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On the need for reference values for culturable bioaerosols : 4 examples of fungal surveys

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SUMMARY
Since September 2000, on medical request, the Regional Unit for Indoor Pollution Intervention (French acronym: CRIPI) conducts indoor pollution analyses in Brussels dwellings, nurseries and schools. In this context, CRIPI is often confronted with visible mouldy surfaces. In practice, fungal investigations often need both a visual screening as well as air samplings in order to complete the diagnosis. Additionally, threshold values can be used to decide whether the presence of a bioaerosol is suspect. In this document, we present four cases of fungal investigations in the indoor environment conducted by CRIPI. Results interpretation was performed by means of the fungal air index developed in the framework of CRIPI. The first case concerns a nursery with a high level of airborne *Penicillium*. The three other cases concern dwellings with abnormal high concentrations of airborne *Aspergillus versicolor*.

KEYWORDS
Airborne mould concentrations, nursery, dwellings

1 INTRODUCTION
For surveys with visible mould contamination on surfaces, one generally agrees that sampling is not needed. The rehabilitation actions are known and can be proposed, at least in a first stage. In case species identification is necessary, e.g. for a specific medical request, sampling and analyses in various matrices (air, dust, water) are indispensable. In general, risk assessment of biological airborne pollutants (IPC, 2004) account for several difficulties. In first instance, the diversity of the current methodological approaches do not allow a consensus on the guideline values. Moreover, the commonly known definition of the guideline limit value (GLV) for indoor air is provided by the WHO (World Health Organisation). The GLV is defined as the concentration below which the concerned air pollutant should have no detrimental effect on the health of the general population. Although the naming may vary from country to country (‘Guideline Limit value’ (GLV), Toxicity Reference values (TRVs) ‘Threshold Limit Values’ (TLV)), all of these limit values are based on available clinical, epidemiological and toxicological data. Currently, numerous chemicals have an associated

GLV. Data on the health effects of culturable bioaerosols are still lacking. In this context, a progressive abolishment of the quantitative environmental approach by most Governmental Agencies is noted, especially for air. However, in practice, a microbiological investigation needs reference values. In order to overcome this problem, one uses concentration values primarily based on environmental data exposures, allowing to compare a specific situation against a set of similar reference situations. These Guide Values (also named "Recommended Values" or "Target level Values") are useful to reveal abnormal levels of specific contaminants, not always visible to the naked eye, in order to propose appropriate remedial measures. However, they differ depending on the country and used methods. The exposure assessment data (IPCS, 2004) are not directly linked to clinical symptoms but may be supplemented with information concerning the hazard of the identified pollutants (CCOHS, 2009). This may help the physician to improve the medical diagnosis and treatment.
In this document, four cases of fungal investigation in the indoor environment in Brussels are presented. The surveys were conducted by the Regional Unit for Indoor Pollution Intervention (French acronym CRIPI). Three out of four cases concerned 'without visible fungal contamination', and among them, only one was equipped with a mechanical ventilation. Result interpretation was performed by means of the fungal air index from CRIPI. The first case concerned a nursery with a high level of airborne *Penicillium.* Literature clearly shows that exposure to indoor air pollution at an early age enhances the risk of acute lower respiratory system infections in children under the age of 5 years (*Bladt et al., 2015*). The three other cases concerned abnormal levels of airborne moulds, especially *Aspergillus versicolor,* in dwellings of patients suffering of respiratory problems.

2 MATERIAL AND METHODS

During each inquiry conducted by CRIPI, several investigations and samplings are systematically performed. Visible mouldy surfaces are sampled using tape and present species are further identified. In addition, dust samples from furniture or dust present in carpets or mattresses are also taken into account. In parallel, air samplings were performed in each room and outdoors using a RCS+ (Biotest®, 80 liters). Strips filled with HS Agar media were used and incubated at 25°C during 5 days to isolate and identify the mesophilic hygrophilic moulds. The first reported case concerned a survey in a nursery. In addition to air samplings, sand from a suspected sandbox was also analysed. Sand samplings were performed in 3 places at 2 levels of depth using a cereal grain sampler. In the laboratory, 1 g of sand was suspended in a physiological tween solution and agitated during 20 min. Afterwards, a 1/10 dilution was plated on Malt Extract Agar Chloramphenicol for the detection of hygrophilic moulds and on M40Y+10%NaCl for the detection of xerophilic moulds. In the three other reported cases, only airborne mould results were taken into account. Results interpretation was performed using the "Airborne Fungal Index of CRIPI" (*Chasseur & al, 2015*)

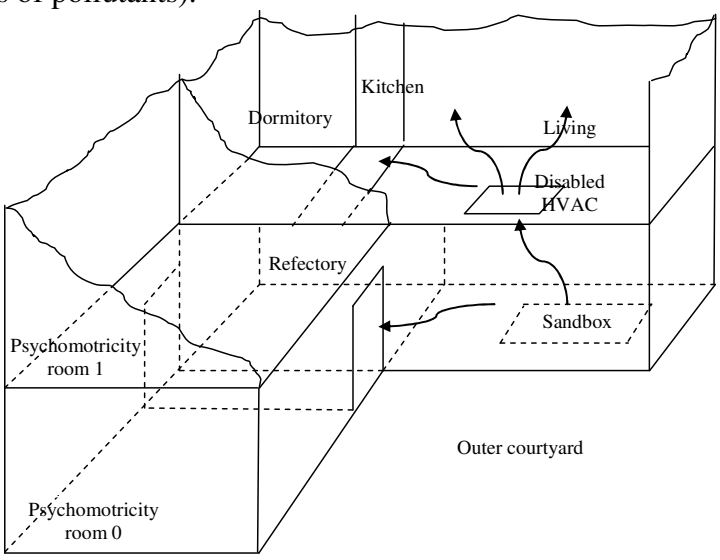
3 RESULTS

A case report of a survey in a nursery

The concerned nursery was located on the first floor of a building and could accommodate 22 children between 3 and 36 months old. No visible mouldy areas and humidity problems were detected. The nursery itself was rather small, with a living and a dormitory separated by a small kitchen. In the living an old and disabled HVAC system (Heating, Ventilation and Air Conditioning) was observed, with air ducts still connected with the ground floor. The rest of the building was dedicated to games and activities for older children: on the same floor as the nursery, there was a psychomotricity room (psychomotricity room 1). On the ground floor a second psychomotricity room (0), a large refectory, and an old indoor pool converted in

sandbox to welcome older children (Figure 1) were located. The sandbox was situated just below the living room of the nursery.

Figure 1: plan of the building with the nursery on the first floor (represent possible dispersal routes of pollutants).



The mould results of a first intervention on December 14th, showed unacceptable airborne concentration levels of *Penicillium spp.* according to the CRIPI Index.

Table 1: results of airborne *Penicillium spp* CFU/m^3) performing during 4 successive interventions.

Successive interventions	**1** December 14	**2** December 22	**3** January 05	**4** March 16	
	Contamination detection	Contamination confirmation	Sources localization	Source confirmation, propagation path	
		In the nursery			
Dormitory	1 450	550	438	3 475	>3 425
Kitchen	1 000	213	2 038	>3 838	>3 288
Living				>5 763	>6 813
- middle	>7 950	2 325	10 625		
- on the left of HVAC	/	/	/	>3 088	>4 663
- on the right of HVAC	/	/	/	>12 063	>24 013
- air outlet of HVAC	/	/	/	>15 438	>13 713
		Extended to other rooms in the building			
Psychomotricity room 1	>12 325	>34 000	>13 175	>10 513	>10 863
Psychomotricity room 0	/	/	/	>12 300	>11 150
Sandbox	/	/	> 30 000	>11 088	>9 250
Refectory	/	/	4 700	>8 300	>5 175
		Outdoor reference			
Outdoor	175	13	175	50	88

Penicillium spp. can originate from several sources, namely: indoor humidity, fruits, cheese, or anthropogenic outside activities. In order to confirm these results, a second survey was conducted promptly after the first one on December 22th. Similar results as for the first survey were obtained as seen in Table 2. However, again, it was not possible to identify the origin of contamination. Therefore, a third survey was conducted on January 5th in order to include the other rooms of the building, especially for the ones located on the ground floor (Table 1,

column 3). After the third survey, it became clear that the origin of the *Penicillium spp.* contamination was located on the ground floor, more specifically in the room with the sandbox. In order to confirm the sand being contaminated by *Penicillium spp.,* an additional sampling campaign was performed on March 16th. The sandbox was sampled at 3 places at the surface and at 3 places at the bottom of the sandbox using a cereal grain sampler. *Penicillium spp.* concentrations were clearly higher at the bottom than at the surface of the sandbox as seen in table 2. This clearly showed the origin of contamination.

Table 2: *Penicillium spp.* concentrations in three sand samplings at the surface and three samplings at the bottom of the sandbox (CFU/g).

	1	2	3
At the surface	9 250	6 400	8 600
At the bottom	250 000	40 000	192 000

During the fourth survey, the air in the nursery was also sampled. These results showed how contaminated *Penicillium* air was introduced in the nursery through the air ducts of the disabled HVAC system (Table 2, column 4, 2 repeats). Concerning the identified pollutant, recent studies have shown that exposure to airborne *Penicillium* is associated with increased peak expiratory flow variability in asthmatic children (*Bundy, 2009*). In this context, the elimination of the contaminated sandbox, followed by a thorough cleaning of all the concerned rooms in the building were the first preventive sanitary measures to protect the health of the young children.

Case report in three patient dwellings

The first case concerned a survey conducted in May, in a dwelling of a patient with asthma. During the survey, no visible mouldy areas nor problems of humidity were noted. The total airborne mould concentration was lower than the outdoor reference. However, *Aspergillus versicolor* indoor concentrations were twice the alert level in the CRIPI Index (Table 3), and the weak outdoor spores concentration measured during the survey reinforces the possibility of an indoor origin.

Table 3: identified and quantified airborne moulds in the first patient's dwelling.

Places	Total moulds	*Aspergillus versicolor*	*Penicillium spp.*	*Cladosporium herbarum*	*Sterile mycelia*
Living	213	**88**	0	50	25
Kitchen	150	0	25	38	75
Bathroom	225	**100**	38	38	63
Patient bedroom	175	50	25	38	50
Outdoor	538	25	13	**250**	**225**

The second survey was conducted in the beginning of June, in a dwelling of a patient suffering from chronic rhino-sinusitis. Among the four presented examples, it is the only case with a mechanical ventilation. As in the previous survey, no visible mouldy areas nor humidity problems were detected. Total mould indoor air concentrations were rather weak except for the patient's office, as seen in table 4. By contrast, outdoor air was very contaminated, but identified species are not the same. *A. versicolor* concentrations of 163 CFU/m^3 were observed in the patient's office as well as in the system outlet of pulsed air, measured in the kitchen (Table 5). Such high concentrations are considered as an alert level in the CRIPI Index. An additional indoor mould species, namely *Cladosporium sphaerospermum* was also detected in the office (213 CFU/m^3), meanwhile *C. herbarum* was more abundant in the outdoor environment. Similar results were found for sterile mycelium (475 CFU/m^3). The patient's office was clearly the source of contamination and the pulsed air system could contribute to the dispersal of fungal spores within the dwellings.

Table 4: identified and quantified airborne moulds of the second case.

Places	Total moulds	*Aspergillus versicolor*	*Penicillium spp.*	*Cladosporium sphaerospermum*	*Cladosporium herbarum*	*Sterile mycelia*
Living	150	0	38	0	13	50
Kitchen (outlet of pulsed air)	250	**163**	0	0	13	75
Patient's bedroom/bathroom	25	13	0	0	13	0
Son's bedroom	50	0	0	0	0	38
Office	**913**	**163**	0	**213**	275	238
Outdoor	2,225	25	13	0	**2,225**	**475**

In this kind of ventilation system, filters (G3) are not very effective, especially for particles smaller than 3 µm (the spore size of *A. versicolor*). Additionally, some indoor contaminants ejected outdoors can be reintroduced in the indoor environment in case the air inlet and outlet are in close proximity. The third survey was conducted in June, in a dwelling of a patient with respiratory problems and pneumonia. In this case, some visible mouldy areas were observed in the bathroom and in the patient's room. However, identified moulds were not the same as the ones observed in the sampled air. On the visible mouldy surfaces, *C. sphaerospermum* and numerous mites were observed. In the indoor air samples, *A. versicolor, Penicillium and Wallemia sebi* prevailed (Table 5). In contrast, they were absent in the outdoor air or present in very low concentrations.

Table 5: identified and quantified airborne moulds of the third case (CFU/m^3).

Places	Total moulds	*Aspergillus versicolor*	*Penicillium spp.*	*Wallemia sebi*	*Cladosporium sphaerospermum*	*Cladosporium herbarum*
Living & kitchen	438	75	50	50	0	63
Bathroom	1764	**513**	**888**	**325**	0	38
Patient's bedroom	2339	**538**	138	**1450**	0	125
Outdoor	613	0	88	0	0	350

The concentration values measured for these mould taxa (Table 5) were several times higher than the one considered as an unacceptable level in the CRIPI Index.

In the three patient's cases mentioned above, *A. versicolor* was present. *Jussila* (2002) showed that *A. versicolor* spores can cause acute inflammation in mouse lungs. The author concluded that such spores may have the potential to provoke adverse health effects, such as infections, in the occupants of moisture-damaged buildings. *A. versicolor* also synthesises important toxic metabolites such as the carcinogenic sterigmatocystin ST (*Piontek & al, 2016*).
In the third dwelling, also an abnormal presence of *Wallemia sebi* was noted. Domestic and workplace exposure to this mould species is known to result in among others an IgE sensitization (*Desroches & al, 2014*). The presence of these taxa in abnormal elevated airborne concentrations should be reported to the physician and they justify rehabilitation measures in order to avoid possible adverse health impacts.

4 DISCUSSION

Up to date, no uniform international standard, with Threshold Limit Values (TLV), has been established. However, several values in relation to recommended concentrations of fungal and bacterial bioaerosols are published. One has to keep in mind that these guide values differ according to countries and used methods. Interpretation of our results combines 3 systems proposed for viable moulds contamination: the indoor/outdoor ratio (in *Rao & al, 1996*), the semi-quantitative approach of *Miller & al (1988)* and the specific index of *Chasseur & al (2015)*.

Concerning the nursery, the main contaminant in the air samples was *Penicillium spp*. For the patient's dwellings, *Aspergillus versicolor* was the dominant species present in air. Our surveys clearly demonstrated that without taking air samples, their presence would go unnoticed. It should be noted that presence of moulds does not always imply contamination. However, in the case of the nursery and the 3rd home indoor airborne mould concentrations were so specific and so high compared to outdoors that there was no doubt. This was not the case for the first and second home. In these cases, the indoor/outdoor ratio was reduced. Therefore, it was important to use a second support of results interpretation, namely the CRIPI Index to refer to specific exposures in similar environments and in a progressive way.
This quantitative approach can also help in locating the source of pollution in case of microbiological concentration gradients, such as in the described nursery.
In any case, 'the air' remains an unstable matrix that is subjected to many factors. In order to ensure (mould) detection, precautionary measures such as closing the windows a few hours before the survey should be taken into account. Also, a multitude of samples should be performed in order to cover different matrices. Last but not least and if necessary, results should be confirmed by performing a second or a third intervention.

5 CONCLUSIONS

The presented examples of mycological surveys show the importance of air samplings and guide values. A mycological investigation cannot be limited to a simple visual observation. Additional air data seem to be indispensable in order to obtain a complete image of the presented indoor air problem and the reported health problems. It is also important to refer to specific reference values, especially in cases of rather low concentrations.

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