be considered in sampling strategies for bioburden exposure assessment. The correlation between lower metabolic activity and higher bacterial contamination (lung cells) reinforces the importance of the correct use of FRPD by workers and adoption of hygienic measures to avoid contamination during their use.

Overall, these findings are to be considered when refining the risk management measures to implement in the waste sorting industry with the aim of controlling workerś exposure by using FRPD. Other risk management measures should be implemented to avoid that workers protection are only dependent on FRPD use, such as decreasing exposure and number of workers exposed. Finally, training and education programs for workers must include information on the proper use of FRPD including adopting the FRPD replacement frequency to avoid contamination.

CRediT authorship contribution statement

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

Declaration of Competing Interest

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

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