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Survey of 1012 moldy dwellings by culture fungal analysis: Threshold proposal for asthmatic patient management

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Abstract

Different countries have tried to define guidelines to quantify what levels of fungi are considered as inappropriate for housing. This retrospective study analyzes indoor fungi by cultures of airborne samples from 1012 dwellings. Altogether, 908 patients suffering from rhinitis, conjunctivitis, and asthma were compared to 104 controls free of allergies. Portuguese decree law no 118/2013 (PDL118), ANSES (a French environmental and health agency) recommendations, and health regulations of Besançon University Hospital were applied to determine the rates of non-conforming dwellings, which were respectively 55.2%, 5.2%, and 19%. Environmental microbiological results and medical data were compared. The whole number of colonies per cubic meter of air was correlated with asthma ($P < 0.001$) and rhinitis ($P = 0.002$). Sixty-seven genera and species were detected in bedrooms. Asthma was correlated to *Aspergillus versicolor* ($P = 0.004$) and *Cladosporium* spp. ($P = 0.02$). Thresholds of 300 cfu/m^3 for *A. versicolor* or 495 cfu/m^3 for *Cladosporium* spp. are able to discriminate 90% of the asthmatic dwellings. We propose a new protocol to obtain an optimal cost for indoor fungi surveys, excluding surface analyses, and a new guideline to interpret the results based on >1000 cfu/m^3 of whole colonies and/or above threshold levels for *A. versicolor* or *Cladosporium* spp.

KEYWORDS
allergies, indoor air, low cost measurement, mold risk, threshold, unworthy dwelling

1 | INTRODUCTION

Microorganisms, mites, insects, pets, and humans live in the same habitats, with the same range of temperature and relative humidity. This biological presence can affect human health.[1] A sterile indoor environment is not possible, nor perhaps desirable.

Musty odors and mold development on walls disturb and socially downgrade inhabitants. Dampness or mold in the home was associated with depression.[2] Moisture in dwellings has indoor (bathing, cooking, condensation due to high relative humidity, and cold walls, etc) or outdoor sources (flooding, water leakage, etc).[3] Observation of moisture and the presence of mold in dwellings have been associated with respiratory disorders,[4,5] such as wheezing[6] or asthma.[7] However, due to insufficient measurement, no causality has yet been clearly demonstrated between indoor exposure and allergenic diseases.

Respiratory difficulties, cough, rhinitis, and conjunctivitis are the main reasons for medical consultation. Now, people associate their degraded dwelling with their clinical symptoms and ask for the mold risk to be evaluated. Consequently, inhabitants seek administrative, judicial, or medical means to improve their dwelling condition. In France, since 1991, Medical Indoor Environment Counselors (MIEC) have been recruited by various structures to lead investigations in patients' dwelling to help them to improve their living conditions and their health.[8]

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No threshold limit values for mold (TLVs) have been given by the World Health Organization (WHO) indoor air quality guideline.[9] Currently, there are no United States of America-Environmental Protection Agency (USA-EPA) regulations or standards for airborne mold contaminants.[10] Different countries (Finland, Belgium, Brazil) have tried to define guidelines to quantify what levels of fungi are considered as inappropriate for dwellings for various kinds of patients compared to the healthy population. Some countries such as Finland have described the measurement protocol but have not defined a threshold.[11,12] In 2017, the Belgian Ministry of Health noticed that for each of its three regions (Flanders, Wallonia, Brussels) there were different criteria to characterize dwellings at risk, but none of them had been chosen as a reference because they were considered unusable (www.health.belgium.be).[13] However, in Brazil,[14,15] two texts from the Brazilian Health Surveillance Agency (Anvisa) from 2000[16] and from 2003[17] were published establishing referential standards for indoor air conditioning with a maximum limit of 750 cfu/m^3 for mold. In France, debate on measurement and mold thresholds began in 2004-2006 with the working group of the French Superior Council of Public Health (CSHPF) which provided rules for dwelling environmental analysis. They were essentially based on classifying moldy surface areas (>3 m² required remediation) and listing at-risk fungi, but no threshold was defined for culture analysis at that time.[18]

At the Besançon University Hospital ((UHB) Besançon, France) we developed our own interpretation reading grid.[19] The cultures of air results are subdivided into four classes and the surface results are summarized in compliance with the same rules as those implemented by the CSHPF.

In 2013, Portuguese decree law no 118 (PDL 118) introduced new criteria concerning the interpretation of air cultures, including the whole fungi count, but the PDL 118 also added specifying limits for every potentially allergenic or toxic species (ie, *Aspergillus versicolor* or *Stachybotrys chartarum*).[20] These criteria provided the most ambitious grid in Europe for interpreting culture results.

In 2016, the French National Agency for Environment Health (ANSES) recommended that the CSHPF list of at-risk fungi be revised and fixed an "abnormal" threshold for culture at >1000 cfu of total fungi/m^3 and/or moldy area surfaces of more than 3 m² as indicating insalubrity for which health authorities should rehouse inhabitants.[21]

The aim of this retrospective study, analyzing 1012 dwellings, was to evaluate the usefulness of measuring the concentration of indoor fungi by culture to advise patients suffering from rhinitis, conjunctivitis, and asthma who are exposed to unhealthy living conditions.

First, our protocol was evaluated from a microbiological point of view.

Second, PDL 118, ANSES recommendations, and our UHB self-guide for air analysis interpretation were used to determine the rate of non-conforming dwellings.

Third, the environmental microbiological results and medical data for each kind of disease were compared.

Practical implications

- Dwellings with >1000 cfu/m^3 in whole molds, either 300 cfu/m^3 of *Aspergillus versicolor* or 495 cfu/m^3 for *Cladosporium* spp. must be considered as dwellings at risk for allergic patients.
- The Portuguese-decree law no 118/2013 could be modified by raising the thresholds of *A. versicolor* from 12 to 300 cfu/m^3 thus lowering the rate of non-standard dwellings from 55.2% to 23.3%.
- Four species out of 67 isolated from dwellings are correlated to asthma: *A. versicolor* (P = 0.004), Cladosporium (P = 0.02), *Aspergillus niger* (P = 0.03), *Alternaria alternata* (P = 0.05).
- Bedroom airborne samples indicate the best level of indoor fungi pollution of a dwelling. Surface analyses are too dependent on sampling practice and should not be used systematically, except for large contaminated surfaces (>3 m²).

Finally, a new guideline was proposed to interpret the results and to obtain an optimal cost for this kind of survey.

2 | METHODS

2.1 | Subjects

Patients suffering from various respiratory diseases (n = 908) seen in private allergology offices and several allergology and pneumology units of Besançon and Dijon University Hospitals ("Bourgogne-Franche-Comté" region) were referred to the "Réseau d'Allergologie de Franche-Comté" (RAFT) (n = 491) and to the "Mutualité Française Bourgogne" (MFB) (n = 417). The RAFT is a structure organized by the regional agency of health ("Agence Régionale de Santé"—ARS) to help in the management of allergic patients by proposing the advice of a dietitian for food allergies or an MIEC to evaluate the sanitary conditions of dwellings and to suggest improvement. In the western part of the "Bourgogne-Franche-Comté" region, the MFB assumed this role for the members of one of the numerous mutual health insurance companies available. The MFB staff includes two MIEC. (See map Figure 1.)

The allergic patients' homes included in the study were inspected and managed by these two networks, and fungi samples were taken by MIEC. Subjects free of allergic respiratory symptoms from two previous studies were used as the control groups ((Hematologic Group (HG) n = 56)[22] and (Composting Group (CG) n = 48)).[23]

2.2 | Environmental survey procedure

The medical and environmental questionnaires, protocol visits, sampling, and analyses were the same throughout the study for all participants. The MIEC went to dwellings and took on average of four

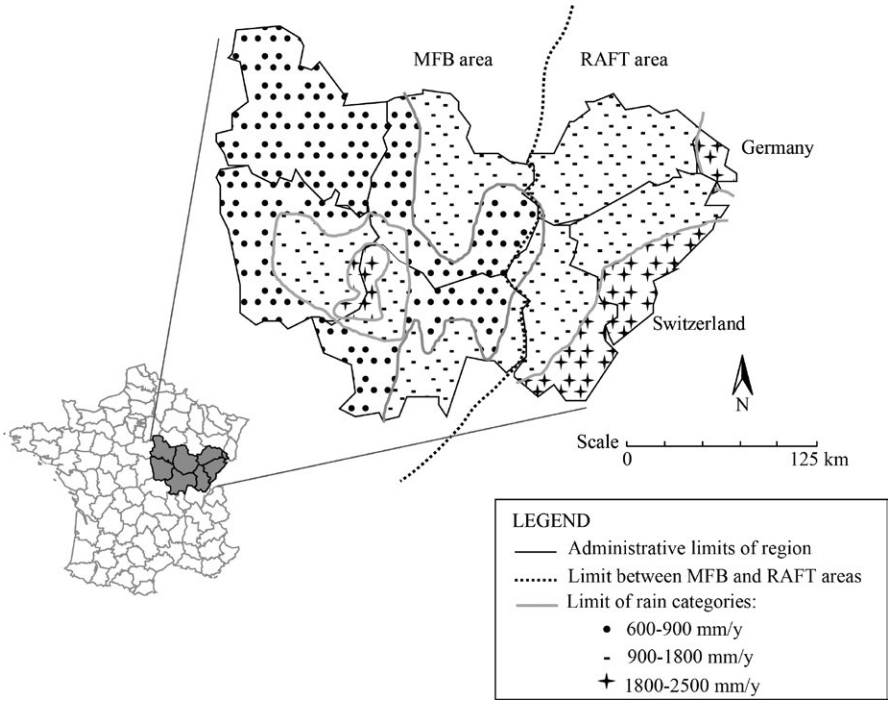

FIGURE 1 Rainfall in sampling area (Burgundy-Franche-Comté)

air samples (100 L each) by impaction (100 L/min) using Mas-100^{TM} device (Merck®, Darmstadt, Germany), a single-stage multi-holed impactor (400 holes/90 mm). At each sampled dwelling, the sampling grid was disinfected beforehand using a 70% alcohol solution and rinsed with sterile water under a class II microbiological security station. For sampling, the device was deposited at a level of 0.8 m on a tripod. The four surface samples (100 cm² each) were taken with wet swabs on surface moisture if present, or 10 cm below the middle of the window (often a damp area) if no surface moisture was visible. Rooms systematically sampled were as follows: bedroom, bathroom, kitchen, and living room. Sampling was carried out with closed windows from 1st October to 30 April, so as to avoid the summer period. Inquiries (visits and questionnaires on flooding, water leakage, aerating, room ventilation habits, etc) were supplemented with different measurements (temperature, relative humidity, mite detection, and gas). In cases where the CO or CO_2 rate was too high, the health authority (ARS) was immediately informed. If the measured temperature was below 18°C and the relative humidity (RH) was higher than 60%, the MIEC advised the inhabitants on how to obtain a temperature between 18 to 22°C with a humidity rate below 40% RH. They also provided other advice in a report on aerating, ventilation, and remediation work necessary to resolve flooding and water leakage. This report was sent to each patient and his medical practitioner.

2.3 | Microbiology lab procedures

Dichloran Glucose 18 (Oxoid®, Basingstoke, UK) with 0.1% chloramphenicol (Sigma-Aldrich®, Steinheim, Germany) was used for each sample and incubated at 20°C for seven days for mold growth. All fungal colonies were identified by macro- and microscopic observation and numbered on each Petri dish.[24-26] The definitive count was obtained by using the "positive hole conversion table" provided by the manufacturer. Results were given in cfu/m^3 of air and in cfu/100 cm² of surface. Mycological results were added to the MIEC recommendations and were sent to both the patient and his/her doctor.

2.4 | Interpretation criteria and thresholds

2.4.1 | UHB criteria

Total fungi air concentrations are respectively considered as low, middle, high, and very high for the contamination classes of 0 to 170 cfu/m^3; 170 to 560 cfu/m^3; 560 to 1000 cfu/m^3; and >1000 cfu/m^3.[19] Interpretations are modulated if high concentrations of any infectious, allergenic or toxic undesirable species (according to CSPHF 2006) are present in relatively high concentrations (up to 50% of the total isolated mold).

Surface concentration is taken into consideration only if the result is >150 cfu/100 cm² in total molds, sampled by wet swab. The culture result is only qualitative and interpreted with the moldy area criteria as proposed by CSHPF. Four classes of moldy surface (called S0, S1, S2, and S3) are defined: S0 no visible mold, S1 < 300 cm², 300 cm² ≤S2 ≤ 3 m², and S3 > 3 m².[18]

2.4.2 | Portuguese decree law no 118/2013 (PDL118)

PDL 118 makes a clear distinction between the various species based on their potential impact on human health.[20] Three conditions are cumulative for considering dwellings at risk for human health:

Seven mold concentrations (*Cladosporium* spp., *Penicillium* spp., *Aspergillus* spp., *Alternaria* spp., *Eurotium* spp., *Paecilomyces* spp.,

and *Wallemia* spp.) ≤500 cfu/m^3 correspond to healthy dwellings, and higher concentrations in the dwelling are considered at risk for human health.

Five more rare genera (including *Acremonium* spp., *Chrysonilia* spp., *Trichothecium* spp., *Curvularia* spp., and *Nigrospora* spp.) <50 cfu/m^3 (if alone) or <150 cfu/m^3 (if many rare species are mixed) are considered compliant. However, above this level, dwellings are classified at risk.

Nine toxic species (*S. chartarum*, *A. fumigatus*, *A. versicolor*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus terreus*, *Fusarium moniliforme*, *F. culmorum*, *Trichoderma viride*) >12 cfu/m^3 are considered dangerous.

A fourth condition regarding three tropical highly pathogenic fungi (class III biohazard) is not applicable in metropolitan France. Only *Cryptococcus neoformans*, a class II b that has been found in Europe, is considered dangerous.

2.4.3 | ANSES recommendations

ANSES states three recommendations[21]:

A culture presenting a concentration in total molds >1000 cfu/m^3 is considered "abnormal" and requires professional intervention for remediation.

The list of the undesirable species is similar to that of the CSPHF 2006: *Acremonium* spp., *Alternaria alternata*, *A. flavus*, *A. fumigatus*, *A. versicolor*, *Aspergillus niger*, *Aureobasidium* spp., *Chaetomium* spp., *Cladosporium sphaerospermum*, *Epicoccum* spp., *Fusarium* spp., *Mucorales (Mucor* spp., *Absidia* spp., *Rhizopus* spp.*)*, *Penicillium* spp., *S. chartarum*, *Trichoderma* spp., *Trichothecium* spp.. Only *Ulocladium* spp. was also added by ANSES.

A moldy area is classified according to its size: low level of contamination if the surface is <0.2 m², average level between 0.2 and 3 m² and high level (substandard home insalubrity) if the surface >3 m².

2.5 | Statistics

Rstudio software (3.2.2 version, Boston, USA) was used for the statistical analyses. A logarithmic conversion was applied to the results obtained by culture after air impaction. A multiple comparison test (after the Kruskal-Wallis test)[27] was used to compare total fungi concentrations between the different groups of dwellings and indoor factors influencing fungal contamination. A linear mixed-effects model incorporating a random effect to overcome the dwelling effect was used to compare total fungi concentrations between the four kinds of rooms.

Logistic regressions were used to explore associations between health data (asthma, rhinitis, and conjunctivitis) and exposure metrics at two levels: in global bedroom contamination and by quantification of different species. For this last part of the analysis, we kept only species present in at least 5% of dwellings. Thus, 12 mold genera and species were included in the model and a stepwise backward selection process was used to find the optimal model. Adjusted odds ratios (aOR) were adjusted on the other species included in the model and calculated using the "odds ratio" package.[28]

3 | RESULTS AND DISCUSSION

3.1 | Description of recruited dwellings

Dwellings situated on the first floor represent <5% and under the roof <2%. Comparisons between the two groups (908 allergic and 104 controls) show no difference based on the kind of dwellings (50% houses, 50% apartments), on the surface area (mean 115 m²), on the flooding and water leakage incidents (around 16%), on ventilation systems (50% active controlled mechanical ventilation (CMV)), on the temperature (a third below 20°C), and on pets (dogs, cats or rodents) equally present in the two groups (around 55%). However, control dwellings were less humid than patient homes (respectively 59% and 12% for the class below 40% RH), contained fewer plants (60% vs 76%), and fewer inhabitants were smokers (17% vs 37%).

3.2 | Sampling and microbiological methods used

MIEC visits are very important for patient management, but sometimes all mold sources cannot be discovered by visual inspection (behind the wallpaper, linoleum or furnishings, carpet, inside interior or exterior walls, in attics, in subflooring).[29] Consequently, a more objective measurement of airborne fungi is needed.

3.2.1 | Swab sampling

Culture swab was systematically lower for MFB than for RAFT (Figure 2A) due to a sampler effect (swab, medium furnished by our lab). Therefore, we decided to stop using this method systematically to evaluate home contamination. In the future, surface sampling by swabbing in an intensive manner will be used only for surfaces >3 m².

3.2.2 | Indoor air sampling

Unlike culture after swabbing, air impaction culture did not differ between MFB and RAFT (Figure 2B). In fact, multiple comparison tests (performed after the Kruskal-Wallis test ($P < 0.001$)) between the different groups (RAFT, MFB, HG, and CG) showed that there were no statistical differences in air culture between dwellings recruited by RAFT or by MFB. Nor were there statistical differences between the HG or CG studies. However, homes with allergenic patients (RAFT and MFB) showed different concentration ranges from those without allergenic people (HG and CG). Thus, as no geographical differences were shown between the two areas of the Franche-Comté region (RAFT: to the east and higher, near the Jura Mountains) and Bourgogne (MFB: to the west and drier plains) (see map Figure 1), we did not take geographical recruitment into account in the analyses presented in the following paragraphs.

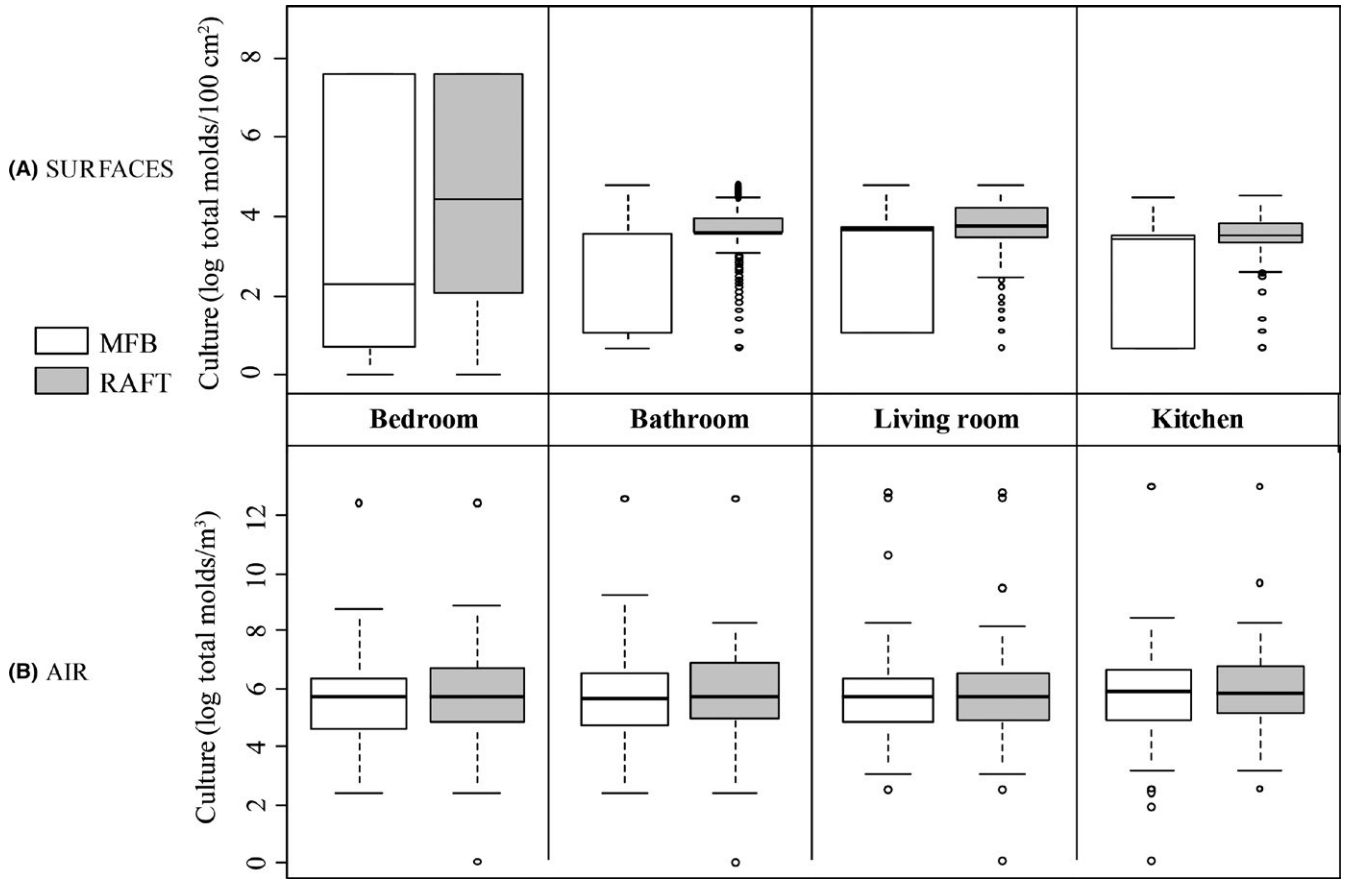

FIGURE 2 Comparison of total fungi concentrations on surfaces and in the air between the MFB and RAFT dwellings and for the 4 kinds of rooms. Boxplot (or box-and-whisker plot) show the distribution of observations. The center value of the graph is the median. The upper and lower sides of the rectangle are the quartiles (first quartile for the lower and third quartile for the upper). The ends of whiskers are greatest and least values excluding outliers which are outside the whiskers and represented by dots

3.2.3 | Outdoor factors influencing fungal contamination

Climatic and geographical position can influence fungal concentrations in indoor air.[30] This is especially the case in subtropical and tropical areas where other species more specific to the tropical zones are isolated in high concentrations (*Curvularia* spp., *Exophiala* spp., *Dreschlera* spp., and *Penicillium decaturense*) in addition to more common species that are also found in continental climates (*Aspergillus* spp., *Penicillium* spp., and *Cladosporium* spp.).[31] Moreover, concentrations of whole fungi in indoor and outdoor air are higher in subtropical and tropical areas than in Europe.[15,32,33]

From a practical point of view, in Europe, the climatic differences between Portugal, France, and Finland are not so important in terms of temperature range, sunshine, and rainy periods[34] (Table 1).[35] In addition, indoor lifestyle (regarding temperature and relative humidity) does not differ in European dwellings very much in winter months. However, indoor fungal contamination can vary greatly for Mediterranean, arid, subtropical, and tropical climates where inhabitants keep their windows open all year round.

It is indisputable that outdoor air influences fungal concentrations in indoor air, more than bacterial communities strongly influenced by the number and the type of occupants living in the home.[36] To avoid this seasonal effect, some authors compare indoor results to outdoor results.[1,30,37,38] It is also true that certain weather conditions, such as snow, can reduce fungi concentrations in outdoor air.[39] Indoor air investigators should interpret indoor / outdoor fungal ratios carefully in case of snow[39] or low winter temperatures in subarctic areas.[30] All in all, the most important factor influencing fungi measurement is whether or not windows are closed. Therefore, like other authors, we take samples only from October 1st to April 30th[19,40-43] so as to avoid outdoor air influence as much as possible. Indeed, it can mask the indoor fungal pollution, in particular that of slow-growing species such as *Cladosporium* spp.[44] To report indoor air pollution and eliminate the season effect, we do not think it is enough to simply subtract outdoor air concentrations species by species[29] or use a ratio of outdoor air to indoor air (I/O) >1 that would be linked to unhealthy dwellings.[39] When interpreting indoor results during the winter, we do not consider outdoor measurements of great significance. Consequently, despite practitioners' insistence, we prefer not to take samples during the summer.

TABLE 1 Climate data from Helsinki to Lisbon

Town	Min temperature (°C)	Max temperature (°C)	Mean temperature (°C)	Rain/year (mm/year)	Sun/year (h/year)	Kind of climate
Helsinki (Finland)	−10	+21	10.5	650	1600-1800	Continental
Lille (France)	+2	+23	10	673	1200-1600	Oceanic
Besançon (France)	0	+25	10.5	992	1600-1800	Continental
Dijon (France)	+2	+23	10.5	768	1600-1800	Continental
Bordeaux (France)	+4	+27	12.7	931	1800-2000	Tempered hot
Lisbon (Portugal)	+8	+27	16.9	691	>2500	Mediterranean

3.2.4 | Indoor factors influencing fungal contamination

The development factors of molds growing indoors are numerous and have been thoroughly listed by Nevalainen et al..[1] Concerning dwellings of our region, we previously described some general dwellings variables associated with an increase in total concentration of fungi in airborne samples.[45] Factors correlated to mold growth were as follows: fewer rooms, high occupant-surface ratio, high number of inhabitants per cubic meter, lack of ventilation, electric heating, apartments located on the ground floor and water damage. Water damage was correlated with an increase in *Penicillium* ($P = 0.03$). However, the following characteristics were not associated with a significant change in the total concentration of fungi: the nature of the sampled room, volume, used to dry washing, presence of a washing machine, presence of windows, air vents in windows, airing time per day, floor covering, presence of plants, outdoor and indoor temperature, indoor hygrometry, geographic zone (urban/rural), age of the house, smoking habits in the dwelling, and presence of pets.[45]

The main causes of increased mold growth in the present study were humidity and water damage, but no statistical difference in air fungal concentration was detected. There was only a statistical difference between the types of dwellings (*P*-value <0.01), with apartments located on the ground floor being more contaminated than others. The discrepancy with regard to the analysis of the previous study[45] is probably due to the more restricted questionnaire on the environmental description of dwellings in the present study.

3.2.5 | Air sampling and room choice

A small but not significant difference in total fungi was observed between rooms of a given dwelling, with higher mold concentrations found in bathrooms (Figure 2B). Bathroom measurements alone appear to be indicative of the contamination level of a dwelling. However, some qualitative differences on isolated genera between a dry room (ie, more *Penicillium* spp.) and a wet room (ie, more *Rhodotorula* spp.) should influence the choice of which room(s) to be sampled. On the other hand, the bathroom is used by each family member only a few minutes per day, whereas the bedrooms are occupied 7 h by adults and up to 10 h by children. Paradoxically, the bedroom is often closed, less ventilated, and cleaned less often than other rooms. It is probably a good idea to continue sampling two rooms (bedroom and bathroom); and if only one is chosen, we believe that the bedroom is the best choice. Beguin and Nolard have suggested investigating the bedroom first and foremost.[46]

3.3 | Comparison of thresholds and guidelines

The distribution into four classes (UHB criteria) of the airborne concentrations in total molds of 1012 dwellings is as follows: low (40.2%), moderate (34.2%), high 6.6%, and very high (19%). Only the last class is considered as not compliant with the UHB criteria.

3.3.1 | PDL 118 vs UHB criteria

Five hundred and fifty-eight rooms (55.2%) do not respect one or several conditions of the PDL 118 (Table 2) against 19% using the UHB criteria. From an institutional point of view, it is not socially manageable to classify more than 50% of dwellings in non-compliance. The high number of non-compliant rooms declared by the PDL 118 is due to the cumulative effect of several conditions: low level needed for *A. versicolor* concentration (12 cfu/m^3) (399 rooms), the whole number of colonies of non-toxic species (>500 cfu/m^3) (268 rooms) (easy to find in 25% of the dwellings), and to a lesser degree the low level needed for *A. fumigatus* concentration (12 cfu/m^3) (72 rooms).

3.3.2 | ANSES recommendations vs UHB criteria

In the present study, only 5.2% of dwellings did not comply with both ANSES recommendations, leading to the rehousing of some inhabitants: >1000 cfu/m^3 (21.5% n = 152/707) and >3 m^2 of moldy surfaces (8.3% n = 59/707) (Table 3).

A discrepancy between visible molds and the low concentration of airborne molds was described in two previous studies.[45,47] This discrepancy analyzed by culture was estimated at 16%, and on the contrary, the presence of a strong airborne concentration with no visible moldy surfaces represented 18% of the samples.[45]

Hidden sources of molds were described by measuring mVOC (microbial volatile organic compounds).[48] In fact, criteria based on simply observing the extent of moldy surfaces in a given dwelling are less reliable and less accurate than measuring concentrations of airborne fungi. Consequently, more objective measures must be

TABLE 2 Comparison of classification of dwellings according to Portuguese decree law no 118 (PDL 118) and University hospital Besançon (UHB) classes (n = 1012)

		Airborne concentrations in total molds: UHB classes			
		Low (<170 cfu/m^3)	Moderate 170 to 560 cfu/m^3	High 560 to 1000 cfu/m^3	Very high >1000 cfu/m^3
PDL 118	Compliant	29.4%	14.8%	0.2%	0.4%
	Not compliant	10.8%	19.4%	6.4%	18.6%

TABLE 3 Comparison of two regulations on dwellings in non-compliance, University hospital Besançon (UHB) classes vs ANSES recommendations (n = 707)

		Airborne concentrations in total molds : UHB classes			
		Low (<170 cfu/m^3)	Moderate 170 to 560 cfu/m^3	High 560 to 1000 cfu/m^3	Very high > 1000 cfu/m^3
Size of moisture surface	0	22%	20.7%	3.4%	7%
	<0.2 m²	8.1%	10.5%	2.7%	4.6%
	0.2 to 3 m²	1.9%	4.3%	1.2%	4.9%
	>3 m²	0.7%	2.4%	0.4%	5.2%

developed and implemented to replace subjective considerations such as odors and the amount of moldy surface area. It seems that ANSES recommendations are excessively high to manage patients with asthma and are more adapted to managing people living in sub-standard dwellings.

3.3.3 | Culture investigations and guideline applicability in France

In the past, French research teams published data on fungi exposure in French dwellings using air cultures (Marseille n = 65, Paris n = 32, Strasbourg n = 61, Nancy n = 90, Paris n = 190, Besançon n = 118, Rennes n = 20, Rennes n = 150).[19,49-55] Most of them were limited to describing essentially four major fungal genera and their species found in dwellings (*Cladosporium* spp., *Penicillium* spp., *Aspergillus* spp., and *Alternaria* spp.).

In Marseille (southern France, Mediterranean climate), *Cladosporium* spp., *Penicillium* spp., *Aspergillus* spp., and *Alternaria* spp. were detected most often,[49] at an order of frequency quite similar to that of Paris (continental climate)[53] and Rennes (western France, oceanic climate).[54] In Strasbourg (eastern France, continental climate), the most common fungi were *Cladosporium* spp., *Aspergillus* spp., *and Penicillium* spp..[51] The only change was in the order of frequency of each of the four most common genera isolated in Nancy (110 km west of Strasbourg),[52] and in Besançon (170 km south-east of Nancy, 210 km south of Strasbourg).[19] The main findings of these previous papers were descriptions of the fungi species present in French dwellings, similar from one area to another, the only change being the order of frequency. Consequently, guidelines determined in one region could be useful in another.

3.3.4 | Other indoor fungi measurements in France

French home investigations were not limited to culture means. QPCR,[54-56] mVOC,[57] and metabarcoding[58] were also used.

We do not believe that it is possible to use the mVOCs measure. Molds do not produce VOC continuously but rather according to their stage of development. The nature and quantity released are also dependent on the nature of the substrate. Another point is that VOC emitted by sources other than mold (up to 100-1000 higher concentrations) are detected in the indoor environment (furniture, paint, wallpaper, etc) thus skewing the analysis.[59]

QPCR requires a prior choice of targeted species. It can quantify both live spores and dead ones, allowing a better estimation of the specific fungal load to which allergic subjects may be exposed.[60]

Although metabarcoding detects all the fungal species present in a given area, systematic quantification at the species level is not possible. The quantification obtained is generally a relative quantification between species. For example, some genera belonging to the mucorales are poorly amplified and thus underestimated. Except for the genus *Epicoccum* spp., the main genera identified in dwellings are the same as in culture. Numerous other rare species have also been identified, including uncultivated species,[58] but time is needed to obtain significantly new results with molecular tools.[61]

3.4 | Correlation between diseases and fungal concentrations in dwellings

We compared the results obtained from 908 patient dwellings and those of 104 non-allergic controls to seek a link between home fungal concentrations and the diseases developed by the patients. Fungi air concentrations were higher for allergenic than

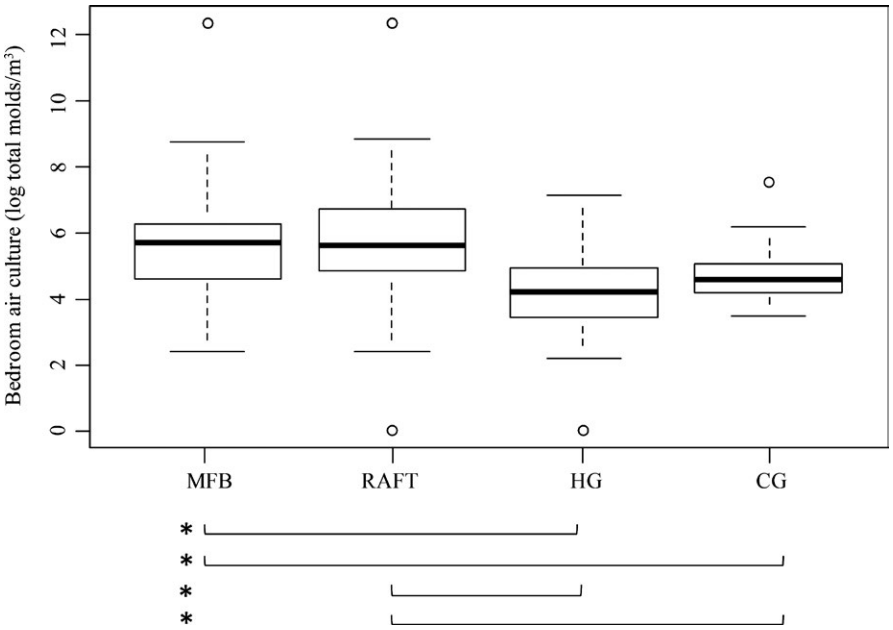

FIGURE 3 Comparison of total fungi concentrations in bedroom air between the four categories of dwellings. Kruskal-Wallis test between the different groups (RAFT, MFB, HG, CG): $P < 0.001$. * Homes with allergenic patients (RAFT and MFB) showed significant differences from those without allergenic people (HG and CG) (multiple comparison test). See Figure 2 for box-plot explanations

for non-allergenic people (logistic regression, $P < 0.001$), and the median values were respectively 285 cfu/m^3 and 89 cfu/m^3 (Figure 3).

Medical diagnoses were established and the disease distribution was as follows: conjunctivitis 101 (11.2%), rhinitis 237 (26.1%), asthma 170 (18.7%), asthma associated with rhinitis 306 (33.7%), and other diseases 100 (11.0%) (nausea, fatigue, hypersensitivity pneumonitis (HP)).

The whole number of colonies per cubic meter of air was correlated with asthma ($P < 0.001$) and rhinitis ($P = 0.002$), but not with conjunctivitis ($P = 0.14$) nor with HP ($P = 0.3$) (Table 4).

In total, 67 genera and species were detected in bedrooms. For species analysis with health data, rare fungi, defined as present in <5% of dwellings, were not added to the analysis. Thus, only 12 species were included in the logistic regression analysis. Asthma was correlated to *A. versicolor* ($P = 0.004$), *Cladosporium* spp. ($P = 0.02$), *A. niger* ($P = 0.03$), and *A. alternata* ($P = 0.05$) (Table 5).

For each of the significantly correlated species, the value thresholds which allowed us to discriminate 90% of the asthmatics were 300 cfu/m^3 for *A. versicolor*, 495 cfu/m^3 for *Cladosporium* spp., and 10 cfu/m^3 for *A. alternata* or for *A. niger*. These values could suggest modifying the recommendations of the PDL 118 on the basis of the observed correlations. Instead of 12 cfu/m^3 of *A. versicolor*, the threshold should be 300 cfu/m^3, 495 cfu/m^3 for *Cladosporium* spp. (included in the non-toxic species), 10 cfu/m^3 for *A. alternata*, and 10 cfu/m^3 for *A. niger* (last two species not included in the PDL 118).

Nevertheless, the number of dwellings concerned by the isolation of *A. alternata* and *A. niger* is not high enough to serve as an indicator of risk. The Mas-100 impactor is incompatible with an optimal sampling of species with large macroconidies (up to 60 μm) and does not allow the systematic isolation of *A. alternata*. The weak frequency of *A. niger* isolation is probably due to the fact that the microconidies are totally smooth (with no ornamentation). This prevents them from remaining airborne for a long time. *Aspergillus fumigatus* should be excluded from the criteria as fungi with mainly an infectious role in immunocompromised patients.

TABLE 4 Logistic regression models of the association between health data (asthma, rhinitis, conjunctivitis, and hypersensitivity pneumonitis) and global bedroom contamination

Asthma	**Yes**	**No**
Global bedroom contamination : mean cfu/m^3 (SD)	1830 (3479)	1019 (2549)
Logistic regression : *P*-value	<0.001	
OR (95% CI)	1.09 (1.05-1.15)	
Rhinitis	**Yes**	**No**
Global bedroom contamination : mean cfu/m^3 (SD)	1706 (3349)	1058 (2638)
Logistic regression : *P*-value	0.002	
OR (95% CI)	1.08 (1.03-1.13)	
Conjunctivitis	**Yes**	**No**
Global bedroom contamination : mean cfu/m^3 (SD)	1731 (3383)	1357 (3001)
Logistic regression : *P*-value	0.14	
OR (95% CI)	1.04 (0.99-1.09)	
Hypersensitivity pneumonitis	**Yes**	**No**
Global bedroom contamination : mean cfu/m^3 (SD)	790 (2015)	1454 (3111)
Logistic regression : *P*-value	0.3	
OR (95% CI)	0.91 (0.71-1.06)	

Some studies did not find any relation between isolated molds and respiratory disease. Although *Cladosporium* spp. and *Penicillium* spp. were the most prevalent fungi in homes of asthmatic and nonasthmatic Swedish children (n = 400), there were no significant

TABLE 5 Logistic regression model, adjusted odds ratios (aOR), and 95% confidence intervals (95% CIs) between asthma and microbial genera and species concentrations

Genera and species selected by model selection process[a]	Asthmatic dwelling: mean cfu/m^3 (SD)	Non asthmatic dwellings : mean cfu/m^3 (SD)	*P*-value	aOR (95% CIs)
Cladosporium spp.	517 (1843)	224 (1029)	0.020	1.12 (1.02-1.2)
Aspergillus versicolor	190 (551)	99 (355)	0.004	1.10 (1.03-1.19)
Alternaria alternata	3 (12)	2 (6)	0.046	1.21 (1.03-1.47)
Aspergillus niger	3 (10)	1 (7)	0.033	1.48 (1.03-2.21)

[a]The other genera and species included in the model before the stepwise backward selection process were *Aspergillus fumigatus*, *Aspergillus glaucus*, *Aspergillus nidulans*, *Aspergillus ochraceus*, *Penicillium* spp., *Wallemia sebi*, *Rhodotorula* spp., and white yeasts. OR were adjusted on the other species included in the model.

differences in the mean fungal concentrations of indoor air between the two groups.[47] Moreover, no association could be found between the spore concentrations of five genera and unidentified yeasts in indoor dust (*Penicillium* spp., *Aspergillus* spp., *Alternaria* spp., *Rhodotorula* spp., *Trichoderma* spp., and yeast) and asthma in children.[62] Similar results were reported in floors, mattresses, dust and the bedroom air of German children's dwellings (n = 397).[43]

On the other hand, *Cladosporium* spp. and *Aspergillus* spp., cultivated from 272 house dust samples, were associated with an increased risk of allergic sensitization in German children.[63] In France, using five qPCR (n = 220 surface samples), quantification of *Cladosporium sphaerospermum* can help to better target social service intervention in moisture-damaged homes (MDH) (MDH vs controls, $P < 0.001$) and quantifying *A. versicolor* DNA could be useful to characterize allergic patient homes (APH) (APH vs controls, $P < 0.05$).[56] Nevertheless, on the same recruitment using air culture in patient homes, our team found significantly higher concentrations of only *Penicillium* spp.[19] Others studies demonstrate that levels of *Penicillium* spp. were a significant risk factor for wheeze in the first year of life[64] and were associated with increased peak expiratory flow variability in asthmatic children.[65] However, serological but not environmental evidence, given in favor of the probable role of *A. versicolor* antigens as major antigens, might enable a species-specific diagnosis of allergic reactions.[66] In a meta-analysis, *Cladosporium* spp., *Alternaria* spp., *Aspergillus* spp., and *Penicillium* spp. were found to be present in higher concentrations in homes of asthmatic patients, exacerbating asthma symptoms in children and adults.[67] In the same way, and with a molecular tool, Reponen and colleagues observed that the sum of three mold species *Aspergillus ochraceus*, *Aspergillus inguis*, *and Penicillium variabile* measured in dust by qPCR for one-year-old children significantly predicted the presence of asthma at seven years of age.[68]

Mendell and colleagues concluded in a meta-analysis that indoor mold was consistently associated with increased asthma development and exacerbation, but specific causative agents have not yet been established.[69] Observed synergistic interactions between numerous microorganisms and insects (fungi, bacteria, and mites) or animal allergens (dogs and cats), and geographical specificity probably explain the difficulties in identifying specific causes affecting health in indoor environments.[70]

While awaiting new data concerning the role of some species in the development of allergic diseases, it is necessary to define new thresholds. So, the results of our study (for *Cladoporium* spp. and *A. versicolor*) could be added to the criteria of 1000 cfu/m^3 of whole molds retained by UHB and ANSES and to the PDL 118 thresholds (for *Acremonium* spp., *Stachybotrys* spp., *A. flavus*, and *A. ochraceus*) to create a new key to interpret the results of air cultures sampled by impaction. Dwellings with more than 1000 cfu/m^3 in whole molds and/or up to 300 cfu/m^3 *A. versicolor*, and/or 495 cfu/m^3 for *Cladosporium* spp., and/or 50 cfu/m^3 for *Acremonium* spp., and/or 12 cfu/m^3 for *Stachybotrys* spp., *A. flavus*, *A. ochraceus* should then be considered at-risk dwellings for hypersensitive inhabitants who could develop asthma or whose already existing asthmatic condition could worsen. With these conditions, 23.3% of dwellings would be considered at risk.

4 | CONCLUSIONS

Asthma is caused by a combination of multiple individual and environmental factors. Its development cannot be explained solely by the presence of one or several specific allergens from fungi, mites, pets, and bacteria. On the other hand, the species of fungi that we described as correlated to asthmatic dwellings are probably markers of particular environmental situations favoring the disease or its exacerbation. As such, they present a risk for patients and require the implementation of measures to protect them (cleaning or rehousing).

Short-term sampling, variable mycological expertise, competition between species on Petri dishes, absence of consideration of the uncultivable species are some of the numerous imperfections of air impaction sampling and culture analyses. Despite their imperfections, these sampling and measurement methods are still commonly used to monitor fungi in indoor air.

Without thresholds and interpretation guidelines, analyzing the domestic environment is a pure waste of time and money. Therefore, we propose a new grid for conducting routine surveys of dwellings fungal contamination. On a European scale, the proposed thresholds can serve as early warnings in continental and subarctic regions, represented in our series by samples taken in Burgundy and in Franche-Comté including the cold Jura mountains.

With regard to couple sampling of air by impaction/culture on DG18, in the present study, we determine which dwelling is at risk by using limited sampling (four air and four surface samples). These indicators on whole fungi concentrations and some specific fungi species are probably more efficient than using chemical compound measurement. Using only one air sample in bedrooms will considerably reduce the survey cost.

Obviously, in the near future, sensors such as the electrostatic dust fall collector (EDC)[71] will make it possible to carry out longer time samples, and molecular tools (qPCR and metabarcoding) to detect and quantify target species.[58,70,72]

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