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"I strongly believe in the rule of compensation. True awards are always in proportion with work and sacrifices."

Nikola Tesla, Serbian inventor

For amazing people I get the privilege to call my family, to you Gordana, Olgica and Draško

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- **Aleksic B**., Bailly S., Draghi M., Oswald IP., Bailly JD., Lacroix M. Développement d'une méthode analytique permettant l'analyse de la toxinogénèse de *Stachybotrys chartarum* sur matériaux, Journée AFSEP, Toulouse, 13th November 2014
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- **Aleksic B.**, Bailly S., Draghi M., Lacroix M., Oswald IP., Robine E., Bailly JD. Production de mycotoxines sur les matériaux de construction par trois espèces fongiques toxinogènes - contaminants communs des environnements intérieurs. Journées Mycotoxines, Toulouse, 15th -16th March 2016

- Lacroix M., **Aleksic B.**, Bailly S., Draghi M., Robine E., Bailly JD. Développement et validation d'une méthode de dosage de mycotoxines par UPLC/MS/MS dans les matériaux d'environnement intérieur. 11ème congrès francophone des sciences séparatives, Paris, 31st March – 2nd April 2015.

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AcN	acetonitrile
ANSES	French agency for food, environmental and occupational health & safety
ANOVA	analysis of variance
ATP	adenosine thiotriphosphate
a_w	water activity
CFUs	colony forming units
$CHCl_3$	chloroform
CV	coefficient of variation
DG18	dichloran glycerol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ERMI	environmental relative mouldiness index
ESI	electrospray ionization source
ESI^+	positive electrospray ionization mode
EtOH	ethanol
EU	European Union
FG	fiberglass
FISH	fluorescence in situ hybridization
FM	epifluorescence microscopy
FWP	painted fiberglass wallpaper
GC	gas chromatography
HPLC	high performance liquid chromatographicy
IC50	concentration inhibiting 50 % of cell viability
IS	internal standard
LC	liquid chromatographicy
LM	light microscopy
LOD	limit of detection
LOQ	limit of quantification
MALDI-TOF MS	Matrix assisted laser desorption/ionization time of flight mass spectrometry
MCT	macrocyclic trichothecenes
MEA	malt extract agar
MeOH	methanol
MF	matrix factor
MPA	mycophenolic acid
MPA-d3	mycophenolic acid - d3
MRM	multiple reaction monitoring
MS	mass spectrometry
MSC	microbial safety cabinet
MSQPCR	mould specific quantitative PCR
MVOC(s)	microbial volatile organic compound(s)
NADP(H)	nicotinamide adenine dinucleotide phosphate
NS	nutritional solution

o-mSTG	o-methyl sterigmatocystin
PBS	phosphate buffer saline
PCA	principal component analysis
PCR	polymerase chain reaction
PD	petri dish
PDA	potato dextrose agar
QC	quality control
RH	relative humidity
RL2	roridin L2
RSD	relative standard deviation
RT	retention time
SBS	sick building syndrome
SD	standard deviation
SEM	scanning electron microscopy
SG	satratoxin G
SH	satratoxin H
STG	sterigmatocystin
TLC	thin layer chromatography
TOX-	non-toxinogenic strain or weakly toxinogenic strain
TOX+	toxinogenic strain
UPLC	ultra performance liquid chromatography
USA	United States of America
VerA	verrucarin A
VerJ	verrucarin J
VOC(s)	volatile organic compound(s)
VWP	vinyl wallpaper
WP	wallpaper

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PART ONE.

Bibliographic review

Context of the study

With new life style today, people spend most of their time inside the buildings and indoor air quality progressively rose as an important concern for human health. There are many described air pollutants, such as: physical, chemical or microbiological pollutants.

Among these, fungi are frequent contaminants with possible important impact on health. Indeed, some species are well known for their ability to produce toxic secondary metabolites named mycotoxins.

These toxic compounds are frequent food contaminants and therefore, their deleterious effect following ingestion have been widely studied and led to the setup of regulations at international level. By contrast, the possible presence of mycotoxins in case of development of toxinogenic moulds on non-alimentary substrates is less documented. However, the frequent identification of potent toxinogenic species in indoor environments, especially following water damages, rose the question of the possible consequences for inhabitants in case of contact with contaminated materials or if toxins can be aerosolized from support to the air and subsequently inhaled.

Our overall objective was to generate data allowing a better risk assessment regarding mycotoxins in indoor environment.

For that, we characterized successively the production of mycotoxins on different building materials, their possible aerosolization and their toxicity on pulmonary cells.

In parallel, we also studied the possibility to use specific volatile organic compounds to identify an active toxinogenesis; as well as the efficacy of a common remediation procedure on the persistence of moulds.

All these results will be presented in distinct chapters following a general introduction on bibliographic data available on moulds and mycotoxins in indoor environments.

1.1 Mould and Mycotoxins in habitats – General introduction

Today, in industrialized countries, people spend most of their time inside the buildings (work, school, home, sports etc.). Therefore indoor air quality presents an important aspect of human health. Many air pollutants can contaminate indoor environments such as: physical, chemical or microbiological pollutants.

Fungi are members of a complex community of biological indoor agents, along with bacteria, viruses and protists, such as amoebae; pollen and other plant material; and even arthropods, such as house dust-mites and ants (Nevalainen *et al.*, 2015). Fungi are omnipresent in nature and more than 100 000 species have been identified. They are found in air, water, soil, plants and many other substrates. However, only a few hundred of them are known to cause diseases (Douwes *et al.*, 2003). Fungi can also be found in indoor environments. They are able to grow on almost all natural and synthetic materials, especially if water activity is sufficient (Haleem Khan and Mohan Karuppayil, 2012). The minimum requirements for temperature, pH, light, and availability of nutrients are usually present in buildings, and therefore, moisture is the key factor regulating microbial growth.

Several hundreds of fungal species can be found in indoor environments (Gutarowska and Akowska, 2002). *Cladosporium*, *Penicilium*, *Aspergillus*, *Alternaria* and *Stachybotrys* are the most found genera in dwellings and other buildings (Verdier *et al.*, 2014).

Fungal development in a building can represent a source of air pollution, more precisely as spores, fragments of hyphae and spores, but also microbial metabolites such as microbial volatile organic compounds (MVOCs) or toxins can be transferred to air following aeraulic solicitations of contaminated supports. Due to its possible health effects, mould contamination of homes is a public health concern.

In a study performed by Hulin et al. (2013), it was estimated that more than 35% of French dwellings were contaminated with moulds. Other available surveys confirm the importance of this problematic. In 2009, the survey on housing conditions in Ile de France, reported that 21% of households surveyed reported traces of moisture on the walls of their homes, humidity and this presented the leading cause of discomfort (IAU, 2009). Similarly, according to ESMHA investigation, an epidemiological study in the Ile-de-France, performed in 2010, 25% of homes surveyed had at least a trace (patch) of dampness or mould presence. Thus, according to the index of contamination build by CSTB, 750 000 housing might be concerned by the presence of visible mould development. It is probably that this number underestimated the reality considering the

fact that the most vulnerable households weren't in condition to be sampled (Host *et al.*, 2010). Moreover, a national campaign by the Observatory of the Quality of Indoor Air revealed that more than 610 000 homes had mouldy surfaces of more than 1 m^2 (Moularat *et al.*, 2008a). The percentage of contaminated homes revealed to be more important in urban areas, probably due to overpopulation. According Host et al. (2010) in Île-de-France, the presence of visible mould was partially linked to overcrowding. They concluded that 63% of dwellings with visible mould were over-occupied, against 15% of dwellings being unoccupied. The results of Rocchi et al. (2015) confirmed the link between occupancy rate and the presence of mould in homes in France.

Nielsen (2002) estimated that proportion of dwellings with mould contamination in Northern Europe and North America varied from 20 to 40% affected buildings. Report for specific countries showed comparable frequency:

- 30-45% in the United Kingdom
- 20-25% in Netherlands
- 20-30% in Finland in USA
- up to 40% in USA
- up to 30% in Canada.

Mould growth only occurs when water conditions are favourable, therefore mainly in water-damaged and humid constructions. Accordingly the major part of the problems in Scandinavia and North America are due to insufficient aeration (Nielsen, 2002). In the USA about 40% buildings suffer from poor indoor air quality (Gutarowska and Piotrowska, 2007).

Such a high frequency of mould contamination goes with direct impact on health of inhabitants or workers. As an example, studies performed in offices in Canada revealed that 30–69% office workers display symptoms typical for sick building syndrome (Miller *et al.*, 1988). Studies concerning Czech Republic and Romania revealed that more than 30% of all surveyed buildings suffered from health implications of fungal growth. In the USA, Mudarri and Fisk (2007) estimated that more than 4.5 million cases of asthma result from exposure to damp and mould damaged buildings. Such health problems have important social and economic consequences since annual economic cost of asthma was estimated at approximately $3.5 billion in USA (Verdier *et al.*, 2014). It was also estimated that fungi are responsible for over 80% of total building material degradation (Gutarowska and Piotrowska, 2007).

The interest in mould exposure in different indoor environments has increased over the last 20 years. Probably the main reason is that exposures to those biological pollutants in occupational and residential environments are associated with a wide range of adverse health effects with

major public health impact, including irritations, inflammations, infectious diseases, acute toxic effects, allergies and other respiratory diseases (inhalation exposure), skin diseases (dermal exposure) and even cancer (Douwes *et al.*, 2003). Moreover in new buildings, increased insulation allowing energy saving, together with poor ventilation and multiplication of steam making apparatus (coffee machine, smoothing iron…) lead to more favourable environment for mould development.

Overall significance of this problem led to publishing guidelines for indoor air quality related to humidity and mould by The World Health Organisation (World Health Organization, 2009).

To further describe the problems related to mould contamination of indoor environment, we will now present parameters influencing their development, markers that can be used to monitor their presence, available data on indoor contamination with a special focus of the situation in Europe, compared to USA and finally, we will describe their possible deleterious effects on health of inhabitants.

1.1.1 Generalities on fungal development

A *fungus* is any member of the group of eukaryotic organisms that includes microorganisms such as yeasts and moulds, as well as macroscopic fungi known as mushrooms. These organisms are classified in a kingdom *Fungi* which belongs to the domain *Eukarya* (Cahagnier, 1998).

In this work the terms “mould” or “fungi” will be related to microscopic fungi. They belong to the division *Eumycota* (Silar and Malagnac, 2013). Fungi are not capable of photosynthesis; they are heterotrophic and use complex organic compounds as sources of energy and carbon (Boullard, 1997). For this, they degrade complex organic matter thanks to the excretion of enzymes and acids in the external medium and they absorb the digested components, all this taking place through the permeable wall of their vegetative system (Silar, 2016). Like bacteria, fungi play an essential role in ecosystems because they are decomposers and participate in the cycling of nutrients by breaking down organic materials to simple molecules. Fungi are most often saprophytic and develop on the inert organic material. However, some can also act as parasites and develop on the living organisms (*Fusarium* in cereal plants or *Aspergillus fumigatus* in animals). Some others are symbiotic and live together in mutual benefit with other organisms (*Neotyphodium* in fescue).

“Perfect fungi” reproduce both sexually and asexually, while imperfect fungi reproduce only asexually (by mitosis) (Figure 1 (Casselton and Zolan, 2002) and Figure 2 (Samiksha, 2016)). In both sexual and asexual reproduction, fungi produce spores that disperse from the parent

organism by either floating on the wind or transported by humans, animals or insects. The most common mode of asexual reproduction is through the formation of asexual spores. Spores allow fungi to expand their distribution and colonize new environments. They may be released from the parent thallus either outside or within a special reproductive "bag" called a sporangium.

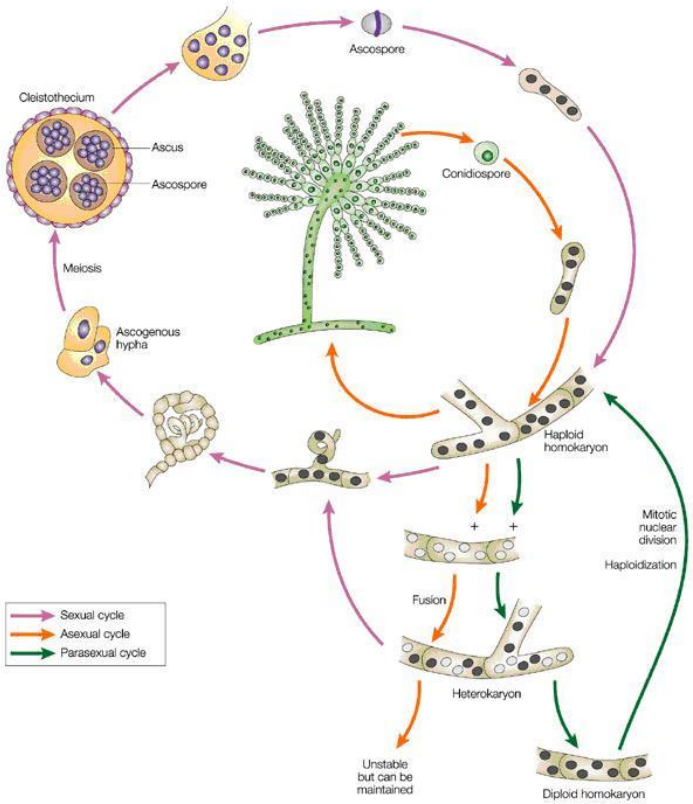

Figure 1: Life cycle of fungi (asexual and sexual reproduction of *Aspergillus nidulans*)

The germination will be triggered by the combined presence of water, light intensity, temperature or the types of nutrients available on substrate. In a general matter, fungi develop more easily on substrates with high carbohydrate/proteins ratio. However, their enzymatic abilities make them able to colonize many substrates, with various compositions. The spores will germinate and then give birth to a first undifferentiated filament, called hyphae, which will lengthen to form a group called mycelium. This set of filaments, more or less branched constitutes the thallus of fungus. In the presence of favourable conditions for sporulation, the mycelium will generate more specialized structures that will produce asexual spores (conidia) or, more rarely, sexual spores. Each mould produces a great number of conidia that are dissemination and resistance structures. The size, shape and colour of conidia vary greatly from one species to another. These elements are one of the parameters used for morphologic identification (Cahagnier, 1998; Pitt and Hocking, 2009; Samson *et al.*, 2010).

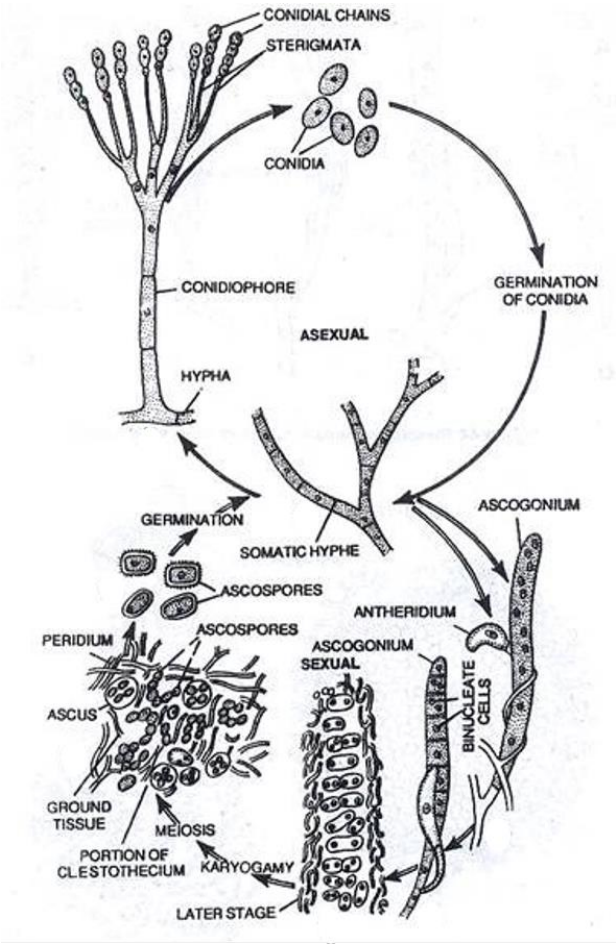

Figure 2: Life cycle of fungi (asexual and sexual reproduction of *Penicillium*)

1.1.2 Fungal development in indoor environments

Broad spectre of fungal species is found in outdoor environments and can be transferred to indoor by people movements, animals, insects or airflows. There is no sterile indoor space (except for special facilities such as surgical block) and the presence of biological contaminants at low concentrations is normal. The contamination problem occurs when concentrations of biological agent(s) arise or increase above certain level.

Probably the first reference about the destructive influence of fungal flora on human dwellings and clothes is found in the 3rd Book of the Bible, Leviticus (Bible, n.d.). Prehistoric times (confirmed by the analysis of rock paintings from Paleolithic caves), archaeological investigations and conservation studies revealed that the destruction of inorganic and organic materials was mainly connected with fungal and actinomycetal biodeterioration activities (Górny, 2004).

Colonization abilities of microorganisms present in indoor environment are determined by the physical and chemical characteristics of building materials. The main factors influencing mould

colonization of indoor environments are moisture, available nutrients and temperature (Haleem Khan and Mohan Karuppayil, 2012). This latter parameter, usually ranging from 18–25°C, promotes the growth of mesophilic fungi and can't be used to control microorganisms' development. .

1.1.2.1 Humidity as a major factor for mould growth

Among the most important factors, the moisture content in the substrate, together with the nutritional substances derived from building materials, initiate the development of microbial colonies. Therefore, the growth of microorganisms depends to the highest degree on the availability of water. Availability of water is usually expressed in terms of water activity (a_w). It corresponds to the ratio of the water vapour pressure exerted by the material to the vapour pressure of pure water at the same temperature, i.e. 1/100 of the equilibrium relative humidity (ERH) for a defined temperature. It is important to note that water availability and temperature are interdependent and, for example, increasing temperature has been found to lead to a reduction in the a_w requirement of moulds (Grant *et al.*, 1989). Several studies have shown that a_w equal to 0.65 is the lowest value necessary to initiate the microbial growth on the material when enough nutrition substances are available (Górny, 2004). Microbial activity and ability to colonize new surfaces increase as the water activity approaches 1, i.e., when water is freely available. Based on a_w parameter, fungal and actinomycetal microorganisms can be categorized according to their ability to initiate the growth on building materials and the order in which they appear on material's surface as primary, secondary, and tertiary colonizers. Combined classification for several fungal and actinomycetal species is summarized by Górny (2004):

- Primary colonizers (aw<0.85): *Alternaria citri, Eurotium amstelodami, E. repens, Aspergillus candidus, A. niger, A. penicillioides, A. restrictus, A. versicolor, Paecilomyces variotii, Penicillium aurantiogriseum, P. brevicompactum, P. chrysogenum, P. commune, P. expansum, P. griseofulvum, Wallemia sebi*;

- Secondary colonizers (aw=0.85-0.90): *Aspergillus flavus, Cladosporium cladosporioides, C. herbarum, C. sphaerospermum, Mucor circinelloides, Rhizopus oryzae*;

- Tertiary colonizers (aw>0.90): *Alternaria alternata, Aspergillus fumigatus, Epicoccum spp., Exophiala spp., Fusarium moniliforme, Mucor plumbeus, Phoma herbarum, Phialophora spp., Rhizopus spp., Stachybotrys chartarum, Trichoderma spp., Ulocladium consortiale, Rhodotorula spp., Sporobolomyces spp., Actinomycetes*.

Microbial investigations on building materials can be also expressed in terms of relative humidity (RH). It appears that building materials become sensitive to microbial colonization when the RH reaches 70% for wooden materials, 85% for gypsum-board and around 90-95% for cementitious and concrete materials. Study conducted by Johansson et al. (2013) provided different ranges of critical RH according to the nature of material. They also highlighted the influence of the temperature, incubation time and criteria used for mould growth assessment on the results of such testing. Adan (1994) showed a significant increase in the development of *P. chrysogenum* during testing on gypsum substrates when changing RH from 86 to 97%. Several authors highlighted that increased humidity favours the germination, the proliferation, and the diversity of mould on building materials. Several studies also showed that RH measurements could be used as a microbial contamination indicator for construction materials in water-damaged buildings. Pasanen et al. (2000) stated that RH of a material describes the water availability for microorganisms better than the moisture content does. Some authors have even developed mathematical models for predicting contamination by moulds, which use RH as a major parameter (Chase *et al.*, 2016; Lugauskas *et al.*, 2003; Thelandersson and Isaksson, 2013).

Some major characteristics of most building materials are high porosity and surface roughness. Thanks to the high porosity, materials have particular abilities for water absorption. When the environment provides high moisture levels, porous materials become supplies of water for microorganisms and offer them a larger growth subsurface (Hoang *et al.*, 2010). In addition, surface roughness and porosity could favour the attachment of nutrients carried by dust and resulting from the activity in buildings (Verdier *et al.*, 2014).

Water damage of homes can occur due to flooding or storm damage, leaks in plumbing, damage of air conditioners, dehumidifiers, humidifiers; as well as because of ice damming on building roofs (Haleem Khan and Mohan Karuppayil, 2012). Each time when the water appears on the surfaces of construction materials or penetrates them through holes and cavities, it can provoke microbial contamination. American investigations reveal that 27-56% of homes have problems with visible fungal contamination of surfaces, and/or bad quality of indoor air. In Europe, this percentage ranges from 12–80% (summarized in Górny, 2004). Microbial contamination of buildings is very often connected with environmental disasters. One of the latest examples is Katrina hurricane in the USA in 2005 (Chew *et al.*, 2006). In those cases, long terms effects provoke serious health outcomes, influencing mostly the families whose dwellings had not been rebuilt, drained or protected against moulds.

1.1.2.2 Impact of material composition and surface biodeterioration

Concerning the nutrition requirements, moulds are very elastic and have several adaptation possibilities. They are able to grow on almost all natural and synthetic materials, especially if they are hygroscopic or wet. These microorganisms reach basic nutrients, rich in carbon and nitrogen, thanks to the decomposition of organic materials. The majority of fungi present in indoor environment are saprophytic, which means that they gather nutrients from dead materials such as: wood, paper, paints, glues, soil, dust, food chips, etc. Moreover, they are able to frequently colonize surfaces consisting of inorganic moist material (glass, fibreglass, metal or concrete) if they are covered with dust, air contaminants or even finger-marks, creating invisible layer of biofilm (Górny, 2004; Samet and Spengler, 2003). Nevertheless, the works of Hoang et al. (2010) and Gutarowska (2010) indicate that cellulose-based materials are more sensitive to contamination than inorganic materials (gypsum, mortar, concrete, etc.) because cellulose can be metabolised by some fungi (Gutarowska, 2010; Hoang *et al.*, 2010).

Wood is highly vulnerable to fungal contamination. *Cladosporium* and *Penicillium* (*Penicillium brevicompactum and Penicillium expansum*) are reported to infest wooden building materials. Kiln dried wood surfaces are more susceptible to fungi (Sailer *et al.*, 2010). Acetylated wooden furniture, wood polyethylene composites, plywood and modified wood products are susceptible to infestation by *Aspergillus, Trichoderma and Penicillium* (Doherty *et al.*, 2011; Thacker, 2004). Inner wall materials used in buildings, such as gypsum board which is hygroscopic, highly favours the growth of *Stachybotrys chartarum* (Haleem Khan and Mohan Karuppayil, 2012). Also, paper and glue used for indoor surfaces are very good substrates for growth of most of indoor fungi. Fiberglass insulation and ceiling tiles support the growth of a number of fungi, among them *A. versicolor, Alternaria, Cladosporium, and Penicillium* species were frequently isolated (Erkara *et al.*, 2008). *Aspergillus* and *Penicillium* grow superficially on painted surfaces. Acrylic painted surfaces can be attacked by *Alternaria, Cladosporium, and Aspergillus* (Shirakawa *et al.*, 2011). Air filters and ventilation ducts can also be colonized by fungi (Noris *et al.*, 2011).

The physiological, biochemical and morphological properties of fungi and their frequent presence make them the most important cause of biodegradation of building materials. While growing on material, fungi produce various substrate-specific enzymes that destroy and/or disintegrate organic materials. Bio-corrosion occurs due to the microbial excretion of organic and inorganic acids. They include a variety of acids as oxalic, citric, gluconic and others formed during respiration. Moreover, biogenic organic acids produced by moulds are considered as one of major damaging agents leading to biodeterioration of stone, rocks, minerals (Gutarowska and Piotrowska, 2007). Such degradation process of homes has major economic importance. Only in

Germany the costs related to mould damages in buildings are estimated to more than 200 million Euro per year (Sedlbauer, 2001).

Among fungi, there are many organisms with strong cellulolytic (e.g., *Trichoderma, Botrytis, Chaetomium, Alternaria, Stemphylium*), proteolytic (e.g., *Mucor, Chaetomium, Aureobasidium, Gymnoascus, Trichoderma, Verticillium and Epicoccum*), and lipolytic properties (e.g., fungi from the previous group plus *Paecilomyces*) (Górny, 2004).

pH range of 5–6.5 observed on most building materials allows the growth of most of the fungi (Hoang *et al.*, 2010; Vacher *et al.*, 2010). Cementitious materials, which are alkaline (pH around 12 - 13) are relatively insensitive to colonisation at early ages. However, over time, the carbonation process reduces the pH of these materials to values around 9, which allows fungal growth. These materials thus become the target of significant contamination (Verdier *et al.*, 2014).

Sufficient light and oxygen are also critical for the growth of fungi in indoor environments (Airaksinen *et al.*, 2004; Haleem Khan and Mohan Karuppayil, 2012; Voisey, 2010).

Degraded building materials then represent sources of small particles easily aerosolized to indoor air, with numerous adsorbed substances, some of them being biologically active.

1.2 Biomarkers of fungal contamination

Evaluation of fungal contamination in indoor air samples to estimate health risks for occupants has been widely reported (Pasanen, 2001; Portnoy *et al.*, 2004). Indeed, the identification of a direct relationship between moulds' exposure and health problems in habitants requires a precise measurement of fungal colonization of indoor environment and several techniques can be used to estimate fungal presence.

Culture-based methods are the traditional approach in mycology, but other methods such as chemical, immunological and PCR-based methods are now also frequently used for examination of indoor environments. All these techniques have advantages and limits, so it seems that combining them should probably allow producing wider picture about fungal flora in indoors (Niemeier *et al.*, 2006; Pitkäranta *et al.*, 2008; Reboux *et al.*, 2009). Méheust et al. (2014) proposed schema below (Figure 3) to summarize methods to detect fungi in indoor environments.

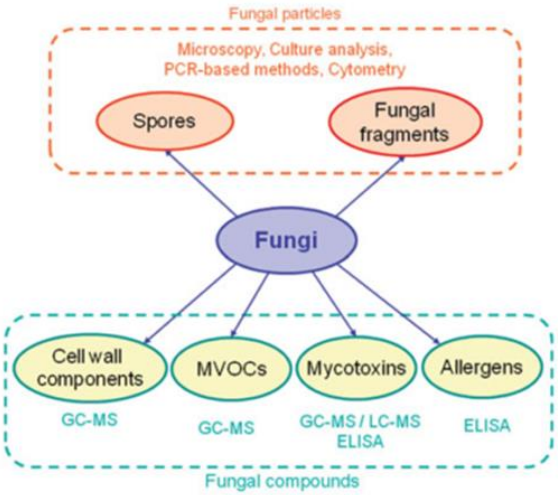

Figure 3: Methods to detect fungi in indoor environments
MVOCs - microbial volatile organic compounds. GC-MS - gas chromatography/mass spectrometry. LC-MS - liquid chromatography/mass spectrometry. ELISA – enzyme-linked immunosorbent assay

A presentation of the possible interest of these different methods will be done in following part of this manuscript. However, the first and major step in estimating the presence of indoor moulds is sampling.

1.2.1 Environmental sampling techniques

The consequences of exposure to fungal particles on health are related to several parameters, such as species of microorganisms, exposure pathway (inhalation or contact with skin/eyes) and environmental conditions, quantitative importance of microbial growth, aerosolization of contaminants, etc. (Verdier *et al.*, 2014). Many authors highlighted that air sampling is not sufficient to describe the entire microflora present inside buildings, especially in water-damaged buildings (Andersen *et al.*, 2011; Lappalainen *et al.*, 2001). Identifying microbial flora on building materials by surface sampling, has been shown to provide additional information about the potential sources of airborne microbial contaminants (Verdier *et al.*, 2014). In addition, species producing mucilaginous spores, that remain attached to substrates, require the use of surface sampling methods to determine full microbial biodiversity. Although microbial contamination present on surfaces is not directly correlated with health troubles of the occupants, French Agency for Food, Environmental and Occupational Health & Safety recommends sampling such communities on building materials, in addition to air sampling, in order to evaluate microbial proliferation indoors (ANSES, 2016).

1.2.1.1 Surface sampling

Surface sampling is used to determine nature of the microbial presence on environmental surfaces to measure the relative degree of contamination and identify the types of present fungi (Cabral, 2010). It can also assess the effectiveness of remediation and clean-up of indoor environments (Méheust *et al.*, 2014). The samples provide the hyphae fragments and the reproductive structures, spores, which may help for identification (Haleem Khan and Mohan Karuppayil, 2012). Different methods exist for sampling microbial populations on materials: swab, bulk, adhesive, contact plate, etc. (Verdier *et al.*, 2014), but the collecting process has not been well standardised yet, which makes it very difficult to compare results (Méheust *et al.*, 2014). Moreover, although many of these methods have been tested to evaluate their collecting efficiency on non-porous and non-absorbent surfaces (glass, steel, plastic, etc.), few studies have concerned construction materials such as concrete, coatings, mortar, and gypsum board, which are porous, rough and more or less dusty materials. The "Mould in the home" working group of the French Agency for Food, Environmental and Occupational Health & Safety has issued methodological recommendations for sampling on surfaces of building materials and suggests the use of at least two of the following surface sampling methods: swab, bulk sampling, adhesive tape and agar contact (imprint methods)(ANSES, 2016). Swab, adhesive and contact plate sampling, along with bulk sampling, are techniques commonly used on the surface of building materials to collect microorganisms and microbial contaminants prior to analysis.

Many authors emphasise the need for standardisation of the protocols for microorganism sampling on construction materials (ANSES, 2016; Bellanger *et al.*, 2009; Brown *et al.*, 2007; Hyvärinen *et al.*, 2002; Santucci *et al.*, 2007). At present, results can be influenced by the operator and many other factors, including the sampling technique itself and its different steps (sampling location, size of sampled surface, pressure applied, conservation of strains, etc.), the analysis method (observation, chemical, molecular, etc.) and/or the chosen culture medium. Moreover, the number of microorganisms collected from a surface is likely to depend on the species and the stage reached in the adhesion and biofilm formation process. This aspect has also been little studied to date (Verdier *et al.*, 2014).

1.2.1.2 Air sampling

Since moulds are associated with various respiratory disorders, air sampling is usually performed in indoor environments. This way of sampling is useful for determining whether the air in homes or workplaces is normal or not in the sense of microbiological composition. Air samples are also taken in hospitals as part of surveillance programs, especially in facilities where fungal presence

can have direct and important health consequences (surgical blocks, areas with immune-compromised patients…) (Méheust *et al.*, 2014).

The concentration of spores in the indoor air is a reliable indicator of the air quality and, indirectly, of some of the health hazards. Assessment of mould contamination of indoor air involves estimation of total number of fungi, classification of mould species and their relative proportion (Gutarowska and Piotrowska, 2007).

Air sampling for fungi can be done by three standard methods including: impactor, liquid impinger, and air filtration methods (Godish and Godish, 2007).

In the impactor method, the air stream passes through a slit into a culture medium and adhesive microscopic slide or tape strip is used to collect the sample (Zhen *et al.*, 2009). The most common impactor samplers (Figure 4) that are used are: Single stage impactor, Andersen, multistage impactor, Burkard, slit samplers, rotorod, casella, SAS, sierra marple impactor and centrifugal samplers. The air flow rate can be from 2 to 180 L/min (Engelhart *et al.*, 2007).

Figure 4: Andersen multistage; Slit-to-agar; Casella, SAS, Centrifugal sampler (from left to right)

The use of impingers is a flexible method for producing samples for a range of laboratory techniques (Méheust *et al.*, 2014). Liquid impingers collect the samples directly into the fluid and the microorganisms are retained in the liquid until they are cultivated on media or evaluated by techniques like biochemical or immunoassays (Haleem Khan and Mohan Karuppayil, 2012). The most common impinger devices that are used are: multistage, AGT– 30, shipe sampler, midget, and micro impingers. The airflow rate can be from 0.1 to 55 L/min and the sampling time varies from minutes to hours. Centrifugal samplers such as Reuter centrifugal sampler, aerojet cyclone are devices with 40–100 L/min air flow rate (Gralton *et al.*, 2011).

The use of high- volume centrifugal samplers is increasing since the air flow rate is often greater than 300 L/min. Collected solution is used for the cultivation of microbes or examination with analytical techniques. Filtration methods allow long-term measurements. Sampling volumes are

usually adapted to measure the fungal concentration. The design of the air samplers is known to influence their efficiencies for measuring airborne microbial concentrations (Yao and Mainelis, 2007). In air filtration sampling most common filters that are used are: glass, polycarbonate cellulose ester and Teflon filters. The air flow rate for this sampling is 1–1000 L/min (Haleem Khan and Mohan Karuppayil, 2012).

1.2.1.3 Dust sampling

Dust is analysed to evaluate presence of fungi or fungal agents that have accumulated with time. This method provides an indication of the microbial agents that may have been airborne. The term ''settled dust'' is usually used to describe the particulate matter that collects on horizontal surfaces, primarily floors. Over a defined time period, vacuum cleaners or suction devices are used to collect dust from a given surface of carpets or hard floors in homes (usually 1–2m^2). This method is commonly performed following moisture damage and/or health complaints in workplace investigations. Furthermore, efficiency of the air treatment in hospitals can be practically indicated by analysing dust in ventilation ducts. One of the main advantages of dust sampling is that this matrix may be analysed by different techniques. Nevertheless, variables such as the type of carpet, vacuum cleaner capture velocity and relative humidity can affect how well dust is removed from the floor (Macher, 2001). Moreover, the inhalable fraction of the dust and the length of the dust accumulation are unknown. Passive airborne dust collection methods, such as electrostatic dust fall collectors, may thus be a low-cost means of assessing long-term fungal exposure in standardizing the time and the surface of dust accumulation (Frankel *et al.*, 2012; Madsen *et al.*, 2012; Normand *et al.*, 2011).

As conclusion, sampling strategies is of major importance since can influence the nature of information given by analysis. The method used may depend on the type of sampled environment. Surface and air sampling are generally used in hospitals to confirm that the care environment is safe, especially in Europe. They are also useful in houses to quantify the exposure and evaluate the biodiversity. Dust is more often sampled in homes because it is an indicator of the past fungal exposure. Even if methods differ slightly depending on the level of fungal contamination, air sampling is the most commonly used technique in indoor environments, as it provides a better characterization of the airway exposure (Méheust *et al.*, 2014).

1.2.2 Methods to characterise fungal population

1.2.2.1 Microscopy

This type of sample examination can give different information. Identification and enumeration of fungal spores collected by spore-trapping samplers (airborne contamination) or tape adhesive samples (surface contamination) or even direct observation of contaminated material can be done (Godish and Godish, 2007). Three types of microscopy are used for identification of fungi – light microscopy (LM), epifluorescence microscopy (FM), and scanning electron microscopy (SEM) (Martinez *et al.*, 2004). Which one will be used depends on sample preparation (Haleem Khan and Mohan Karuppayil, 2012).

Fungal identification can be achieved thanks to the observation of specific morphological features. But it should be noted that such identification requires specific skills (ANSES, 2016; Santucci *et al.*, 2007). Samson performed detailed descriptions of fungi and his identification method based on morphological observations is widely used (Samson *et al.*, 2010; Verdier *et al.*, 2014). Nevertheless, identification of the fungal spores is usually difficult, mainly because just a small number of fungal spore types can be identified with confidence at generic level, and significant genera such as *Aspergillus* and *Penicillium* differentiation is very difficult. Moreover, as previously mentioned, high level of expertise is required (Mandal and Brandl, 2011). Some of disadvantages of this method can be that the procedures are laborious, complicated and time consuming (Méheust *et al.*, 2014).

Quantitative measurement of microbial forms on samples is based on direct counting (spores, fungal propagules etc.) (Verdier *et al.*, 2014). General advantage of this method is that the counted number of particles in conjunction with counted microscopic area and the volume of air or surface/weight of solid sample, can give the total number of particles in sample (Martinez *et al.*, 2004). While microscopy allows quantification of living as well as dead microorganisms (Douwes, 2005), including both culturable and non-culturable cells in the total count, its disadvantage is that aggregation of spores can affect the accuracy of total counting (Martinez *et al.*, 2004).

Recently, studies in microbiology started using epifluorescence microscopes for this purpose. The principle is based on the irradiation with specific wavelengths of light of a fluorochrome, which is fixed to the DNA. The advantages of this method are rapidity and representative determination of the concentration of spores in a fluid (Verdier *et al.*, 2014). In addition, the use of such a device to study building materials could be attractive because the observations do not require a transparent substrate. Protocols for determination of fungal contamination by those methods, directly in contaminated facilities, are developed and used as previously described in work of Méheust

(2012), but some adaptation are still needed. Finally, electron microscopes have been used as well. Like epifluorescence microscopes, these devices do not need a transparent substrate and are therefore commonly used for microbial investigation on building materials. Observations of surfaces may give information about eventual damages of materials due to the fungal development (Gadd *et al.*, 2014; Wiktor *et al.*, 2009). The technique can also be used to estimate the number of fungal propagules (Andersson *et al.*, 1997) or to observe the fungal growth directly on the substrate through SEM observations (Verdier *et al.*, 2014). On the other side, SEM has higher magnification than LM or FM. It can be used to identify mould spores and has the ability to detect even the small actinomycete spores and virus particles (Cabral, 2010). SEM is useful for determining surface characteristics, especially for mould spores, but is not routinely used for microorganisms' identification.

1.2.2.2 Culture-based methods

Culture-based methods can be used to determine qualitative and quantitative information on viable and culturable fungi in almost all types of sample. Those methods are widely used and recommended. Results are nevertheless influenced by many factors, particularly incubation conditions (such as culture medium, temperature) and competition between species (Méheust *et al.*, 2014). Also, collection process should be done with many precautions, in order not to underestimate fungal number (Martinez *et al.*, 2004). Among others, culture medium has a major impact on fungal growth. Some selective media can be used to isolate selected species/genera by promoting their growth (Mandal and Brandl, 2011; Sharma *et al.*, 2010). In work of Samson et al. (2010) it is recommend to use a specific culture media depending on the type of analysis and the microorganisms that will be studied (Samson *et al.*, 2010). Malt extract agar (MEA) has a high sugar content and its water activity is favouring fast-growing species, whereas dichloran glycerol (DG18) allows more diversity but doesn't support the growth of some fungi because of its low water activity (Chao *et al.*, 2002a; Martinez *et al.*, 2004; Méheust *et al.*, 2014; Yang and Heinsohn, 2007). Temperature is another important factor, and therefore Sabouraud media are incubated at 30–37 °C (for detection of fungal pathogens), whereas MEA and DG18 are incubated at the temperatures below 28 °C (for saprophytic ones) (Martinez *et al.*, 2004; Méheust *et al.*, 2014).

Quantitative evaluation is achieved by counting the number of active Colony Forming Units (CFUs) developed on a chosen medium. It represents the number of spores or mycelium fragments initially present and can be related to volume, mass or surface of the sample. The culturing of microorganisms on specific media is the traditional method of identifying fungi to the species level (Pitt and Hocking, 2009; Samson *et al.*, 2010). Different microorganisms (Figure 5) can be identified thanks to different characteristics of mycelium and spore appearance, size, shape,

pigmentation etc. Culture-based analysis is one of the most economical ways of identifying moulds at species level (Verdier *et al.*, 2014). Nevertheless, it is relatively time-consuming, usually requiring at least a seven-day incubation period, and it absolutely needs to be performed by highly qualified personnel (Méheust *et al.*, 2014).

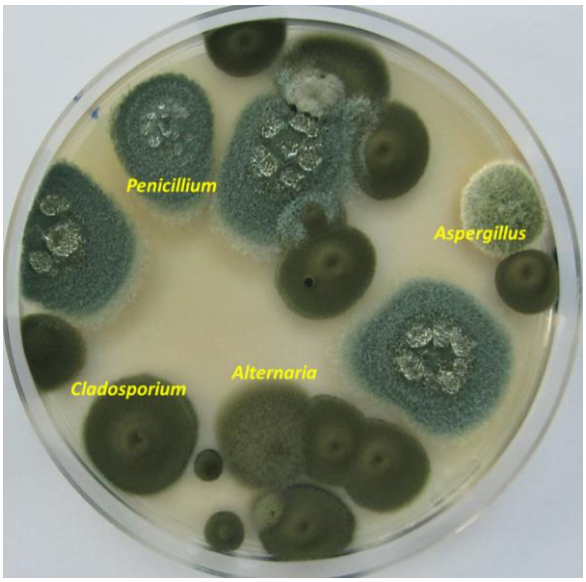

Figure 5: Variety of fungal species growing on MEA (Photo: S. Bailly)

In last years, microbiologists have agreed that the exclusive use of culture-based methods may not be sufficient to characterise a contaminated area with high accuracy. These methods are usually more sensitive to sampling procedure than other methods (Reboux *et al.*, 2009). Also using this method just part of complete microbial flora present in sample is determined (ANSES, 2016; Santucci *et al.*, 2007; Verdier *et al.*, 2014). In particular, they detect active forms that are capable of growth; but not slow-growing microorganisms (viable non-culturable) or inactive and non-viable forms, although these can be allergenic and/or irritant (Méheust *et al.*, 2014).

One sampling campaign by Santucci et al. (2007), performed in-situ, showed that fungal flora identified after culture-based methods following swab and imprint sampling were different and more complete than those found by direct observations from adhesive tapes. Indeed, if 87 % of the genera identified by direct observations were also found after culturing, only 42% of genera identified by culturing were also identified by direct observation (Santucci *et al.*, 2007).

It is known that quantitative assessment tends to underestimate populations and especially inter-species ratios. For example, many studies have shown that concentrations of some species were much higher when quantified by molecular methods than when assessed by culture methods (Hospodsky *et al.*, 2010; Meklin *et al.*, 2004; Yamamoto *et al.*, 2011). It shouldn't be forgotten that important advantages of qualitative assessment are the possible isolation and preservation of strains.

1.2.2.3 PCR-based methods

The use of molecular techniques as methods for studying of microorganisms in the environment has increased significantly with polymerase chain reaction (PCR) amplification and DNA sequence analysis (Peccia and Hernandez, 2006). Methods using recombinant DNA are based on the isolation of specific DNA sequences in order to target a particular phenotype, which presents the signature of group of microorganisms. Since K. Mullis invented it in the 1980s, PCR has become one of the most essential tools in most of the studies about microorganisms. The reason relies in the fact that PCR-based methods enable the detection, identification and even quantification of microorganisms present in variety of sample types. The process is based on the use of two primers, which function is to bind to a DNA region that is specific to a species or a larger group (Verdier *et al.*, 2014).

PCR methods have been used to detect and quantify microorganisms from various environments (Haas *et al.*, 2010; Millon *et al.*, 2006; Norbäck and Cai, 2011; Pinheiro *et al.*, 2011). PCR can be used for human clinical samples, like blood, urine, feces, in order to obtain a positive identification of the infectious organisms in a short time period (Yang and Rothman, 2004). This technology has also been used for biological contaminants in indoor environments (Bellanger *et al.*, 2009; Nonnenmann *et al.*, 2012; Vesper *et al.*, 2005). Commercial licenses are available for the DNA sequences for several fungal species commonly found in damp indoor environments (US EPA, 2004).

Mould Specific Quantitative PCR (MSQPCR), a DNA-based mould analysis method, was created for 130 moulds (Vesper *et al.*, 2011). In the USA, national sampling campaign using MSQPCR in homes produced a scale for comparing the mould contamination in homes, called the Environmental Relative Mouldiness Index (ERMI) (Vesper *et al.*, 2007a). This index showed to be useful for the characterization of homes of severely asthmatic children (Vesper *et al.*, 2008).

These methods have been used in many studies in different indoor environments. They have been applied in studies in schools (Simoni *et al.*, 2011), in hospitals (Morrison *et al.*, 2004; Pitkäranta *et al.*, 2008), in homes (Bellanger *et al.*, 2009; Fairs *et al.*, 2013; Godish and Godish, 2006; Iossifova *et al.*, 2008; Kaarakainen *et al.*, 2009; Méheust *et al.*, 2012, 2013b; Meklin *et al.*, 2007; Nonnenmann *et al.*, 2012; Pitkäranta *et al.*, 2011; Vesper *et al.*, 2013, 2005, 2007b), in shopping centres (Yap *et al.*, 2009) and in day-care centres (Cai *et al.*, 2011, 2009). Moreover it is used in agricultural environments (Zeng *et al.*, 2006) and even in hotel rooms (Norbäck and Cai, 2011).

Molecular techniques can be expensive but they offer rapid (generating results in a few hours) (Martinez *et al.*, 2004; Stetzenbach *et al.*, 2004) and sensitive assessment of fungal presence (Verdier *et al.*, 2014). They are sensitive enough for the detection of specific microorganisms,

which are slowly growing and difficult to cultivate, and furthermore they have capacity of species-specific identification (Goebes *et al.*, 2007). For instance, a work on fungal contamination of moisture-damaged dwellings, by Bellanger et al. (2009), found *Stachybotrys chartarum* on 21 samples using real time-PCR while only one was isolated with a culture-based method. On the other hand, no distinction is made between viable and dead cells and this could also participate to such differences (An *et al.*, 2006; Mandal and Brandl, 2011). Moreover, the results may be strongly impacted by contamination during or after sampling due to the high sensitivity of the method and special care has to be taken to avoid such problem.

This approach also requires some preliminary knowledge on the organisms that are suspected to be present, and available data bank to select DNA sequences and the corresponding primers. Some authors have selected more specific regions such as ITS (internal transcribed spacer) for fungi. This implies that PCR should be coupled with other methods in order to add a degree of specificity.

1.2.2.4 Cytometry

Several studies have used cytometry methods for the detection of fungi in indoor environments. In flow cytometry, a suspension of cells is passed rapidly through a capillary in front of a measuring window. Light emitted from a source is scattered by particles in the liquid and several particles characteristics such as size, shape, biological and chemical properties can be measured simultaneously (Mandal and Brandl, 2011). This method can have some interference from other particles that can stain such as plant and animal fragments (Martinez *et al.*, 2004). On the other side, flow cytometry technique is used to detect fungal propagules labelled by fluorescence in situ hybridization (FISH) (Prigione *et al.*, 2004). Fluorescent intercalating agent, that is not specific for fungi, is used to stain DNA (as propidium iodide). It allows differentiation of fungal propagules from other particles present in samples (Méheust *et al.*, 2014). Combination of FISH and flow cytometry can result in a more powerful analysis of air samples (Mandal and Brandl, 2011).

Solid-phase cytometry is also useful method to quantify fungal spores in environmental samples (Méheust *et al.*, 2013b). By combining flow cytometry and epifluorescence microscopy principles, highly dynamic detection range is achieved and moreover results are obtained within a few hours (Méheust *et al.*, 2014). Moreover solid-phase cytometry based on immunofluorescent labelling was used for detection of specific fungi. For example, Vanhee et al. (2009) quantified *Aspergillus fumigatus* in air samples.

Advantages of cytometry for use in environmental microbiology are the quantity and quality of the data it provides in short time, even its use is still not highly applied. One of the major drawbacks presents the lack of "stainability" of most fungal propagules since they proved to be stainable or fluorescent in a small percentage only (Prigione *et al.*, 2004). Moreover it is

insufficiently specific to particles of fungal origin and must be supported by additional methodologies.

1.2.2.5 MALDI-TOF

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is spectroscopic technique that is more and more used for the identification of microorganisms such as bacteria or fungi and recently has been introduced as tool for the analysis of bioaerosols. They are mainly identified by measurements of highly abundant proteins in linear positive mode in a mass range of mass per charge (m/z) of 2000–20 000 Da. Generated fingerprint mass spectra are compared with reference spectra by dedicated software and compared with stored known profiles. This method is suited for identification of microorganisms on at least genus, partially species, or even subspecies level (Ulrich *et al.*, 2016).

Main advantages are that species diagnosis by this procedure is much faster, more accurate, and cheaper than up-to-now standard procedures based on immunological or biochemical tests. In combination with an impactor sampler, bioaerosols directly deposited on a target have been analysed (Kim *et al.*, 2005). Using instrumental improvements it is even possible to obtain a mass spectrum of a single airborne particle allowing on-line measurements and analysis without the need of prior cultivation (Kleefsman *et al.*, 2008).

Main disadvantage of this method is that MALDI-TOF MS reference spectra are not well known and poorly available for many species. Also, filamentous fungi are considered as being technically more difficult to differentiate than yeasts or bacteria for example, because of different cell wall/membrane compositions. It was shown by several authors that in the case of fungi more complex and elaborate protein extraction methods are needed (Clark *et al.*, 2013; Ulrich *et al.*, 2016).

In overall conclusion, considering all presented methods and approaches, it is essential to distinguish two analytical approaches: targeting specific species or analysis of the overall population.

Several methods are available for sampling and analysing fungal presence on building materials but the obtained results are usually directly linked to the chosen method: for example, specific culture medium could promote the growth of one species and therefore lead to some species being overlooked. Concerning sampling and analysis processes, particular attention must be paid to the handling of samples.

Great needs for methodological standardisation were pointed out by many authors. Since in published data various methods can be found for quantitative assessment, it makes the comparison of results very difficult. Moreover, to evaluate fungal contamination of building materials and eventual health risk assessment for occupants, criteria such as surface coverage, amount per square meter, toxicity potential, etc. should be uniformed and standardised.

1.2.3 Methods to assess global fungal development

Environmental samples can also be analysed in order to assess global fungal contamination or exposure. Moreover, some cell components and fungal metabolites may be specifically quantified to characterize the risk for occupants since some of them are well known to be associated with adverse health effects.

Several chemical methods can be used to estimate the metabolic activity and moreover the toxic potential of a microbial population on a substrate. Measurement of such chemical components may have two aims:

- Measurement of fungal presence - measurement of the chemical components that form the mycelium cells for fungi (ergosterol, chitin) (Gutarowska, 2010) (Pasanen *et al.*, 1999; Szponar and Larsson, 2000), or adenosine triphosphate (ATP) which is an energy-producing molecule, or polysaccharides of the cell walls (b-D-glucan) (Andersson *et al.*, 1997). Quantification of those components can give information about the quantity of microorganisms or indicate the type of microorganism. It should be noted that these methods can be applied on microorganisms that are in an inactive form.
- Measurement of fungal metabolism - measurement of chemical compounds such as MVOCs, mycotoxins and other metabolites produced or emitted during fungal development on material (Bloom *et al.*, 2009b; Peitzsch *et al.*, 2012b; Polizzi *et al.*, 2012b). This approach is an indirect method for assessing the metabolic (or biological) activity of fungi, so the precise estimation of the microbial population is not accurate. This type of method is more used to assess the quantity of potentially dangerous compounds and estimate pathogenic potential that microbial population presents for health. In several cases it has been proven that there is no significant correlation between the presence and level of fungal species and targeted (expected) compounds (Bloom *et al.*, 2009a, 2009b; Polizzi *et al.*, 2009). Moreover, it is well known that a single species can produce various metabolites (Andersen *et al.*, 2002) and that production can occur at specific stage of fungal growth (general opinion is that secondary metabolites are produced in the latest stages of growth.

Chromatography (high performance liquid, gas, thin layer) and mass spectrometry are the main analytical methods employed for evaluation of the chemical compounds/components from contaminated surfaces.) (Verdier *et al.*, 2014).

1.2.3.1 Measurement of cell wall components: Ergosterol and glucans

Ergosterol

Methods based on measurements of chemical components forming the mycelium cells like ergosterol and chitin can determine the both, active and inactive mycelium (Choi *et al.*, 2014; Gutarowska and Piotrowska, 2007; Robine *et al.*, 2005, 2006). Determination of ergosterol content is a widely applied method of microbial growth monitoring in food industry studies as well as in studies on building materials. Ergosterol is the major sterol in cell membranes of hyphae and spores of filamentous fungi and in yeast's cellular membranes (Haleem Khan and Mohan Karuppayil, 2012). It is absent or occurs in very small quantities in plant cells. This method can provide a good estimation of present fungal biomass (Pasanen *et al.*, 1999, Gutarowska and Akowska, 2002). Nevertheless, it is considered that the ergosterol estimation depends on many factors, some of them being the microorganism species and age, the type of material, the moisture content and the growth conditions. By contrast, temperature does not seem to have a significant effect on ergosterol production (Verdier *et al.*, 2014).

Ergosterol, can be used as indicator of fungal contamination in building material, dust and even air, in case of long-term sampling (Gutarowska and Piotrowska, 2007; Martinez *et al.*, 2004). However, it gives no information on the species that are present (Portnoy *et al.*, 2004). Ergosterol is generally analysed by liquid-phase chromatography (Robine *et al.*, 2005) or by high-resolution gas chromatography/mass spectrometry (Dales *et al.*, 2010; Gutarowska and Piotrowska, 2007; Park *et al.*, 2008; Pitkäranta *et al.*, 2008). The amount of ergosterol can be correlated by established mathematical calculations to the number of colony-forming units (CFU) of mould-contaminating building materials. Ergosterol content in spores is estimated to ranges from 1.7-5.1 pg/spore depending upon fungal species and subsequent spore size (Miller and Young, 1997).

Glucans

The term glucan is defined as a polysaccharide that is a polymer of glucose. If related to fungi, the term glucans is restricted to polysaccharides made of glucose joined through β-1,3 or β-1,6 linkages (Martinez *et al.*, 2004).

(1-3)- β-D-Glucan is a biologically active, most abundant and well-studied constituent of the fungal cell wall that has been shown in several studies to be related to respiratory health disorders (Douwes, 2005; Méheust *et al.*, 2014).

For detection of glucans, a very sensitive method, the Limulus amebocyte lysate (LAL) test is applied. It can detect quantities as small as a few picograms in samples (which corresponds to the presence of a few tens of fungal spores) (Bex *et al.*, 2011; Bex and Squinazi, 2006; Crawford *et al.*, 2009; Foto *et al.*, 2005). The glucan content can vary according to the fungal species (Seo *et al.*, 2007). This biological active poly-glucose molecule may represent up to 60% of the weight of the fungal cell wall (Tischer *et al.*, 2011). Main disadvantage of using glucans as prediction of fungal presence is that they are not specific to moulds and are also present in vegetable cells (including pollen) and some bacteria (Méheust *et al.*, 2014). Therefore, the level of fungal exposure is likely to be overestimated by using (1,3)- β -D-glucan as indicators.

(1–3)- β -D glucans can be detected in house dust (Adhikari *et al.*, 2010; Iossifova *et al.*, 2007; Reponen *et al.*, 2010) and different building material samples (Adhikari *et al.*, 2009; Seo *et al.*, 2008, 2009). The exposure to glucans is often assessed in homes (Dales *et al.*, 2010) and in workplaces (Adhikari *et al.*, 2011; Hansen *et al.*, 2012).
Evidences suggest that these agents play a role in bioaerosols-induced inflammatory responses resulting respiratory symptoms (Srikanth *et al.*, 2008). Several epidemiological studies have reported that those components have strong immune-modulating effects (Méheust *et al.*, 2014). Field studies performed in the indoor environments suggested a relation with respiratory symptoms, airway inflammation, lung function and atopy in exposed individuals (Douwes *et al.*, 2003; Haleem Khan and Mohan Karuppayil, 2012; Weinmayr *et al.*, 2013).

1.2.3.2 Measurement of ATP

Adenosine triphosphate (ATP) is the most important biological fuel in living organisms. It plays a central role as an intermediate carrier of chemical energy linking the catabolism and biosynthesis in microbial cells. Assay using ATP has been recognized as very good and reliable method for estimating the total microbial biomass in almost all types of environmental samples (Kim *et al.*, 2011; Mandal and Brandl, 2011). ATP bioluminescence is a method based on a light generating reaction with luciferin and firefly luciferase. Emitted light from the reaction is proportional to the ATP concentration contained in the total biological contaminants. Techniques using the ATP bioluminescence have been used to quickly access and monitor microbial contamination on surfaces, especially in food industries. Advantages of this method are rapidness, easiness to perform, affordable price and it is able to detect cultivable as well as non-cultivable organisms (Mandal and Brandl, 2011). Combination of an aerosol condensation system with an ATP-bioluminescence system has been developed to detect ATP from aerosols in real-time and to determine the existence of airborne microbes within 10 min (Lee *et al.*, 2008). However, this

measurement is not specific to fungi and only gives information of the global microbial load of one sample/surface.

1.2.3.3 Measurement of secondary metabolites: MVOCs and mycotoxins

MVOCs

There are numerous sources for indoor volatile organic compounds (VOCs) such as the outdoor air, the activities of people living and working inside the building, and the building materials and furniture themselves. In the 1990s, it was shown that indoor mould can also be a source of VOCs (World Health Organization, 2009). It was shown by Pasanen et al. (1997) and Claeson et al. (2002), and more recently by Moularat et al. (2008c, 2008d) that indoor fungi can produce VOCs while growing on building materials and that some of found compounds exclusively belong to fungal metabolism.

Microbial volatile organic compounds (MVOCs) are secondary metabolites produced by fungi during their growth and emitted into the environment. Indoors MVOCs may have strong and unpleasant odours and exposure to these components has been linked to some health problems. It is suspected that exposure to high concentrations of some of the VOCs can have toxic effects, while exposure to lower concentrations is suspected to trigger sensory irritation (Gminski *et al.*, 2010). Exposure to MVOC may lead to acute respiratory and neurological troubles (Araki *et al.*, 2012; Burton *et al.*, 2008; Wålinder *et al.*, 2005).

MVOCs compounds comprise a great diversity of chemical structures including alkenes, alcohols, ketones, aldehydes, terpenes, ethers, esters (Joblin *et al.*, 2010; Moularat *et al.*, 2008d). Among MVOCs generally issued, alcohols (hexanol, 2-ethyl-1-hexanol, 1-butanol, 3-methyl-1-butanol, 2-methyl-1-propanol, 2-terpineol), terpenes (limonene), sesquiterpenes (thujopsene, cedrene, farnesene), ketones (acetone, butanone, pentanone, 2-hexanone, 3-octanone), furans (pentylfuran), dimethyl disulfide, aldehydes, aromatic compounds (1,3-dimethoxybenzene), as well as ammonia and various amine compounds have been identified (ANSES, 2016; Gao and Martin, 2002).

It should be noted that volatile compounds are only produced by actively growing fungi, but from the beginning of their development (Moularat *et al.*, 2008c).

It is estimated that more than 500 different MVOCs have been identified so far (Van Lancker *et al.*, 2008). Usually, sampling methods for those compounds involve capture onto carbon-based or TENAX adsorbents, followed by gas chromatography/mass spectrometry analysis (Joblin *et al.*, 2010; Moularat *et al.*, 2011). Other methods include computer detection of volatile compounds, so-called electronic nose (Kuske *et al.*, 2005). The method involves the application of neural

networks for the identification of individual volatile compounds and their measurement (Keshri *et al.*, 1998).

Main factors that are influencing production of MVOCs are substrate on which fungi grow and the environmental conditions (Reboux *et al.*, 2011), as well as fungal species (Moularat *et al.*, 2008c).

Use of the MVOCs as fungal biomarkers has often been obstructed by their low concentrations, unless the environment is heavily contaminated (Schleibinger *et al.*, 2005), and by the lack of specificity of most of the compounds emitted (Moularat *et al.*, 2008d; Polizzi *et al.*, 2012a; Schleibinger *et al.*, 2008; Schuchardt and Kruse, 2009). Therefore, some studies suggest that MVOC analysis can rely on the detection of several compounds forming characteristic patterns (Claeson *et al.*, 2007; Moularat *et al.*, 2008d). An index of fungal contamination based on MVOC measurements was recently used to evaluate the exposure of French population to moulds at homes (Hulin *et al.*, 2013; Moularat *et al.*, 2011). This chemical index of fungal contamination has been developed by Moularat et al. (Moularat *et al.*, 2008d) and is calculated as a function of the source and specificity of each VOC emitted by moulds.

Mycotoxins

Mycotoxins are secondary fungal metabolites, that include a chemically diverse group of molecules that cause a toxic response at low doses in vertebrates (Bennett and Klich, 2003; Jarvis and Miller, 2005; Reboux, 2006). They are non-volatile with a molecular weight below 1500 Da (Nielsen, 2003).

These compounds are mostly known as frequent food contaminants and their toxicity has been widely studied in case of ingestion. However, some of them also demonstrated toxicity by inhalation or dermal exposure.

The presence of toxinogenic fungi in the buildings does not necessarily mean that mycotoxins are present. Nevertheless, many potentially toxigenic fungi have been isolated from building materials and even from air samples in buildings with moisture problems, where the residents have suffered from nonspecific symptoms possibly related to mycotoxin production, such as cough, irritation of eyes, skin, respiratory tract, joint ache, headache and fatigue (Bonetta *et al.*, 2010; Boutin-Forzano *et al.*, 2004; Gottschalk *et al.*, 2008).

The analytical methods for detecting mycotoxins include biological assays, analytical chemistry assays, and immunoassays. No single method has been recommended for all mycotoxins, either on building materials, in air or in settled dust (Martinez *et al.*, 2004).

Identification and measurement of mycotoxins often require advanced analytic instrumentation, such as gas chromatography/mass spectroscopy and liquid chromatography/mass spectroscopy (Bloom *et al.*, 2007a, 2007b; Polizzi *et al.*, 2009; Tuomi *et al.*, 2000). Competitive enzyme-linked immunosorbent assay (ELISA) tests and array biosensors have also been developed (Brasel *et al.*, 2005a; Charpin-Kadouch *et al.*, 2006).

Martinez et al (2004) highlighted some of the problems related to determination of mycotoxin contaminations of indoor environments. Biological assays tend to be sensitive but nonspecific. Analytical chemistry assays and immunoassays are specific, but given the hundreds of possible mycotoxins, it is not clear for which specific mycotoxins one would select to assay. Many studies evaluating mycotoxins in air are qualitative because of the limitations of air sampling (requiring high-volumetric sampling) and analytical methods (primarily biological assays).

High performance liquid chromatographic (HPLC) methods provide a comprehensive quantitative and qualitative analysis of mycotoxins but because of high sensitivity of this method, extensive procedures for samples' cleaning are required, which is time and money consuming. Moreover, these methods rely on the availability of pure standards to quantify the level of contamination of samples.

Since many mycotoxin-producing fungal species produce, simultaneously, several different mycotoxins and several mycotoxins can be produced by different fungal species (Cabral, 2010) measurement of mycotoxins do not always allow the correspondence with fungal species.

1.3 Fungal contamination of indoor environments

Many studies conducted in various countries reveal that more than 600 fungal species may occur in indoor environments including homes (water-damaged, mould-damaged, damp and even homes without visible mould or moisture problems), schools, university facilities, offices, work buildings, hospitals, day centres, care centres, archives etc.

Among all fungi, the most frequent genera were *Aspergillus (A. versicolor, A. fumigatus, A. niger, A. ochraceus, and even A. flavus,), Penicillium (P. chryysogenum, P. brevicompactum, P. oslonii and others), Cladosporium, Alternaria* and *Stachybotrys.* Also some genera like *Basidiospores, Cheatomium, Aureobasidium, Acremonium, Trichoderma, Rhizopus* and *Mucor* were found in different investigations.

However, summarization of bibliographic data collected by different research groups in different countries is complicated since every performed study had its own specificity, either in terms of sampling methods and performed analysis or in terms of interpretation of results, implying that overall comparison is not possible.

To have wide picture about mould contamination problem in indoor environments and to evaluate subsequent risk for occupants related to this problem we have chosen to collect data from surveys published during last 15 years in European Union (EU) and United States of America (USA). Indeed, since last years revealed the most comprehensive examinations of fungal contaminations in indoor environment and in parallel since significant changes in climatic conditions and indoor conditions (type of buildings, different materials, lower aeration of homes etc.) were observed in the past, it is not sure that data published more than 15 years ago are still valid for comparison with data obtained more recently.

In tables below we summarized collected results and some specific points will be further discussed and compared.

Table 1: Fungal contamination of building materials in indoor environments in EU countries

Nature of location	Country	Nb of locations/samples	Method	Genera	Species	Year	Reference
archives	Portugal	swabs from contaminated surfaces in 3 archives	culturing (MEA, DG18, mycobiotic agar)	*Cladosporium spp, Penicillium spp, Aspergillus spp, Stachybotrys spp*	*A. niger, A. versicolor, A. fumigatus*	2011	Pinheiro et al.
archives and museums	Poland	6 museums & archives: swab samples of shelves, walls, historical objects	culturing (YES, Czapek agar)	*Aspergillus spp, Penicillium spp, Cladosporium spp, Alternaria spp*	*Aspergillus versicolor, A. fumigatus, A. niger, A. flavus, A. ochraceus, A. parasiticus, C. herbarum, P. carheum*	2012	Gutarowska et al.
buildings (MD)	Denmark	>5300 samples from building materials with visible mould growth in various buildings (homes, offices, schools etc.)	culturing (V8 medium)	*Penicillium spp, Cheatomium spp, Acremonium spp*	*Aspergillus versicolor, Cladosporium sphaerospermum*	2011	Andersen et al.
buildings (MD)	Germany	15 samples: 3wallpapers, 10 walls, 2 gypsum boards	culturing (Sabouraud agar)	*Penicillium spp, Aspergillus spp, Stachybotrys spp*		2006	Gottschalk et al.
buildings (WD)	Finland	16 samples: 2 WD buildings (before and after remediation); 2 control buildings	ITS sequencing and culturing	*Aspergillus spp, Eurotium, Penicillium spp*		2011	Pitkaranta et al.
buildings (WD)	Norway	11032 samples: 58% concrete, 27% wood, 10.6% gypsum board, 6.5% wallpaper, 6.8% wind barrier	Optical microscopy	*Cladosporium spp, Penicillium/Aspergillus spp*		2012	Nunez et al.
buildings (WD+MD)	Sweeden, Lund	WD and mould infested building materials: 2 gupsyum board papers, 1 mineral wool, 1 masonite	culturing (DG18, MEA)	*Stachybotrus spp, Penicillium spp, Cladosporium spp, Aspergillus spp, Cheatomium spp*	*A. penicillioides*	2003	Wady et al.
dwellings	United Kingdom	83 energy efficient homes	culturing (MEA)	*Aspergillus/Penicillium spp, Cladosporium spp, Ulocladium/Alternaria/Epicoccum spp, Fusarium spp and Trichoderma spp*		2016	Sharpe et al.
dwellings (health problems of occupants)	France, Lorraine	90 dwellings from Lorraine patients: 1080 swab samples: 4 rooms/home: 2 humide (bathroom, kitchen), 2 dry (living room, patient room). 3 samples / room: ventilation, window frame, comer opposite to heating	culturing (Sabouraud chloramphenicol agar)	*Aspergillus spp, Penicillium spp, Alternaria spp, Rhizopus spp*	*Aspergillus fumigatus, A. niger, A. versicolor, A. flavus*	2014	Rivier et al.
dwellings (health problems of occupants)	France, Marseille	100 dwellings, patients with respiratory diseases: 458 wall samples (schotch)	direct mycroscopic	*Cladosporium spp, Penicillium spp, Stachybotrys spp, Aspergillus spp*	*C. sphaerospermum, S.chartarum, A.versicolor*	2004	Boutin - Forzano et al.
dwellings (MD)	France (Eastern)	2 swab samples (1 bedroom, 1 bathroom) / home: 30 moisture damaged homes 25 allergic patient homes 55 paired control homes	culturing (MEA, DG18)	*Cladosporium spp, Aspergillus spp, Penicillium spp, Alternaria spp, Stachybotrys spp*	*MDH: C. sphaerospermium, P. chrysogenum, A. versicolor APH: C. sphaerospermium, P. chrysogenum, A. versicolor, Alternaria alternaria Ctrl: C. sphaerospermium, P. chrysogenum, A. versicolor*	2009	Bellanger et al.
dwellings (MD)	France, Marseille	149 homes of total 400 patients from MIEC (Medical Ind. Env. Counselor)		*Cladosporium spp, Aspergillus spp, Ulocladium spp, Acremonuim spp, Stachybotrys spp, Penicillium spp*		2009	Speyer - Olette et al.
dwellings (MD)	Poland	34 dwellings, wall samples	culturing (CYA)		*Penicillium chrysogenum, Aspergillus versicolor, A. niger, A. flavus, Stachybotrys chartarum*	2010	Gutarowska et al.
dwellings (MD)	Poland	22 dwellings with wisible mould: scrapings from mouldy surfaces (22 samples)	culturing (MEA, Oatmeal agar for Stachybotrys)	*Cladosporium spp, Aspergillus spp, Penicillium spp, Ulocladium spp, Acremonium spp*	*Aspergillus versicolor, C. cladosporioides, P. chrysogenum, U. chartarum and Acremonium charticola*	2016	Ježak et al.
dwellings (MD+health problems of occupants)	France (Eastern)	2 swab samples(1 bedroom, 1 bathroom)/home: 30 moisture damaged homes 25 allergic patient homes 55 paired control homes	qPCR	*Cladosporium spp, Aspergillus spp, Penicillium spp, Alternaria spp, Stachybotrys spp*	*MDH: C. sphaerospermium, Aspergillus versicolor, P. chrysogenum, Alternaria alternata, S. chartarum APH: C. sphaerospermium, A. versicolor, P.chrysogenum, Alternaria alternata, S. chartarum Ctrl: C. sphaerospermium, A. versicolor, P. chrysogenum, Alternaria alternata, S. chartarum*	2009	Bellanger et al.
dwellings (MD+health problems of occupants)	France (Eastern)	surface samples by swabbing from 118 dwellings: 32 visible mould, 27 allergic patients, 59 control buildings	culturing (MEA, DG18)	*Penicillium spp, Cladosporium spp, Aspergillus spp*	*A. versicolor, A. niger, A. ochraceus, A. fumigatus*	2009	Reboux et al.
dwellings (WD+MD)	Belgium	7 WD homes for at least 1 squared metar - 25 wallpapers (cut away), 25 mycelium (scraped off with scalpel), 2 silicone (cut away)	culturing (PDA, MEA) + sequence analysis	*Penicillium spp, Aspergillus spp, Cheatomium spp, Cladosporium spp*	*P.chrysogenum, A.versicolor*	2009	Polizzi et al.

Table 1: Fungal contamination of building materials in indoor environments in EU countries (continued)

dwellings (WD+MD)	Sweeden	57 water damaged dwelings: 100 samples : 40 gypsum boards, 37 wood based samples, 7 linoleum flooring, 6wallpapers, 10 other	culturing (MEA)	*Stachybotrus spp. Aspergillus spp. Chaetomium spp. Penicillium spp.*		2009b	Bloom et al.
elderly care centers	Portugal, Lisbon	swabs from 4 elderly care centers	culturing (MEA)	*Penicillium spp, Aspergillus spp, Chrysosporium spp*	*A. niger*	2014	Viegas et al.
hospital	France, Dijon	1301 samples - biocontact applicator	culturing (Sabouraud chloramphenicol plates)	*Search and found Aspergillus spp.*		2010	Foumel et al.
hospital	France, Dijon	64 samples	culturing (Sabouraud chloramphenicol plates)	*Aspergillus spp, Penicillium spp, Arthrographis*	*Aspergillus fumigatus, A. niger, A. versicolor, A. flavus,Sterile mycelium*	2007	Sixt et al.
hospital	France, Grenoble	surface samples (contact plates; wet swabs)	culturing (Sabouraud chloramphenicol agar) - microscopy	*Penicillium spp, Cladosporium spp, Aspergillus spp*		2002	Faure et al.
hospital	France, Paris	78 swab samples in patient rooms + 45 swab samples in other sites	culturing (MEA)	*Aspergillus spp, Cheatomium spp, Penicillium spp, Fusarium spp*		2001	Alberti et al.
hospital	Greece	3 hospitals : 634 premoistured cotton-tipped sticks	culturing (Sabouraud dextrose agar+chloramphenicol+ gentamicin)	*Candida spp, Aspergillus spp, Penicillium spp, Alternaria spp*	*C.albicans, parapsilosis Aspergillus niger, A. flavus*	2002	Panagopoulou et al.
wine cellars	Austria	36 wine cellars from 20 vintners - sterile swabs: 62 wall swabs 23 wooden barrels	culturing (DG18, MEA) and qPCR sequencing	*wall: Penicillium spp, Cladosporium spp, Aspergillus spp* *barrels: Penicillium spp, Cladosporium spp, Basidiomycetes spp, Aspergillus spp*	*A. versicolor*	2010	Haas et al.

Fungal contamination of building materials in different indoor environments in EU was studied in more than 10 countries, predominantly Scandinavian and west European countries (Table1). The most examined sites are dwellings, representing almost 45 % of surveys. They are followed by buildings and hospitals that represent each 20 % of performed examinations. Mostly mould damaged dwellings or dwellings with occupants reporting health problems were studied. Number of studied environments, samples or even type of samples are very variable among studies.

Methods used for investigation of fungal contamination are mostly based on culturing (84 % of studies). Sometimes this method is supplemented by quantitative PCR (4 studies). In three studies direct counting on microscope was performed, and in two of them that was the only method used.

Almost all surveys, with one exception, determined the most predominant fungal genera. Many of them also went to the identification of the most predominant species in tested environments. The most predominant fungal genera are *Penicillium, Aspergillus* and *Cladosporium*. They are always found in indoor environments. In almost 30% of studies *Stachybotrys* was found as well. Other detected genera were *Alternaria, Cheatomium, Basidiospores, Acremonium, Trichoderma, Rhizopus, Mucor,* and *Aureobasidium*.

Regarding the species found in samples, *A. versicolor, A. fumigatus, A. niger,* and even *A. flavus* were the most predominant *Aspergillus* species, being found in more than 30 % of surveys which determined fungi up to species level. *Penicillium* genus was mostly represented by *P. chrysogenum*. Finally, for *Cladosporium* the most found species was *C. sphaerospermum* whereas for *Stachybotrys* it is *Stachybotrys chartarum* that was always identified.

Table 2: Fungal contamination of building materials in indoor environments in USA

Nature of location	Country	Nb of locations/samples	Method	Genera	Species	Year	Reference
dwellings	California, Placentia	31 residentials: 150 wall and ceiling cavity samples	counting microscopically, cultivation (MEA, corn meal agar)	*Aspergillus spp, Penicillium spp, Stachybotrys spp*	*Stachybotrys-like spores in 25% of the samples by counting method, S. chartarum colonies were only detected on 2% of samples on MEA and 6% of samples on CMA.*	2003	Spurgeon et al.
dwellings (health problems of occupants)	Indiana, Muncie	2 building with occupants health concerns; 2 buildings with structural damage - WSACI (wet spray-applied cellulose insulation)	qPCR		*Penicillium chrysogenum, Stachybotrys chartarum, Aspergillus versicolor, Cladosporium cladosporioides*	2006	Godish and Godish
dwellings (WD)	Louisiana, New Orleans	7 homes with WD after Katrina	microscopic counting + cultivation (MEA, corn meal agar)	*Aspergillus spp, Penicillium spp*		2007	Schwab et al.
dwellings (WD)	Maryland, Pocomoke City	5 buildings with possible SBS history of WD: tape liftor + bukl sample	culturing	*Stachybotrys spp, Aspergillus spp, Penicillium spp, Cladosporium spp*	*S. chartarum, A. niger, A. ustus, P. aurantiogriseum*	2005	Shoemaker and House
dwellings (WD)	Texas, Houston	200 homes with history of water incursion: surface, wall cavity air samples		*Search for Stachy: 58,5% homes positive for Stachybotrys spp*		2005	Kuhn et al.
hotel (WD)	Ohio, Cincinnati	14 bulk samples of materials	culturing and non-culturing techniques	*culturing: Acremonium spp, Alternaria spp, Aspergillus spp, Penicillium spp, Stachybotrys spp* *Non culturing techniques all same but S.chartarum found in 13/14 samples*	*Aspergillus niger, A. sydowii, S. chartarum*	2001	Trout et al.

In the USA, bibliographic data are less numerous (Table 2). Dwellings, mostly water-damaged ones, were analysed in 5 of the 6 considered studies. As for EU studies, number of buildings and samples as well as types of samples were variable.

Same types of methods were applied, culturing being also the most used one. Concerning genera, besides mentioned *Aspergillus spp.* and *Penicillium spp.*, in USA *Stachybotrys* is one of the predominant genera. More interestingly, *Stachybotrys chartarum* was the most predominant species, followed by *Aspergillus niger*. Of course, these results should be taken into account together with fact that only 6 surveys were at our disposal. However, it could be related to differences in building materials used in different countries but also to the fact that, in USA, surveys were generally conducted following natural disasters that led to water damage of many buildings.

Table 3: Fungal contamination of air in indoor environments in EU countries

Nature of location	Country	Nb of locations/samples	Method	Genera	Species	Year	Reference
archives	Portugal	air samples from 3 archives	culturing (MEA, DG18, mycobiotic agar)	*Cladosporium spp, Penicillium spp, Aspergillus spp, Stachybotrys spp*	*A. niger, A. versicolor, A. fumigatus*	2011	Pinheiro et al.
archives	Portugal	archive of the University: 12 air samples	culturing	*Penicillium spp, Fusarium spp, Aspergillus spp*	*P. griseofulvum, F. solani, A. fumigatus*	2013	Nunes et al.
archives and museums	Poland	6 museums & archives	culturing (YES, Czapek agar)	*Aspergillus spp, Penicillium spp, Cladosporium spp, Alternaria spp*	*A. versicolor, A. fumigatus, A. niger, A. flavus, A. ochraceus, A. parasiticus, C. herbarum, P. carheum*	2012	Gutarowska et al.
children daycare centers	France, Paris	26 children daycare centers	culturing (MEA)	*Cladosporium spp, Penicillium spp, Aspergillus spp, Stachybotrys spp*		2011	Roda et al.
dwellings	EU: Nicosia, Budapest, Dublin, Helsinki	lobbies of buildings	culturing (PDA)	*Cladosporium spp, Penicillium spp, Hyalodendron, Aspergillus spp, Alternaria spp*		2010	Bernasconi et al.
dwellings	Finland	18 buildings without visibe mould damage (except in insulation)	culturing (MEA)	*Cladosporium spp, Aureobasidium spp, Aspergillus spp*	*Aspergillus versicolor*	2002	Pessi et al.
dwellings	France, Paris	homes of 190 newborne babies without health problems: 190 air samples	culturing (MEA)	*Cladosporium spp, Penicillium spp, Aspergillus spp*		2008	Dassonville et al.
dwellings	Lithuania	14 dwellings		*Penicillium spp, Aspergillus spp, Cladosporium spp, Alternaria spp*		2002	Gorny and Dutkiewicz
dwellings	Poland	> 100		*Penicillium spp, Aspergillus spp, Mucor spp*	*P. italicum, P. fuscum, P. onobense; A. fumigatus, A. niger, A. ochraceus*	2002	Gorny and Dutkiewicz
dwellings	Poland		culturing	*Aspergillus spp, Penicillium spp*		2004	Gorny
dwellings	Sweeden	8 airborne samples from 8 homes	culturing (MEA)	*Stachybotrys spp, Penicillium spp, Mycelia sterila, Cladosporium spp*		2007b	Bloom et al.
dwellings (health problems of occupants)	France, Lorraine	90 dwellings from Lorraine patients: 4 rooms/home: 2humide (bathroom, kitchen), 2 dry (living room, patient room)	culturing (MEA, DG18)	*Aspergillus spp, Penicillium spp, Dematies, Alternaria spp, Cladosporium spp*	*Aspergillus fumigatus, A. niger, A. versicolor, A. flavus; Eurotium herbatorium*	2014	Rivier et al.
dwellings (health problems of occupants)	Germany	Childrens' bedroom: 199 homes (children with alergy) 200 homes (children without)	culturing (DG18)	*Aspergillus spp, Penicillium spp, Cladosporium spp*		2004	Jovanovic et al.
dwellings (health problems of occupants)	Germany, Leipzig	200 rooms of babies with health problems	culturing (DG18)	*Penicillium spp, Cladosporium spp, Aspergillus spp*	*P. chrysogenum, brevicompactum; A. versicolor, (sporadicly flavus)*	2002	Muller et al.
dwellings (health problems of occupants)	Spain, Barcelona	22 homes of patients allergic to mould: 431 saples	culturing	*Cladosporium spp, Penicillium spp, Aspergillus spp, Alternaria spp*	*C. herbarum, C. cladosporioides; P. chrysogenum, P. frequentans, P. funiculosum; Aspergillus niger, A. versicolor, A. flavus, Alternaria alternata*	2006	Gomez de Ana et al.
dwellings (MD or health problems of occupants)	France	500 air samples from 500 rooms in 128 dwelllings (problems with moulds, or consulted doctor with symptoms related to housing conditions), 59 control buildings	culturing (MEA, DG18)	*pb homes: Aspergillus spp, Penicillium spp, Cladosporium spp Ctrl: Penicillium spp, Aspergillus spp, Cladosporium spp*	*P.chrysogenum, P. olsonii, A. versicolor, A. glaucus, A. fumigatus, A. niger, A. ochraceus, A. nidulans, A. candidus, A. flavus, C. cladosporioides, C. herbarum*	2008	Roussel et al.
dwellings (MD or health problems of occupants)	France (Eastern)	118 dwellings: 32 visible mould, 27 allergic patients, 59 control buildings	culturing (MEA, DG18)	*Penicillium spp, Cladosporium spp, Aspergillus spp*	*P. chrysogenum, P. oslonii, P. brevicompactum, A. versicolor, A. fumigatus, A. niger*	2009	Reboux et al.
dwellings (MD)	Austria	29 apartments without visible mould growth, 37 with visible mould growth	culturing (MEA, DG18)	*No VMG: Cladosporium spp, Penicillium spp, Aspergillus spp VMG: Penicillium spp, Aspergillus spp, Cladosporium spp*		2007	Haas et al.
dwellings (MD)	Finland	3 moisture and mould damage homes 2 nor or minimal mould damage		*Penicillium spp, Cladosporium spp, Aspergillus spp, Stcahybotrys spp*	*A. fumigatus, A. versicolor, A. niger*	2005	Meklin et al.
dwellings (MD)	Germany, Leipzig	1165 samples from: 415 rooms from mouldy homes 750 rooms without mould	culturing (DG18)	*Mouldy: Penicillium spp, Aspergillus spp, Cladosporium spp No mouldy: Cladosporium spp, Penicillium spp, Aspergillus spp*		2003	Herbarth et al.

Table 3: Fungal contamination of air in indoor environments in EU countries (continued)

dwellings (WD or health problems of occupants)	France	2 air samples (1 bedroom, a bathroom) / home from: 30 water damaged homes 25 allergic patient homes 55 paired control homes	culturing (MEA, DG18)	*Cladosporium spp, Aspergillus spp, Penicillium spp, Alternaria spp*	*MDH: P.chrysogenum, A. versicolor, C. sphaerospermium* *APH: P. chrysogenum, C. sphaerospermium, A. versicolor* *Ctrl: C. sphaerospermium, P. chrysogenum, A. versicolor*	2009	Bellanger et al.
dwellings (WD)	Finland	36 samples from wd homes 36 samples from control homes	culturing (MEA, DG18)	*Penicillium spp, Aspergillus spp, Cladosporium spp*	*A. versicolor*	2001	Hyvarinen et al.
dwellings (WD)	France	3 air samples (1 bedroom, a bathroom) / home from: 30 moisture damaged homes 25 allergic patient homes 55 paired control homes	qPCR	*Cladosporium spp, Aspergillus spp, Penicillium spp, Alternaria spp, Stachybotrys spp*	*Cladosporium sphaerospermium, Aspergillus versicolor, P. chrysogenum, Alternaria alternata, S. chartarum*	2009	Bellanger et al.
dwellings (WD)	Not specified	25 air filtres from homes and offices	culturing	*Stachybotrys spp, Cladosporium spp, Mucor, Penicillium spp, Aspergillus spp*	*S.chartarum*	2002	Johanning et al.
dwellings (WD)	Sweeden	37 air samples from 23 locations: 5 offices, 4 appartmants, 3 schools, 3 care facilities, 2 children daycare center, home, swimming hall, public transport vehicle, green house, emergency exit in tunel	culturing (MEA)	*Penicillium spp, Cladosporium spp, Aspergillus spp, Stachybotrys spp.*		2009b	Bloom et al.
elderly care centers	Portugal, Lisbon	4 elderly care centers	culturing (MEA)	*Penicillium spp, Aspergillus spp*	*A. candidus, A. fumigatus, A. niger*	2014	Viegas et al.
hospital	Chech Republic	Transplant intesive care unit: 7 rooms	culturing	*Cladosporium spp, Penicillium spp, Mucor spp*		2006	Vackova
hospital	France	laminar airflow room convetional room archive room	culturing (Sabouraud agar)	*Cladosporium spp, Penicillium spp, Aspergillus spp*		2013a	Meheust et al.
hospital	France	2 hematological units	culturing (MEA, Savouraud)	*Penicillium spp, Aspergillus spp, Bjerkandera adusta, Cladosporium spp*		2009	Sautour et al.
hospital	France, Dijon	1301 samples - biocontact applicator	culturing (Sabouraud chloramphenicol)	*Search and found Aspergillus spp.*	*A. fumigatus*	2010	Foumel et al.
hospital	France, Dijon	208 samples	culturing (Sabouraud chloramphenicol)	*Aspergillus spp, Penicillium spp*	*A. fumigatus, A. versicolor*	2007	Sixt et al.
hospital	France, Paris	20 samples in patient rooms 14 samples in other sites	culturing (MEA)	*Aspergillus spp*	*A. fumigatus, A. flavus*	2001	Alberti et al.
hospital	Greece	3 hospitals	culturing	*Aspergillus spp, Rhizopus spp, Mucor spp, Alternaria spp*	*A.niger, A. flavus, A. fumigatus*	2002	Panagopoulou et al.
hospital	Italy, Genoa	1758 samples from differents rooms in 10 hospitals	culturing (Sabouraud with chloramphenicol)	*Penicillium spp, Cladosporium spp, Aspergillus spp, Rhizopus spp*		2006	Perdelli et al.
hospital	Portugal, Porto	18 rooms	culturing (DG18)	*Penicillium spp, Aspergillus spp*		2008	Araujo et al.
laboratory	Germany	1 laboratory building	culturing (DG18)	*Aspergillus spp, Penicillium spp, Wallemia, Cladosporium spp*		2002	Engelhart and Exner
monastery library	Poland, Jasna Gora (Bright hill)	stacionary (andersen) +personal bioaerosol samplers	culturing (MEA)	*Aspergillus spp, Penicillium spp, Cheatomium*	*A. versicolor, A. niger, C. elongatum*	2011	Harkawy et al.
office buildings	EU: Nicosia, Budapest, Dublin, Helsinki	39 offices in 4 EU cities, modern buildings without mould	culturing (PDA)	*Cladosporium spp, Hyalodendron, Penicillium spp, Aspergillus spp, Alternaria spp*		2010	Bernasconi et al.
office buildings	Italy	240 air samples: ventilation shafts, fan coil nuts	culturing (Rose-Bengal Agar with chloramphenicol)	*Penicillium spp, Cladosporium spp, Aspergillus spp, Mycelia sterila*		2010	Bonetta et al.
offices (MD)	Finland	14 offices (mould damaged) - 46 samples, 20 controls - 56 samples	culturing (MEA, DG18)	*MD: Penicillium spp, Cladosporium spp, Aspergillus spp* *Non MD: Penicillium spp, Cladosporium spp, Aspergillus spp*	*A. versicolor, A. fumigatus, A. penicillioides, A. sydowii, A. niger*	2007	Salonen et al.

Table 3: Fungal contamination of air in indoor environments in EU countries (continued)

schools	Finland	32 schools (24 MD schools, 8 control): 12 wood buildings (143 samples), 12 concrete buildings (351)	culturing (MEA, DG18)	*Penicillium spp, Cladosporium spp, Aspergillus spp*	*A. versicolor, Stachybotrys chartarum*	2002	Meklin et al.
schools	Finland	39 air samples from 2 schools (index and control)	culturing (MEA, DG18)	*Penicillium spp, Cladosporium spp, Aspergillus spp*		2002	Meklin and Nevalainen
schools	Greece, Athenes	7 classrooms / school, 2 schools	culturing (MEA)	*Penicillium spp, Cladosporium spp, Aspergillus spp*		2013	Dorizas et al.
schools	Portugal	20 schools: 3-4 classroom / school	culturing (MEA)	*Penicillium spp, Cladosporium spp, Paecilomyces spp, Aspergillus spp*	*A. fumigatus*	2014	Madureira et al.
schools	Portugal, Lisbon	10 gymnasiums: 50 air samples	culturing	*Cladosporium spp, Penicillium spp, Aspergillus spp, Mucor spp*		2009	Viegas et al.
schools	Sweeden, Uppsala	8 schools (suspected asthma): 23 classrooms	culturing (MEA, DG18)	*Cladosporium spp, Sterile mycelia*		2007	Kim et al.
university	Italy		culturing	*Aspergillus spp, Cladosporium spp, Mucor spp*		2010	Di Giulio et al.
university	Poland	9 university rooms, 59 sample points	culturing	*Cladosporium spp, Penicillium spp, Aspergillus spp*	*C. herbarum, P. chrysogenum, P. viridicatum, P. expansum, A. versicolor, A. niger, A. flavus*	2007	Stryjakowska-Sekulska et al.
university	Slovenia, Ljubljana	27 faculty rooms: 4 fast food restaurants, 1 cultural centre, 7 health centres, 2 hospitals	culturing (MEA, Oxoid CM3 agar)	*Penicillium spp, Aspergillus spp, Cladosporium spp, Alternaria spp*		2008	Zorman and Jersek
wine cellars	Austria	36 wine cellars from 20 vintners: 72 samples of air	culturing (DG18, MEA) and qPCR sequencing	*Penicillium spp, Aspergillus spp, Cladosporium spp*		2010	Haas et al.

Studies focusing on fungal flora in indoor air of EU buildings are numerous (Table 3). With 50 surveys available in the considered period, it is the most frequent subject of analysis compared to analysis of materials or dust. Country giving the most attention to this problematic seems to be France, since 24 % of surveys were realized in that country. It is followed by Portugal (12 %) and Finland and Poland, representing 10 % of studies each. Predominant locations are dwellings (42 %), usually those where occupants reported health problems (almost 30 % of all dwellings) or those where water-damages (23 %) or mould-damages (14 % of all dwellings) were observed. Other types of buildings often studied are hospitals and schools (including university facilities).

Once again, type and number of samples varied significantly, making difficult any direct comparison.

It is interesting that culturing remains the most frequently used method, 92 % of studies.

As observed on building materials, in indoor air the most predominant fungal genera are *Penicillium, Cladosporium* and *Aspergillus.* At the species level, *Aspergillus fumigatus, A. versicolor, A. niger, Penicillium chrysogenum, P. brevicompactum* and *C. sphaerospermium* are the most frequent ones.

Therefore, it appears that results of surveys done on indoor air are quite in agreement with those done on materials, since the same predominant genera/species were observed. So it suggests that fungal particles present in indoor air could be generated from mouldy materials even if air analysis was rarely done together with material observation. By contrast, *Stachybotrys*, which was frequently identified on materials, especially in water-damaged buildings is never reported in indoor air, suggesting a weak aerosolization ability of this fungal genus, probably in relation with its mycelial organization.

There are four studies that differentiated results from different seasons. Research group of Haas et al. (2007) found *Penicillium* to be most prevalent in spring, fall and then winter. *Cladosporium* was found to be predominant in summer and spring and *Aspergillus* in summer and winter. Proceeding in Healthy Building by Sautour (2009) showed predominance of *Penicillium* in autumn, *Cladosporium* in spring and summer and *Aspergillus* during whole year. Bonetta et al. (2010) found *Penicillium* to be the most predominant species during wither and then spring; but Gomez de Ana et al. (2006) found this species to prefers winter, then autumn, summer and finally spring. This last study showed same results for *Cladosporium* as for *Penicillium* and finally they found summer and spring as two seasons when *Aspergillus* is the most predominant. It should be noted that direct comparing cannot be done since all those studies, monitoring fungal prevalence during seasonal variation, did not have same conditions, meaning country, method, type of building etc. However, the variations observed within seasons also highlight the direct interaction between indoor and outdoor air, the latter being a potent source of spores that can enter indoors and further develop if humidity is favourable.

Table 4: Fungal contamination of air in indoor environments in USA

Nature of location	Country	Nb of locations/samples	Method	Genera	Species	Year	Reference
dwellings	Atlanta, GA	600 indoor air samples from 50 homes witout water or mould damages	culturing	*Cladosporium spp, Penicillium spp, Aspergillus spp*	*C. cladosporioides, P. sclerotiorum, P. brevicompactum, A. niger*	2004	Horner et al.
dwellings	Florida	53 air samples from 18 homes withoud visible mould	spore counting	*Aspergillus/Penicillium group, Ascospores, Basidiospores, Cladosporium spp*		2008	Codina et al.
dwellings	Ohio, Cincinnati	17 homes: 6 witouth mould or water damage, 11 minor mould or water damage	mould specific qPCR (MSQPCR)	*Cladosporium spp, Aspergillus spp, Penicillium spp, Aureobasidium spp, Eurotium, Epicoccum*	*C. cladosporioides, C. herbarum, Aspergillus penicillioides, A. versicolor, A. niger, P. brevicompactum, P. chrysogenum, Eurotium chevalieri, Aureobasidium pullulans, Epicoccum nigrum*	2007	Meklin et al.
dwellings	Ohio, Cincinnati	4 homes without mould or water problems, 4 rooms/home	microscopic spore counting	*Penicillium/Aspergillus group, Basidiospores, Ascospores, Cladosporium spp*		2009	Crawford et al.
dwellings	Ohio, Cincinnati	6 typical homes (no visible mould)	microscopic spore counting + culturing (MEA)	*Aspergillus/Penicillium group, Ascospores, Cladosporium spp, Basidiospores*		2006b	Lee et al.
dwellings	USA (all over the country)	9619 samples from 1717 homes	culturing	*Cladosporium spp, Penicillium spp, nonsporulating fungi, Aspergillus spp, Stachybotrys spp*	*A.versicolor, A. fumigatus, A. niger, A. falvus, S. chartarum*	2002	Shelton et al.
dwellings	USA: Kentucky and Ohio, Cincinnati	samples from homes that were free from moisture damage or visible mold.	culturing (triplicate International Streptomyces Project agar 2)	*Aspergillus/Penicillium spp, Cladosporium spp*		2006a	Lee et al.
dwellings (health problems of occupants)	Indiana, Muncie	2 building with occupants health concerns; 2 buildings with structural damage - WSACI (wet spray-applied cellulose insulation)	Spore count by Burkord + culturing (MEA, DG18)	*Aspergillus/Penicillium spp, Cladosporium spp*		2006	Godish and Godish
dwellings (health problems of occupants)	Massachusetts, Boston	405 homes of asmatic children	culturing	*Penicillium spp, Cladosporium spp, Aspergillus spp*		2005	Stark et al.
dwellings (health problems of occupants)	Massachusetts, Boston	494 rooms - homes of babies from women with history of asthma and allergy	culturing (DG18)	*Penicillium spp, Cladosporium spp,Aspergillus spp*		2013	Behbod et al.
dwellings (health problems of occupants)	Massachusetts, Boston	496 homes of babies with familial predisposition for development of asthma (based	culturing (DG18)	*Penicillium spp, Cladosporium spp, non-sporulating, Aspergillus spp*	*A.versicolor, A. niger, A. ochraceus*	2003	Chew et al.
dwellings (health problems of occupants)	Missouri, Kansas city	27 air samples from 31 homes of asmatic children	DNA (ITS sequencing)	*Aspergillus spp, Epicoccum, Cladosporium spp*	*A.versicolor, A. vitricola*	2014	Rittenour et al.
dwellings (health problems of occupants)	Missouri, Kansas city	31 homes of asmatic children	spore counting	*Cladosporium spp, Aspergillus/ Penicillium, Alternaria spp*		2014	Rittenour et al.
dwellings (health problems of occupants)	Missouri, Kansas city	31 samples from 31 homes of asmatic children	culturing	*Cladosporium spp, Penicillium spp, Aspergillus spp, Alternaria spp*		2014	Rittenour et al.
dwellings (health problems of occupants)	New York, Buffalo	50 homes of asmatic children 49 control	culturing + non-culturing methods	*Pb homes: Cladosporium spp, Penicillium spp, Aspergillus spp Control: Cladosporium spp, ascospores, Penicillium/Aspergillus spp, Basidiospores*		2011	Jones et al.
dwellings (health problems of occupants)	New York, Syracuse Audit study	103 infant's homes at risk of asthma	culturing	*Penicillium spp, Cladosporium spp, Aspergillus spp*		2010	Rosenbaum et al.
dwellings (health problems of occupants)	USA (Middle West)	88 homes of asmatic children 85 control homes	culturing (MEA)	*Asmatic: Cladosporium spp, ascospores, Myxomycetes, Basidiospores, Alternaria Control: Cladosporium spp, Penicillium spp, Basidiospores*		2012	Meng et al.
dwellings (health problems of occupants)	USA (North East)	1000 fungal air samples from baby room and living areas (to predict relation between allergen exposure and development of asthma)	culturing (MEA/DG18)	*Cladosporium spp, Penicillium spp, Aspergillus spp*		2001	Ren et al.
dwellings (health problems of occupants)	USA, 7 cities: 44 Boston, 40 Bronx, 54 Chicago, 95 Dallas, 48 NY, 52 Seattle, 81 Tucson	homes of children - 414 mould sensitive children	culturing (DG18)	*Cladosporium spp, Penicillium spp, Aspergillus spp, Alternaria spp*		2004	O'Connor et al.

Table 4: Fungal contamination of air in indoor environments in USA (continued)

dwellings (WD)	California	residence with water leek	culturing (cellulose, DG18)	*Penicillium spp, Cladosporium spp, Alternaria spp, Aspergillus spp*	*P.chrysogenum, P. crystosum, P. brevicompactum, C. cladosporioides, Alternaria alternata, Aspergillus versicolor*	2003	Morey et al.
dwellings (WD)	Louisiana, New Orleans	20 WD homes after Katrina	culturing (MEA,CYA)	*Aspergillus spp, Penicillium, Cladosporium spp, Basidiospores*	*A.niger, A. versicolor, A. flavus, A. fumigatus, Stachybotrys chartarum*	2007	Rao et al.
dwellings (WD)	Louisiana, New Orleans	29 airbonre mould samples from 13 homes after Katrina	microscopic spore counting	*Aspergillus/Penicillium spp, Cladosporium spp, Curvularia*		2007	Schwab et al.
dwellings (WD)	Louisiana, New Orleans	6 flooded homes. 2 minimaly or no flooded	culturing	*Aspergillus/Penicillium spp, Cladosporium spp, Stachybotrys spp*		2006	Solomon et al.
dwellings (WD)	Texas	7 homes with *Stachybotrys* - 40 air samples from 16 rooms		*Stachybotrys spp, Alternaria spp, Cladosporium spp, Ascospores, Penicillium/Aspergillus spp*		2005b	Brasel et al.
dwellings (WD)	Texas, Houston	200 homes with history of water incursion: room air		*Search for Stachybotrys spp - Air samples positive for Stachybotrys in 9,6% rooms*		2005	Kuhn et al.
dwellings (WD)	USA: Ohio and Louisiana, New Orleans	2 WD homes (Ohio), 3 homes after huricane Katrina (NO): Indoor samples in mouldy rooms	spore counting by the Button Samples	*Aspergillus/Penicillium spp, Cladosporium spp, Stachybotrys spp*		2007	Reponen et al.
dwellings (WD+health problems of occupants)	Ohio, Cincinnati	144 homes of kids with rhinitis, allergy (homes damaged by Katrina, CCAAPStudy)	microscopic spore counting	*Penicillium/Aspergillus spp, Cladosporium spp, Basidiospores*		2006	Osborne et al.
hospital	Illinois, Chicago	842 samples	culturing (MEA) + ELISA (search for *A.fumigatus* , protein marquer)	*Aspergillus spp, Penicillium spp, Mucor spp*	*A.niger, A. candidus, A. flavus, A. fumigatus, A. versicolor, P.brevicompactum, P. chrysogenum, M. racemosus*	2005	Curtis et al.
hospital	Minnesota, Minneapolis	1523 indoor air samples	culturing (inhibitory mould agar)	*Search for Aspergillus spp, found in 27-66% of samples dependin on room location*	*A.niger, A.fumigatus, A.flavus*	2007	Falvey and Streifel
hospital	Minnesota, Minneapolis	32 rooms: patient care units; intensive care units; indoor reference; lobby		*search and found for Aspergillus spp*	*A.niger, A. fumigatus, A. flavus*	2007	Falvey and Streifel
hotel (WD)	Ohio, Cincinnati	12 samples from 10 floor hotel	culturing (DG18, Commeal agar)	*DG18: Penicillium spp, Aspergillus spp, Cladosporium spp. Cellulose agar: Penicillium spp, Cladosporium spp, Aspergillus spp, Stachybotrys spp*		2001	Trout et al.
offices	USA (all over the country)	44 office buildings	microscopic spore counting	*Cladosporium spp, Basidiospores, Smuts, Penicillium/Aspergillus spp, Ascospores*		2006	MacIntosh et al.
offices	USA (Base study)	100 office buildings: 2200 samples: 22-28 samples (2sampling duration:2-5min; 2sampling periods:morning, afternoon, 4indoor, 2outdoor) / building	culturing (MEA) and 44 samples from 44 buildings - spore counting	*CFU: Cladosporium spp, Penicillium spp, Aspergillus spp, spores: Cladosporium spp, Ascospores, Basidiospores, Penicillium/Aspergillus spp*		2007	Tsai et al.
schools	Michigan	64 elementary and middle school classrooms	non-viable method, microscopic spore counting	*Aspergillus/Penicillium spp, Cladosporium spp*		2007	Godwin and Batterman

Like in EU countries, in the USA, air is the most studied compartment of indoor environments (Table 4). Almost 80 % of examined locations were dwellings. Half of them have been subjects of examination because of health problems of occupants, and 25 % have been studied because they were water-damaged. Once again, comparison of results is complicated by strong variation on number and kind of samples. In 62 % of studies, culturing was used in order to determinate fungal flora. In 29 % of studies direct spore counting was performed.

As previously observed, the most predominant fungal genera were *Penicillium, Cladosporium* and *Aspergillus*. Moreover, *Alternaria, Stachybotrys* and *Basidiospores* were found as well. It is important to note that less than one third of studies (29 %) determined fungi up to species level. Nevertheless, when determined, the most

predominant species were *Aspergillus versicolor, A. fumigatus, A. niger, Penicillium brevicompactum, P. chrysogenum* and *Stachybotrys chartarum.*

The identification of *Stachybotrys chartarum* in indoor air in some studies done in the USA is a great difference with EU data. Sampling or type of dwelling (strong water damages in the USA) could participate to such observation.

One study of air samples taken during different seasons (Rosenbaum *et al.*, 2010) showed that *Penicillium* was most found in summer, equally in spring and fall and the least in winter. *Cladosporium* was found in same prevalence during summer and fall, and little less in spring. In this study, *Aspergillus* was found most during fall, then summer, winter and at least in spring.

Table 5: Fungal contamination of dust in indoor environments in EU countries

Nature of location	Country	Nb of locations/samples	Method	Genera	Species	Year	Reference
daycare centres	Sweeden	swab dust from 70 rooms: 11 daycare centres (allergen-avoidance) + 11 controls	qPCR	*Penicillium/Aspergillus spp*	*Stachybotrys chartarum*	2009	Cai et al.
daycare centres	Sweeden	21 day care centres (risk of dampness, visible damage and mould growth). 103 rooms: cotton swabs (settled dust) + Petri dishes (airborne dust)	qPCR	*Aspergillus/Penicillium spp*	*Stachybotrys chartarum*	2011	Cai et al.
dwellings	Denmark	27 living rooms	culturing (DG18)	*Aspergillus spp, Penicillium spp*	*A. niger, A. fumigatus, A. flavus*	2012	Madsen et al.
dwellings	Denmark, Copenhagen	airborne settled dust from living room, with electrostatic dust cloths from 27 homes	MALDI-TOF	*Penicillium spp, Aspergillus spp, Cladosporium spp*	*P.glabrum, P. brevicompactum, P. camemberti, A. versicolor, A. niger, C. herbarum, Stachybotrys chartarum*	2016	Madsen et al.
dwellings	England	11 homes no visible mould growth	MSQPCR		*Epicoccum nigrum, Aureobasidium pullans, Cladosporium herbarum, Penicillium brevicompactum*	2005	Vesper et al.
dwellings	Finland	homes without mould problems: rug dust from 3 apartments and 2 homes	qPCR	*Penicillium /Aspergillus spp, Cladosporium spp*	*Aureobasidium pullulans, Aspergillus penicilloides, A. niger, A. fumigatus, C. cladosporioides, C. herbarum, P. brevicompactum, P. chrysogenum, Stachybotrys chartarum*	2009	Kaarakainen et al.
dwellings	Finland	homes without mould problems: rug dust	qPCR	*Cladosporium spp, Penicillium/Aspergillus spp*	*C. cladosporioides, C. herbarum; Aureobasidium pullulans, Aspergillus peniciloides, A. niger, A. fumigatus, P. brevicompactum, P. chrysogenum, Stachybotys chartarum*	2009	Kaarakainen et al.
dwellings	Finland	homes without mould problems: vacumed dust	qPCR	*Penicillium/Aspergillus spp, Cladosporium spp*	*C. cladosporioides, C. herbarum, Aureobasidium pullulans, Aspergillus peniciloides, A. niger, P. brevicompactum, P. chrysogenum, Stachybotrys chartarum*	2009	Kaarakainen et al.
dwellings	Portugal (Central)	28 homes: 7 dust samples	culturing	*Aspergillus spp, Penicillium spp, Mucor spp, Rhizopus spp*	*A. niger, fumigatus, flavus*	2014	Sousa et al.
dwellings	UK	vacumed dust from 10 pillows	culturing (Sabouraud agar)		*Aspergillus fumigatus, Aureobasidium pullulans, Rhodotorula mucilaginosa*	2006	Woodcock et al.
dwellings (health problems of occupants)	Germany	Childrens' bedroom: 199 homes (children with alergy) 200 homes (children without)	culturing (MEA, DG18)	*Aspergillus spp, Penicillium spp, Cladosporium spp*		2004	Jovanovic et al.
dwellings (health problems of occupants)	Sweeden	198 homes (children with asthma/allergy) 202 homes (non symptomatic)	culturing (V8 agar)	*Penicillium spp, Alternaria spp, Aspergillus spp*		2014	Choi et al.
dwellings (WD or MD)	Germany, Hamburg	Visible mould or water damaged dwellings: 11 carpet dust samples from 8 households	culturing (DG18)	*Search for Aspergillus versicolor - found in 91% samples*		2002	Engelhart et al.
dwellings (WD)	Finland	2 WD buildings (1before and 1 after remediation); 2 control buildings - 8 settled dust samples	qPCR	*Penicillium spp, Cladosporium spp*	*P. chrysogenum group, C. cladosporioides group and C. herbarum group*	2011	Pitkaranta et al.
hotel	EU, hotels: Spain (5), Italy(4), Sweeden (3), France(3), Portugal (2), UK(2), Norway (2), Denmark, Germany, Poland, Estonia, Island (1)	69 hotel rooms from 20 countries - swab sampling of door frames	qPCR	*Aspergillus/Penicillium spp*	*A.versicolor, Stachybotrys chartarum*	2011	Norback et al.
libraries	Poland, Jasna Gora (Bright hill)	settled dust and sterile cotton swabs from monastery library	culturing	*Aspergillus spp, Penicillium spp*		2011	Harkawy et al.
libraries and archive	Poland	settled dust in storerooms from 4 libraries and 1 archive	culturing(MEA)	*Penicillium spp.*	*Trichothecium laxicephalum Alternaria tenuis, Aspergillus repens*	2011	Karbowska-Berent et al.
nursing homes	Finland	2 nursing homes	culturing (MEA,DG18), qPCR, sequencing	*Penicillium spp, Sphaeropsidales spp, Aspergillus spp, Acremonium, Stachybotrys spp*	*Aspergillus penicilloides, A. versicolor, A. sydowii, A. ochraceus*	2008	Pitkaranta et al.
offices (MD)	Finland	47 settled dust samples (offices - 15 MD buildings). 126 settled dust samples (offices - 40 control buildings)	culturing (MEA)	*MD: Penicillium spp, Cladosporium spp, Aspergillus spp NonMD: Penicillium spp, Cladosporium spp, Aspergillus spp*	*Aspergillus versicolor*	2007	Salonen et al.

Table 5: Fungal contamination of dust in indoor environments in EU countries (continued)

offices (MD)	Finland	47 settled dust samples (offices - 15 MD buildings). 126 settled dust samples (offices - 40 control buildings)	culturing (DG18)	*MD: Penicillium spp, Cladosporium spp, Aspergillus spp NonMD: Penicillium spp, Cladosporium spp, Alternaria spp, Aspergillus spp*	*Aspergillus versicolor*	2007	Salonen et al.
schools	EU: Italy (Siena, Udine), France(Reins), Norway (Oslo), Sweeden (Uppsala), Denmark (Arhus)	6 units from 5 coutries: 2 settled dust samples / classroom	culturing	*Cladosporium spp, Penicillium spp, Aspergillus spp, Alternaria spp.*	*Aspergillus versicolor*	2011	Simoni et al.
schools	EU: Italy (Siena, Udine), France(Reins), Norway (Oslo), Sweeden (Uppsala), Denmark (Arhus)	6 units from 5 coutries: 2 settled dust samples / classroom	DNA analysis	*Aspergillus/Penicillium DNA*	*A. versicolor, Stachybotrys chartarum*	2011	Simoni et al.
university	Italy	3 new buildings (settled dust, plates with nutrient agar)	culturing (nutrient agar; Sabouraud dextrose agar)	*Aspergillus spp, Cladosporium spp, Mucor spp*		2010	Giulio et al.

Twenty-three studies coming from EU countries were performed on dust in different indoor environments (Table 5). Contrary to studies on materials and air, more than 50 % were done in dwellings that did not signalled water- or mould-damage and the objective was therefore usually to get information of "normal" contamination of indoors. Dust was collected in different manner with variable number of collected samples. It is interesting to note that, contrary to material and air samples, dust samples were analysed as often by culturing and molecular methods (56 % and 43 % of studies, respectively). Besides three already described predominant genera, *Penicillium, Aspergillus* and *Cladosporium*, in dust, *Alternaria* can be found as well. Very important is that most of the studies performed the identification at the species level. The most predominant species were *Aspergillus versicolor* and *Stachybotrys chartarum*. The presence of the latter could result from degradation of supports by cellulolytic activity of fungus and subsequent formation of relatively big size particles that rapidly sediment (and were subsequently only rarely found in case of air analysis). *Aureobasidium pullulans, Aspergillus niger, Penicillium brevicompactum, Cladosporium cladosporioides* and *C. herbarum* were also very often found.

Only one study estimated seasonal variations and showed that in winter the most predominant were *Penicillium* and *Aspergillus*, in spring *Cladosporium*, *Aspergillus* and *Penicillium*; in summer *Cladosporium* and *Penicillium* and finally in fall *Penicillium* followed by *Cladosporium* (Pitkäranta *et al.*, 2008).

Table 6: Fungal contamination of dust in indoor environments in USA

Nature of location	Country	Nb of locations/samples	Method	Genera	Species	Year	Reference
dwellings	Atlanta, GA	settled dust samples from 50 homes witout water or mould damages	culturing (MEA; DG18)	*Cladosporium spp, Penicillium spp, Aureobasidium spp, Aspergillus spp*	*C.cladosporioides, Aureobasidium pullulans, A.niger, P. chrysogenum*	2004	Horner et al.
dwellings	Ohio, Cincinnati and Cleveland	19 and 26 homes no visible mould	MSQPCR		*Epicoccum nigrum, Aureobasidium pullans, Cladosporium herbarum, Alternaria alternata*	2005	Vesper et al.
dwellings	USA	dust sample from bedrooms and living rooms per home from 1096 homes	MSQPCR	*Aspergillus spp, Cladosporium spp, Penicillium spp, Epicoccum spp, Stacybotrys spp*	*A. penicilloides, A. niger, A. fumigatus, A. versicolor, C. cladosporioides, C. herbarum, C. sphaerospermum, Aureobasidium pullulans, Epicoccum nigrum, Eurotium amstelodami, P. brevicompactum, S. chartarum*	2007b	Vesper et al.
dwellings (health problems of occupants)	California, San Diego	living room and bedroom samples from : 93 homes (child with asthma) + 28 control homes	MSQPCR		*Aureobasidium pullulans, Cladosporium cladosporioides, Eurotium group, Cladosporium herbarum, Epicoccum nigrum*	2013	Vesper et al.
dwellings (health problems of occupants)	District of Columbia, Washington	50 rural homes (childrens health center study)	qPCR	*Cladosporium spp, Alternaria spp, Mucor, Penicillium spp, Aspergillus spp*	*C. cladosporioides, P. brevicompactum, Aspergillus niger, A. versicolor, A. flavus, S.chartarum*	2012	Nonnenmann et al.
dwellings (health problems of occupants)	Massachusetts, Boston	397 samples	culturing (DG18)	*nonsporulating fungi, Penicillium spp, Cladosporium spp, Aspergillus spp, Aureobasidium*	*Aspergillus versicolor*	2003	Chew et al.
dwellings (health problems of occupants)	Massachusetts, Boston	412 samples from homes of babies from women with history of asthma and allergy	culturing (DG18)	*Aspergillus spp, Penicillium spp, Cladosporium spp*		2013	Behbod et al.
dwellings (health problems of occupants)	Massachusetts, Boston	76 homes (child with asthma)- dust from living room, 34 control homes - dust from living room and bedroom	MSQPCR		*Aureobasidium pullulans, Cladosporium cladosporioides, Eurotium group, Cladosporium herbarum, Epicoccum nigrum*	2013	Vesper et al.
dwellings (health problems of occupants)	Massachusetts, Boston	405 children with asthma	culturing (CFU/g dust)	*Aspergillus spp, Penicillium spp, Cladosporium spp*		2005	Stark et al.
dwellings (health problems of occupants)	Missouri, Kansas city	30 dust samples from 31 homes of asmatic children	DNA ITS sequencin	*Leptosphaerulina spp, Phoma spp, Aspergillus spp, Cladosporium spp*	*L. chartarum, P. medicaginis, A. vitricola, A. versicolor, A. penicillioides*	2014	Rittenour et al.
dwellings (health problems of occupants)	Missouri, Kansas city	60 homes (children with asthma) -bedrooms of kids 22 control homes	MSQPCR		*Aureobasidium pullulans, Cladosporium cladosporioides, Eurotium group, C.herbarum, Epicoccum nigrum*	2013	Vesper et al.
dwellings (health problems of occupants)	Ohio, Cincinnati	297 indoor dust samples (vacumed) from babies homes	MSQPCR	*Cladosporium spp, Aspergillus spp, Penicillium spp, Epicoccum, Wallemia*	*C.cladosporioides, C. herbarum, C. sphaerospermum, Aureobasidium pullulans, Alternaria alternata, Epicoccum nigrum, Eurotium chevalieri, Mucor racemosus, A. penicillioides, A. niger, A. fumigatus, A. flavus, A. versicolor, P. brevicompactum, Stachybotrys chartarum*	2008	Iossifova et al.
dwellings (health problems of occupants)	USA	12 homes from HAA study. Settled dust	culturing	*Alternaria spp, Aureobasidium spp, Penicillium spp, Cladosporium spp, Epicoccum spp, Aspergillus spp*		2016	Hanson et al.
dwellings (health problems of occupants)	USA	12 homes from HAA study. Settled dust	PCR (ITS sequencing)	*Alternaria spp, Aureobasidium spp, Cladosporium spp, Epicoccum spp, Penicillium spp, Aspergillus spp*		2016	Hanson et al.
dwellings (health problems of occupants)	USA	settled dust samples (1083) from AHHS study	MSQPCR	*Aspergillus spp, Cladosporium spp, Penicillium spp*	*Aspergillus penicillioides, Eurotium amstelodami, Aureobasidium pullulans, Epicoccum nigrum, Cladosporium cladosporioides*	2016	Vesper et al.
dwellings (MD)	Ohio, Cincinnati	18 homes with visible mould 19 control homes	qPCR	*Cladosporium spp, Aspergillus spp, Penicillium spp, Alternaria spp*	*C. cladosporioides, Aspergillus penicillioides, A. niger, A. versicolor, P. brevicompactum, P. chrysogenum, Alternaria alternata*	2004	Meklin et al.

Table 6: Fungal contamination of dust in indoor environments in USA (continued)

dwellings (MD)	USA: California, Los Angeles and Louisiana, New Orleans	5 mould contaminated homes after hurricane Katrina	culturing (MEA;DG18; CYA)	*MEA: Aspergillus spp, Cladosporium spp, Penicillium spp, Trichoderma DG18: Penicilliium spp, Aspergillus spp, Mycelia sterila CYA: Cladosporium spp, Aspergillus spp, Mycelia sterila, Penicillium spp*		2009a	Bloom et al.
dwellings (MD)	USA: California, Los Angeles and Louisiana, New Orleans	5 mould contaminated homes after hurricane Katrina	qPCR	*DNA-sequences: Aspergillus spp, Penicillium spp, S.chartarum*		2009a	Bloom et al.
dwellings (WD)	Colorado	178 spore trap from: 43 homes: 18 no WD, 14 WD>6months, 11 WD<6months	MSQPCR	*no WD and WD>6: Cladosporium spp, Penicillium/Aspergillus spp, Basidiospores WD<6: Cladosporium spp, Basidiospores, Ascospores, Penicillium/Aspergillus spp*		2012	Van Dyke et al.
hospital	USA	12 floor dust samples 6 surface dust samples 9 carpet dust	QPCR	*Search for Aspergillus spp, found in 23/27 samples*	*A.versicolor, A. fumigatus, A. niger, A. ustus, A. sydowii, A. flavus*	2004	Morrison et al.
offices	Massachusetts, Boston	4 office buildings, 21 offices	culturing (MEA, DG18)	*Penicillium spp, Cladosporium spp, Aspergillus spp*		2002b	Chao et al.
offices	West Virginia	328 foor dust and 326 chair dust	culturing (MEA, DG18, cellulose)		*Aureobasidium pullulans, Epicoccum nigrum, Alternaria alternata, Stacybotrys chartarum*	2008	Park et al.

A similar number of studies are available from USA (Table 6). Among 22 studies, 86 % were done in dwellings, mostly those where occupants reported health troubles (63 % of all dwellings). Location from where dust was collected, as well as type of collector and number of samples varied between studies. Nevertheless, used method to analyse collected samples was either molecular-based (64%) or culturing.

As in EU, the most found genera are *Penicillium, Aspergillus* and *Cladosporium*. Once again as in EU, many studies performed determination of species. Beside already identified as most predominant species in dust, *Aspergillus versicolor*, *A. niger, Aureobasidium pullulans, Penicillium brevicompactum, Cladosporium cladosporioides* and *C. herbarum*, an usual presence of *Epicoccum nigrum* and *Aspergillus penicillioides* was also reported. Surprisingly, *Stachybotrys chartarum* was much less observed, being determined just in several studies.

1.3.1 Comparison of mould contaminations observed in EU and USA - Conclusion

We have chosen to investigate results available from European Union (EU) and USA. In the EU, Scandinavian countries demonstrated the strongest interest in fungal contamination of indoors, which could be related to humid and cold climate in that part of Europe.

As demonstrated in literature analysis, some differences can indeed be highlighted probably mostly due to the circumstance of analysis. As an example, the differences in the prevalence of *Stachybotrys chartarum* between continents could be directly related to the nature of building materials (importance of wood) and the climatic or environmental (storm and water-damages) leading to survey.

This fungal species is also a good example of the interest of studying simultaneously different indoor compartments to get an accurate vision of real indoor contaminants. Indeed, air analysis usually does not allow the identification of this species that is however often found when analysing materials and dust.

This bibliographic analysis also demonstrates the difficulty to deeply compare results due to the great diversity of sampling procedures, nature of samples, and methods of analysis used.

For instance, choice of method influenced final results. Firstly, if culturing was applied, chosen culture medium had sometimes influence of fungal species that will be detected (Bloom *et al.*, 2009b). This is normal and expected, since every species has its own requirements for optimal growth. To overcome this problem several mediums should be tested (Park *et al.*, 2008; Pitkäranta *et al.*, 2008; Rao *et al.*, 2007; Salonen *et al.*, 2007) or this method should be completed with some others as PCR-based methods (Haas *et al.*, 2010; Madsen *et al.*, 2016; Trout *et al.*, 2001) or direct microscopy counting (Godish and Godish, 2006; Lee *et al.*, 2006; Spurgeon, 2003). Chosen method is a very important factor since in some studies two methods revealed different results (Bellanger *et al.*, 2009; Trout *et al.*, 2001). It is especially case with species difficult to cultivate on medium when some faster developing ones are present.

This reinforce the importance of setting up standardized procedures to be used in the different cases: intervention in case of water- or mould-damaged home, intervention in case of reported health troubles or evaluation of global indoor contamination in quality control procedures.

However, some common features can also be drawn and especially on the most predominant genera and species. In particular, it seems very important to note that among most frequent species, some are well known to be potent mycotoxin producers (*A. versicolor, A. fumigatus, P. chrysogenum, P. brevicompactum* and *S. chartarum)*. Since most of studies were conducted following appearance of health troubles of inhabitants, this observation point out the possible implication of these toxic compounds.

1.4 Health problems related with exposure to mould contaminations

Main route of exposure to fungi in indoor environments is inhalation. However, dermal exposure shall also be taken in consideration especially for workers involved in remediation works after water damage for instance. Ingestion could also be of interest, particularly for very young children.

The nature of health problems may vary going from infection (mycoses) to allergy or intoxication. They can be related to either the exposure to moulds' part (mycelium, spores etc.) or fungal metabolites (MVOC, mycotoxins).

Usually, due to way of exposure, symptoms are mostly concerning respiratory tract but, in some cases, more complex syndromes or other organs can also be affected.

Deposition of bioaerosols into the human respiratory tract depends on the shape, size, density, chemical composition and reactivity of particles. The majority of particles with a diameter greater than 10 µm, and up to 80% of particles with diameters between 5-10 µm, are trapped in the nasopharyngeal region. Submicronic particles, especially those with size below 0.1 µm, are able to penetrate deeper in to the lungs. Several studies showed that microbial particles with diameters below 2.5 µm (size of fungal spores present in indoor environment or fungal fragments), if inhaled, are the most dangerous for human health. It was shown that they have abilities to avoid numerous defence systems in the respiratory tract (e.g, ciliated epithelium, mucus, saliva, etc.), therefore they can be sources of exposure to very reactive compounds derived from microorganism propagules (Cabral, 2010; Górny, 2004).

The impact of indoor moulds on health will be presented by type of syndrome after what, a special focus will be made on the possible implication of MVOCs and mycotoxins in the appearance of such disorders.

1.4.1 Types of health problems

Respiratory symptoms and lung function impairment are probably the most widely studied among health effects related to exposure to mould contamination. Generally, occupational respiratory diseases result from airway inflammation caused by specific exposures to allergens, pro-inflammatory agents or even mycotoxins. Based on the underlying inflammatory mechanisms and subsequent symptoms, there is clear distinction between allergic and non-allergic respiratory

diseases. Non-allergic respiratory symptoms reflect a non-immune-specific airway inflammation, whereas allergic respiratory symptoms reflect an immune-specific inflammation in which various antibodies (IgE, IgG) play a major role in the inflammatory response (Douwes *et al.*, 2003).

1.4.1.1 Respiratory diseases

Respiratory diseases have been found to be associated with exposure to water-damaged, damp or mould damaged indoor environments. They include chronic rhinosinusitis, invasive, and allergic pulmonary aspergillosis (ABPA), hypersensitivity pneumonitis, allergic rhinitis including allergic fungal rhinitis, and sinusitis, asthma, conjunctivitis, and sarcoidosis. It has been estimated that 21% of asthma in the USA is attributable to dampness and mould exposure. Exposure to mould odours at home increased the risk of developing asthma in children by 2.4 times, and exposure to mould in workplace increased the risk of new-onset asthma by 4.6 times. A study of adult asthma found that those who developed occupational asthma were significantly more likely to have been exposed to water-damage buildings and mould in working environments (Hope, 2013). In the data available in USA, the national annual cost of asthma that is attributable to dampness and mould exposure in the home is estimated to be $3.5 billion per year (Mudarri and Fisk, 2007).

Sinusitis due to inflammation of paranasal sinuses is reported in homes with visible mould or water damage. Damp concrete floors increased the risk of itching, burning or irritated eyes and irritated stuffy or running nose. One study (Bush, 2008) showed that exposure to airborne fungal spores was associated with persistent cough in infants whose mothers had asthma. Mucous membrane irritation syndrome is characterized by symptoms such as rhinorrhea (running nose), nasal congestion and sore throat, and irritation of nose and eyes. This syndrome is common not only in agricultural environments, but also found in people exposed to damp buildings (Brewer *et al.*, 2013; Lanier *et al.*, 2012).

Serious respiratory infections in immune-compromised persons may be result of exposure to several fungal species that are commonly found in indoor environments such as *Aspergillus spp*. and *Fusarium spp*. People with impaired immune system who spend most of their time in indoor environments contaminated by moulds may develop serious fungal infections. Asthma and chronic obstructive pulmonary disease are disorders among persons potentially infected with *Aspergillus*. In cystic fibrosis or asthma patients, *Aspergillus spp.* can provoke invasive or semi-invasive pulmonary aspergillosis and pulmonary aspergilloma (Haleem Khan and Mohan Karuppayil, 2012).

1.4.1.2 Allergy

First suspicion, that there must be link between respiratory exposure to fungal components and allergy was described long time ago, in 1873 (Haleem Khan and Mohan Karuppayil, 2012). In opposite of some other well-known allergens, like pollen, these allergies are not seasonal. Several fungal genera, commonly present in the indoor environment are capable of producing substances with allergenic potential (Horner *et al.*, 1995). Indeed, allergenic proteins have been identified in 23 fungal genera. Moreover, at least eighty mould genera have been shown to provoke type I allergies, especially in susceptible persons (Simon-Nobbe *et al.*, 2008). Most common species known to be allergens include *Alternaria alternata, Aspergillus fumigatus* and *Cladosporium herbarum* (Hossain *et al.*, 2004). *Alternaria* and *Cladosporium* are considered to be the most important fungal airborne allergens found in outdoor air, whereas *Aspergillus* and *Penicillium* have been widely recognized as most important fungal indoor air allergens (Fischer and Dott, 2003). It is reported that approximately 2-6% of the general population, particularly in developed countries, is allergic to fungi (Żukiewicz-Sobczak and Zukiewicz-Sobczak, 2013).

The different symptoms of fungal allergic reactions are related to the size of inhaled particles. Moreover, it has to be noted that fungal cells don't need to be active (alive) for causing health disorders as allergens (Hossain *et al.*, 2004). Main fungal allergens are cell wall components like (1–3)-β-D glucan and glycoproteins. These allergens become airborne when materials contaminated with mould are aerosolized (Adhikari *et al.*, 2011; Haleem Khan and Mohan Karuppayil, 2012; Iossifova *et al.*, 2007; Seo *et al.*, 2009).

The symptoms of allergy appear when an individual is exposed to an allergen, which lead to an IgE-mediated hypersensitivity response that triggers eye irritation, rhinitis and upper airways irritation, sinusitis characterizing allergic syndrome (Bush *et al.*, 2006; Goh *et al.*, 2007; Żukiewicz-Sobczak and Zukiewicz-Sobczak, 2013). The major allergic illnesses caused by mould exposure are allergic asthma followed by allergic sinusitis, allergic rhinitis, broncho-pulmonary mycoses, and hypersensitivity pneumonitis (Cho *et al.*, 2014; Mendell *et al.*, 2011; Piecková and Wilkins, 2004).

- *Allergic asthma*: Due to small size of fungal spores, they may reach the alveolar surface inducing chronic inflammation of the lung tissue (Denning *et al.*, 2006, 2014). Today, an obvious link between asthma and fungal sensitization was described by several authors (Dannemiller *et al.*, 2014; Karvonen *et al.*, 2014; Meszaros *et al.*, 2014; Seo *et al.*, 2014; Sharpe *et al.*, 2015). In one study in the USA, performed in asthmatic patients, up to 80% of the individuals showed clear sensitization to moulds. The most important mould, frequently implicated in allergic asthma are *Alternaria*, *Aspergillus*, *Cladosporium*,

Epicoccum, *Aureobasidium* and *Penicillium* (Simon-Nobbe *et al.*, 2008). In a study on patients with a history of respiratory arrest, 91% had positive skin prick test for *Alternaria alternata* whereas that proportion was only 31% for the matched control subjects with asthma and no history of respiratory arrest. Thus, sensitization to moulds especially to *A. alternata* may be involved in severity of asthma in children and young adults (Haleem Khan and Mohan Karuppayil, 2012).

- *Allergic rhinitis* is characterized by sneezing, rhinorrhea, pruritus and nasal obstructions. It can be provoked by a wide number of fungal species, with, *Aspergillus* (Stark *et al.*, 2005), *Alternaria, Cladosporium* and *Penicillium* (Zuraimi *et al.*, 2009). The symptoms of allergic rhinitis caused by mould are usually hard to distinguish from those induced by inhalation of dust, pollen, animal and insect allergens (Haleem Khan and Mohan Karuppayil, 2012).
- *Allergic broncho-pulmonary mycoses* is most frequently caused by *Aspergillus fumigatus*, which may grow in the bronchial lumen, leading to a persistent bronchial inflammation inducing bronchiectasis in asthmatic patients (Knutsen and Slavin, 2011).
- *Allergic sinusitis*, or more precisely allergic fungal sinusitis, is a subtype of chronic rhinosinusitis characterized by type I hypersensitivity. Sinusitis is often preceded by rhinitis and rarely occurs without it. Common signs and symptoms involve nasal obstruction, plugged nose, and pain in the face. The presence of fungus may contribute to this illness (Thorp *et al.*, 2012). *Aspergillus* and *Alternaria* are connected to allergic sinusitis (Simon-Nobbe *et al.*, 2008).
- *Atopic dermatitis*, classified as an allergic disease, is a chronic inflammatory disease of the skin that is associated with high levels of fungal allergens (Johanning *et al.*, 2014; Wang *et al.*, 2007). The overall prevalence of dermatitis in school-age children is estimated to be high as 15 to 20 % (Baumer, 2008).
- *Hypersensitivity pneumonitis* is a complex syndrome caused by an exaggerated immune response to the inhalation of a large variety of particles, among these, the most frequent being fungi (Millon *et al.*, 2014). *Aspergillus* and *Penicillium* species have been the most associated with hypersensitivity pneumonitis (Millon *et al.*, 2014; Simon-Nobbe *et al.*, 2008). Typical symptoms include cough, fever, chills, weight loss, body aches, presenting an immunologic reaction to the inhaled substance (Færden *et al.*, 2014).

1.4.1.3 Sick Building syndromes (SBS)

Sick Building Syndrome (SBS), as described in United Kingdom/European terminology, or building related symptoms as it is referred to in the United States, has been described as a group of symptoms of unclear aetiology. In broad terms, these symptoms can be divided into mucous

membrane symptoms related to eyes, nose and throat; dry skin; general symptoms of headache and lethargy. More precisely, the most common SBS symptoms include fatigue, nausea, headache and dizziness, irritability, concentration and memory problems, irritated eye, nose and throat mucosa, skin reddening, asthma-like symptoms: breathlessness and cough attacks (Gutarowska and Piotrowska, 2007). These symptoms are common in the general population. What makes them part of SBS is a temporal relation with work in, or occupation of, a particular building. Therefore most of the symptoms usually vanish soon after leaving the problematic area (Haleem Khan and Mohan Karuppayil, 2012). SBS is more clearly recognised in the office environment (Crook and Burton, 2010). However, similar problems could occur, and have been reported, in schools, hospitals, care homes and homes (Cabral, 2010). In Nordic countries, their definition for SBS also attributes health problems associated to bad quality of indoor air in domestic dwellings, especially those associated with water damage.

SBS was first identified in the 1970s and from that period, various biotic and abiotic agents where suspected to contribute to that syndrome (Chao *et al.*, 2002b; Horner *et al.*, 2004).

Mould exposure has been considered as a significant contributor to the SBS health issues, but some studies question these results and conclusions (Crook and Burton, 2010). In some countries (USA, Canada, Scandinavian countries) research programmes have been launched to explain more precisely the relationship between SBS symptoms and microbial contamination of air (Gutarowska and Piotrowska, 2007).

For instance, a review of lower respiratory symptoms in 80 office buildings investigated by the US National Institute of Occupational Safety and Health (NIOSH) found an association between moisture and debris in the ventilation systems and adverse respiratory health effects (Crook and Burton, 2010). In the study of Sahakian et al. (2009), 4345 adult residents were surveyed to examine potential associations between building dampness and respiratory ill health. A significant positive association was found.

In many studies (Cabral, 2010; Herbarth *et al.*, 2003) it was shown that in buildings where abnormally high concentration of fungal flora (*Aspergillus, Penicillium, Stachybotrys*) was observed, typical SBS were reported by occupants. Moreover, after extensive repairing of the buildings (water infiltrations were checked, contaminated building materials were removed, walls were cleaned and cleansed, ventilation air ducts and equipment were cleaned) symptoms disappeared and microbiological analysis revealed a significant decrease in indoor fungal concentrations.

SBS has a very important economic impact since it has been estimated that annual cost resulting from health and building problems related to mould contamination was about US$1.4 billion for the USA (Crook and Burton, 2010).

1.4.2 Implication of fungal metabolites on health problems

As said previously, some disorder related to aerial mould exposure may be linked to the exposure to fungal particles (spores, mycelium) but also to fungal molecules as observed for allergy. Fungal metabolites may also participate to some of the health problems reported previously and particularly MVOCs, emitted by moulds during their development, and mycotoxins.

1.4.2.1 MVOCs

MVOC exposure has been associated with an increased risk for development of allergies and allergic asthma. Inhalation of those aerial bio-pollutants can cause sensory irritation, bronchoconstriction, or pulmonary irritation (Pestka *et al.,* 2008; Rolle-Kampczyk *et al.,* 2008). Moreover, one of commonly found MVOC, 2-ethyl-1-hexanol, is already known as irritant to the upper airways and provoker of asthma (Claeson *et al.*, 2007; Thrasher and Crawley, 2009; Wålinder *et al.*, 2001). Data from different studies indicated that a mixture of MVOC may have synergistic detrimental effects on health of occupants (Fischer and Dott, 2003).

Toxicological and exposure data are summarized in review by Korpi et al. (2009) for the 15 MVOCs most often analysed and reported in water- and mould-damaged buildings. Absorption takes place predominantly through the lungs. Most of MVOCs are quickly metabolized and excreted in the urine and bile. The MVOCs generally do not accumulate in tissues. The most obvious health effect in humans, related to MVOC exposure, is eye and upper-airway irritation, potentially due to stimulation of the trigeminal nerve (sensory irritation mechanism). However, in human experimental exposure studies, symptoms of irritation have appeared at MVOC concentrations several orders of magnitude higher than those measured indoors (single MVOC levels in indoor environments have ranged from a few ng/m^3 up to 1 mg/m^3). On the other hand, the toxicological database is poor even for these 15 major MVOCs (2-methyl-1-propanol, 3-methyl-1-butanol, 3-methyl-2-butanol, 2-pentanol 3-octanol, 1-octen-3-ol, 2-octen-1-ol, 3-methylfurane, 2-hexanone, 2-heptanone, 3-octanone, 2-methylisoborneol, 2-isopropyl-3-methoxy-pyrazine, geosmine, dimethyl disulfide).

Nasal provocation studies in a healthy individual using a relatively high concentration of the 1-octen-3-ol (10 mg/m^3), caused eye irritation and headache associated with an increase in lysozyme, myeloperoxidase, and eosinophilic cationic proteins in nasal lavage fluid obtained post-

challenge (Wålinder *et al.*, 2001). A 3-methylfuran (1 mg/m^3) nasal provocation challenge caused an increase in myeloperoxidase and lysozyme (Wålinder *et al.*, 2005). In vitro studies using broncho-alveolar cells incubated with several MVOCs (2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol and 1-pentanol) resulted in increased histamine release (Larsen *et al.*, 1998). Finally, in a mould-sensitized individual, inhalation challenge to 3-methylfuran (1 mg/m^3) was demonstrated to induce both an early and a late airway response, immediate obstructive airways reaction, followed by long-lasting bronchial obstruction (Wålinder *et al.*, 1998).

Moreover, some studies highlighted a positive association between certain MVOCs and SBS (Sahlberg *et al.*, 2013).

1.4.2.2 Mycotoxins

Mycotoxins are not released to the atmosphere since they are not volatile. However, they are present in spores and mycelium fragments that can be aerosolized and inhaled (Brasel *et al.*, 2005a; Reponen *et al.*, 2007). As conidia are usually smaller than 5 µm (Reponen *et al.*, 2001), they can penetrate deeply in human respiratory system.

In healthy occupants, these particles are destroyed by immune cells. However, residues may remain on epithelium surface, and subsequently enter the blood stream. It is likely that the amount of mycotoxins that enter our organism by inhalation is much lower than that ingested since mycotoxins are primary considered as frequent food contaminants. However, due to time spent in homes and offices, the exposure to those bio-pollutants in contaminated indoor environments could result in chronic or sub-chronic intoxications, and participate in some extent to some of the reported troubles related to mouldy environment and especially SBS (Cabral, 2010).

For instance, many authors have reported negative effects on health of occupants of water-damaged buildings in relation with *Stachybotrys chartarum* exposure. This fungal species is known to be able to produce several highly cytotoxic toxins belonging to the family of macrocyclic trichothecenes.

The first association between respiratory troubles and *Stachybotrys'* mycotoxins was made in late '90s. *S. chartarum* became the focus of attention following reports of its association with idiopathic pulmonary hemorrhage in infants in Cleveland, Ohio (Dearborn *et al.*, 1999).

Hodgson et al. (1998) studied building-related illness in Florida where patients' reported SBS-like symptoms within weeks after moving into the contaminated building. Researchers concluded that inhalation of mycotoxins, including satratoxins G and H produced by *S. chartarum* was likely responsible for the outbreak. Similarly, Johanning et al. (1996) implied that certain mycotoxins

(satratoxin H and spirocyclic lactones from water-damaged material) were the cause of respiratory and immune problems in occupants of “sick” buildings. Tuomi et al. (2000) studied water-damaged Finnish dwellings and identified mycotoxins (satratoxins G and H, T-2 toxin, and the aflatoxin precursor sterigmatocystin) in bulk samples (Hossain *et al.*, 2004).

As reported in tables 7, 8 and 9, the presence of mycotoxins was demonstrated any times on building materials, house dusts and even in indoor air samples. Beyond macrocyclic trichothecenes, sterigmatocystin is also frequently observed in indoors. It is a polyketide mycotoxin known to be produced by several fungal species, among which *A. versicolor,* one of the most frequent indoor contaminant. STG shares part of its biosynthetic pathway with aflatoxins and is considered as one of the last stable intermediate of the aflatoxin synthesis pathway. It was demonstrated that sterigmatocystin was 80-folds more toxic on A549 lung cell line compared to Hep-G2 liver cells, indicating a specific susceptibility of pulmonary cells to this mycotoxin and rising the question of the possible consequence of indoor contamination with that compound (Bünger *et al.*, 2004).

Table 7: Toxin contamination of building materials in indoor environments in USA and EU countries

Nature of location	Country	Nb of location/samples	Detected toxin (+ eventual prevalence)	Quantity/Concentration	Year	Reference
buildings (MD)	Germany *	15 samples: 3 wallpapers, 10 walls, 2 gypsum boards	Satratoxin G and Satratoxin H on wallpaper	Satratoxin G: 27-9650 ng/cm² Satratoxin H: 20-12000 ng/cm²	2006	Gottschalk et al.
dwellings (WD)	Belgium *	7 water damaged homes: 20 wallpapers(cut away), 25 mycelium (scraped off with scalpel), 2 silicone (cut away)	Roquefortine C (in 24 samples) Chaetoglobosin A (in 13 samples) Sterigmatocystin (in 10 samoples) Aflatoxin B1 (in 4 samples)	Roquefortine C: 0.1-7.2 ng/cm² Sterigmatocystin: 0.063-778.4 ng/cm² Cheatoglobosin A:1.16-13.83 ng/cm² Aflatoxin B1: 0.11-0.332 ng/cm²	2009	Polizzi et al.
dwellings (WD)	Finland	9 water damaged residences + 2 public buildings: 14 wood based-, 10 mineral fibers, 6 paper based-, 5 gypsum board, 7 other materials	Emodin: 20-71.4 % (in the different materials) Enniatin B: 17.7-80 % Beauvericin: 7.1-80 Sterigmatocystin: 10-80 % Cheatoglobosin: 10-50 % Mycophenolic acid: 14,3-16,7 % Satratoxin G: 14,3-60 % Satratoxin H: 14,3-60 % Ochratoxin A: 7,1-20 %	Emodin: 45-140 ng/g material Enniatin B: 0.44-0.78 ng/g Beauvericin: 0.11-70 ng/g Sterigmatocystin: 21-110 ng/g Cheatoglobosin: 83 ng/g Mycophenolic acid: 67-91 ng/g Satratoxins G and H: Not quantified Ochratoxin A: 8.3-32 ng/g	2011	Taubel et al.
dwellings (WD)	France, Arles	15 flooded dwellings with *Stachybotrys* contamination - swab samples of mouldy walls	Macrocyclic trichothecenes	40 +/- 50 ppb macrocyclic trichothecenes	2006	Charpin-Kadouch et al.
dwellings (WD)	Sweeden	31 different locations, homes with water damage: 39 gypsym papers, 8 wood based materials, 3 concrete, 6 papers, 7 others	Sterigmatocystin (in 25 samples) Trichodermol (in 35 samples) Verrucarol (in 28 samples) Satratoxin G (in 5 samples) Satratoxin H (in 4 samples)	Sterigmatocystin: 1.9-1100 pg/mg of material Trichodermol: 3,4-18000 pg/mg Verrucarol: 7,7-600 pg/mg Satratoxins G and H: Not quatified	2007	Bloom et al.
dwellings (WD)	Sweeden *	100 samples (from 57 water damaged dwelings) : 40 gypsum boards, 37 wood based samples, 7 linoleum flooring, 6wallpapers, 10 other	Sterigmatocystin (in 22 % of samples) Trichodermol (in 53 % of samples) Verrucarol (in 30% of samples) Satratoxin G (in 5 % of samples) Satratoxin H (in 4 % of samples) Gliotoxin (in 2 % of samples)	Sterigmatocystin: 4.9-1000 pg/mg of sample Trichodermol: 0.9-8700 pg/mg Verrucarol: 8.8-17000 pg/mg Satratoxins G and H: Not quantified Gliotoxin: 0.43-1.12 pg/mg	2009	Bloom et al.
hotel	USA, Ohio, Cincinnati	18 samples of contaminated materials	Satratoxins and roridins detected in 8 of 18 samples		2001	Trout et al.
kindergarten (WD)	Denmark, Copenhagen	7 gypsum wallboards	Stachybotryamide (in 6 samples) Stachybotrylactam (in 7 samples) Stachybotrydial (in 6 samples) Satratoxin H (in 2 samples) Satratoxin G (in 1 sample) Roridin L2 (in 3 samples) Roridin E (in 3 samples) Atranone A (in 5 samples) Atranone B(in 5 samples) Dolabellanes (in 5 samples)	Stachybotryamide: 170-180 ng/cm² of material Stachybotrylactam: 140-1040 ng/cm² Stachybotrydial: 170-540 ng/cm² Satratoxin H: 140 and 180ng/cm² Satratoxin G 140 ng/cm² Roridin L2: 35-55 ng/cm² Roridin E: 17 - 130 ng/cm² Atranone A: 0.031-8.7 ng/cm² Atranone B: 0.38-3.1 ng/cm² Dolabellanes: 0.18-5.3 ng/cm²	2016	Došen et al.

* Surveys that determined predominant fungal genera (species)

Toxin contamination of building materials in different indoor environments in EU and in USA was described in 10 surveys (Table 7), predominantly in European countries. Usually examined locations are dwellings, mostly after water damage.

It is important to note that there are often some differences in the toxins that were investigated and the known presence of the producing fungal species in indoor environments. This observation is also true for toxins investigated in dust (Table 9). For instance, toxins such beauvericin or enniatins are mainly produced by *Fusarium* species, that are only rarely reported as indoor contaminants. This observation is probably related to the availability of analytical methods usable for toxins that may be produced by indoor moulds. Indeed, the nature and levels investigated often require the use of very sensitive, specific methodologies and also may be limited due to the lack of pure standards commercially available.

Nevertheless, sterigmatocystin and macrocyclic trichothecenes are searched for and found on materials, in agreement with the finding that both *A. versicolor* and *S. chartarum* were frequent contaminants of indoor

building material, especially in case of uncontrolled increase of moisture. If these results strongly suggest that moulds can produce their mycotoxins during the colonization of indoor material, the lack of systematic correlation between toxin presence and mould presence make impossible the set-up of dose-response relationship.

It also makes very difficult to compare the different concentrations of toxins that are reported since they may be related to the surface that was analysed, the nature of the fungal contamination as well as the analytical method used.

Table 8 : Toxin contamination of air in indoor environments in USA and EU countries

Nature of location	Country	Nb of location/samples	Detected toxin (+ eventual prevalence)	Quantity/Concentration	Year	Reference
dwellings (MD)	USA, Texas	7 homes with *Stachybotrys* contamination: 40 air samples from 16 rooms	Macrocyclic trichothecenes	from <10 to >1300 pg/m³ of sampled air	2005	Brasel
dwellings (WD)	Belgium *	20 air samples from 7 homes	Roquefortine C (in 1 sample) Chaetoglobosin A (in 3 samples) Sterigmatocystin (in 3 samoples) Aflatoxin B1 (in 5 samples) Aflatoxin B2 (in 4 samples) Roridin E (in 3 samples) Ochratoxin A (in 3 samples)	Roquefortin C: 1.009-4 ng/m³ of air Sterigmatocystin: 0.0034-1.7674 ng/m³ Cheatoglobosine A: 0,0067-3,4205 ng/m³ Aflatoxin B1: 0,0024-0,1463 ng/m³ Aflatoxin B2: 0,0003-0,0211 ng/m³ Roridin E: 0,0031-0,082 ng/m³ Ochratoxin A: 0.0115-0.228 ng/m³	2009	Polizzi et al.
dwellings (WD)	France, Arles	15 flooded dwellings with Stachybotrys contamination + 9 control dwellings	Macrocyclic trichothecenes	0,62 +/- 0,65 ppb macrocyclic trichothecenes 0,29 ppb macrocyclic trichothecenes in control	2006	Charpin-Kadouch et al.
dwellings (WD)	Germany	1 air sample from wd dwelling	Satratoxins G and H	Satratoxin G: 0.25 ng/m³ of air Satratoxin H: 0.43 ng/m³	2008	Gottschalk et al.
dwellings (WD)	Sweeden *	37 samples from 22 locations	Trichodermol (in 26,2 % of samples) Verrucarol (in 21,6 %) Sterigmatocystin (in 40,5 %) Gliotoxin (in 2,7 %) Satratoxin G (in 5,4 %) Satratoxin H (in 5,4 %) Aflatoxin B1 (in 5,4%)		2009	Bloom et al.
dwellings (WD)	Sweeden *	8 airborne cultivable fungal particles from 8 homes	Trichodermol (in 4 samples) Sterigmatocystin (in 1 sample) Verrucarol (in 2 samples)	Trichodermol: 330-1900 pg/cm ² of agar Sterigmatocystin: 130 pg/cm ² Verrucarol: 250-2900 pg/cm²	2007	Bloom et al.

* Surveys that determined predominant fungal genera (species)

If we look at the few studies performed on mycotoxins' presence in indoor air (Table 8), we can see that macrocyclic trichothecenes were sometimes identified. Once again, this is not really in agreement with data on fungal contamination of indoor air since most often, *S. chartarum* was not reported as a contaminant of air, probably in relation with the difficulty to aerosolize fungal particles. Thus, such finding may suggest that these toxins could be aerosolized in the absence of fungal particles, maybe due to their excretion from mycelium and adsorption on small particles.

It has to be noted that only few data are available, probably in relation with the levels that have to be determined and that requires very sensitive methods of analysis.

Table 9: Toxin contamination of dust in indoor environments in USA and EU countries

Nature of location	Country	Nb of location/samples	Detected toxin (+ eventual prevalence)	Quantity/Concentration	Year	Reference
dwelings (MD or WD)	Germany, Hamburg *	11 carpet dust samples from 8 households	Sterigmatocystin in 2 samples	Sterigmatocystin: 2 and 4 ng/g dust	2002	Engelheart et al.
dwelings (MD)	Germany	43 mould damaged buildings 50 control buildings	Ochratoxin A in 5 samples from MD homes and in 8 samples from control homes	In MD homes: 0.23-23.9 ng/g in control homes: 0.23-14.6 ng/g	2016	Fromme et al.
dwelings (MD)	USA, LA, New Orleans *	5 mould contaminated homes after hurricane Katrina	Verrucarol Sterigmatocystin	Verrucarol: 0.6-18 pg/mg dust Sterigmatocystin: 16-28 pg/mg	2009	Bloom et al.
dwelings (WD)	Belgium *	27 dust samples	Roquefortine C (in 2 samples) Sterigmatocystin (in 9 samoples) Aflatoxin B1 (in 1 sample) Aflatoxin B2 (in 7 samples) Ochratoxin A (in 7 samples)	Roquefortine C: 0.1-7.2 ng/cm² of dust sample Sterigmatocystin: 0.063-778.4 ng/cm² Aflatoxin B1: 0.11-0.332 ng/cm² Aflatoxin B2: 0.019-0.253 ng/cm² Ochratoxin A: 0.008-0.773 ng/cm²	2009	Polizzi et al.
dwelings (WD)	Finland	9 wd residences + 2 public buildings: 7 dust bags, 13 floor dust, 7 settled airborne dust	Emodin: 28,6-100 % in different types of dust samples Enniatin B: 100 % Beauvericin: 61.5-71.4 % Sterigmatocystin: 7.7-14.3 % Cheatoglobosin A: 15.4 % Mycophenolic acid: 14.3 %	Emodin: 0.24-84 ng/g dust Enniatin B: 0.63-9.4 ng/g Beauvericin: 0.05-1.6 ng/g Sterigmatocystin: 1.3 ng/g Cheatoglobosin A: 3100 ng/g Mycophenolic acid: not quantified	2011	Taubel et al.
dwelings (WD)	France, Arles	15 flooded dwellings + 9 control dwellings: floor dust from mouldy rooms, cloth moistened with PBS buffer	Macrocyclic trichothecenes	21.9 +/- 35.89 ppb (in wd dwellings) 0.31 ppb (in control)	2006	Charpin-Kadouch et al.
dwelings (WD)	Sweeden	8 settled dust in 4 WD homes	Sterigmatocystin (in 1 sample) Trichodermol (in 2 samples) Verrucarol (in 2 samples)	Sterigmatocystin: 17 pg/mg sample Trichodermol: 2.4 and 3.4 pg/mg Verrucarol: 19 and 43 pg/mg	2007	Bloom et al.
dwellinngs	Germany	200 control buildings	Sterigmatocystin (in 4 samples) Deoxynivalenol (in 18 samples) Gliotoxin (in 1 sample)	Sterigmatocystin: 3.2-108 ng/g DON: 176-8100 ng/g Gliotoxin: 15-110 ng/g	2016	Fromme et al.
kindergarten (WD)	Denmark, Copenhagen	11 setlled dust samples	Stachybotryamide (in 2 samples) Stachybotrylactam (in 10 samples) Stachybotrydial (in 11 samples) Roridin E (in 2 samples) Atranone A (in 2 samples) Dolabellanes (in 2 samples)	Stachybotryamide: 0.08-0.09 ng/cm² of dust Stachybotrylactam: 0.07-0.23 ng/cm² Stachybotrydial: 0.07-0.18 ng/cm² Roridin E: 0.006-0.01 ng/cm² Atranone A: 0.02-2.7 ng/cm² Dolabellanes: 0.025 and 0.5 ng/cm²	2016	Došen et al.
schools (WD)	Finland	225 settled dust samples from 22 primary schools (WD schools and control schools)	control: Emodin (19.5 % of samples) Enniatin A (1.2 %) Enniatin B (34.2 %) Enniatin B1 (7.3 %) Griseofulvin (2.4 %) WD: Alamethicin (1.9 %) Emodin (16.5) Enniatin A (1%) Enniatin B (24.3%) Enniatin B1 (9.7%) Trichodermol (1%) Verrucarol (1.9 %)	control: Emodin (up to 30 pg/cm ³) Enniatin A (0.2) Enniatin B (12.7) Enniatin B1 (12.1) Griseofulvin (24.3) WD: Alamethicin (up to 31.7 pg/cm³) Emodin (4.6) Enniatin A (8) Enniatin B (16.9) Enniatin B1 (33.9) Trichodermol (3.1) Verrucarol (4)	2012	Peitzsch et al.
schools (WD)	Netherland	238 settled dust samples from 20 primary schools (WD schools and control schools)	control: Beauvericin (4 % of samples) Emodin (30.7 %) Enniatin A (2.4 %) Enniatin B (31.5 %) Enniatin B1 (7.3 %) Trichodermol (0.8 %) Verrucarol (0.8 %) WD: Beauvericin (8.6 % of samples) Emodin (22.9) Enniatin A (5.7 %) Enniatin B (35.2 %) Enniatin B1 (13.3 %) Sterigmatocystin (1.9 %)	control: Beauvericin (up to 9.6 pg/cm ³) Emodin (14.3) Enniatin A (0.2) Enniatin B (2.3) Enniatin B1 (0.3) Trichodermol (6) Verrucarol (6.6) WD: Beauvericin (up to 4 pg/cm³) Emodin (16.4) Enniatin A (5.14) Enniatin B (6.3) Enniatin B1 (11.3) Sterigmatocystin (5)	2012	Peitzsch et al.
schools (WD)	Spain	212 settled dust samples from 24 primary schools (WD schools and control schools)	control: Beauvericin (4.4 % of samples) Emodin (17.4 %) Enniatin B (17.4 %) Enniatin B1 (8.7 %) Trichodermol (2.9 %) Verrucarol (1.5 %) WD: Beauvericin (0.7 % of samples) Emodin (20.4) Enniatin B (8.8 %) Enniatin B1 (6.1 %) Griseofulvin (1.4 %)	control: Beauvericin (up to 0.58 pg/cm³) Emodin (16.5) Enniatin B (1.2) Enniatin B1 (4.3) Trichodermol (2.21) Verrucarol (1.4) WD: Beauvericin (up to 0.34 pg/cm³) Emodin (316) Enniatin B (0.7) Enniatin B1 (2.4) Griseofulvin (8.8)	2012	Peitzsch et al.

* Surveys that determined predominant fungal genera (species)

Twelve studies (Table 9) reported determination of mycotoxin presence in dust, mostly in dwellings, even if one reported data in schools in three different European countries. As said previously for materials, one can question the choice of toxins that were investigated (Peitzsch *et al.*, 2012b). Since some are mostly produced by fungal species that are phyto-pathogens and that contamination of indoor dust could be more the consequence of outdoor air income than related to fungal development of indoor environment.
Nevertheless, once again, sterigmatocystin and macrocyclic trichothecenes were found in dust, in agreement with the presence of producing species in building.

So, to date, more and more evidences suggest that mycotoxins may contaminate indoor environments and therefore participate to some of the troubles observed in inhabitants of mouldy homes. However, the simultaneous presence of many biological and chemical contaminants in indoor air makes often difficult to establish a direct correlation between the presence of mycotoxins and health problems in occupants (Creppy, 2002; EU, 1993; Nielsen, 2003; World Health Organization, 2009). Moreover, it is complicated to draw conclusion from the levels of mycotoxins that were reported on the different compartment of indoor environments (material, dust, air) since no dose-effect relation are available for most of analysed compounds and since no data about quantities that could effectively be inhaled are available.

Finally, there are numerous studies reporting either the presence of fungal contaminant or mycotoxins in indoor environments. However, only few of them studied both, making possible at least a correlation between the two parameters. It seems that, to allow a better risk assessment of possible health consequences of the presence of toxinogenic moulds in indoor environment, studies aiming to follow the process from production of the toxic compounds on materials and their subsequent transfer to air are still required.

Aims of the study

Within this overall context, our study aimed to provide new insights in the evaluation of problems that could result from fungal contamination of indoor environments.

For that, we have chosen to study the different parts of this complex problem, using three toxinogenic species that are known as frequent indoor contaminants: *A. versicolor, S. chartarum* and *P. brevicompactum.*

Firstly, we studied the ability of these species to colonize and produce their respective toxins on different building materials.

In a second step, we analysed the ability of produced toxins to be aerosolized from contaminated support and their transfer to air according to different aeraulic solicitations.

Finally, we characterized their cytotoxicity on human pulmonary cells and compared it to that observed on digestive cells in order to compare effect that could be related to inhalation *vs* ingestion.

In parallel, we also tried to identify markers that could be used to monitor an active toxinogenesis in indoor environment by studying the MVOCs that are emitted during toxinogenesis.

We also tested the efficacy of one of the most used antifungal treatment on the elimination of one of our contaminant on different materials.

To reach these objectives, some specific methodologies were developed and will be further detailed in the coming parts.

PART TWO.

Experimental work

CHAPTER ONE.

Fungal development and toxinogenesis after contamination of indoor materials of interest with chosen fungal strains

1.1 Introduction

In order to determine fungal potential to develop on different types of materials, commonly used in indoor furnishing, and their subsequent ability to produce toxins during that period, we worked on three fungal species: *Aspergillus versicolor, Penicillium brevicompactum* and *Stachybotrys chartarum.*

The choice was made based on three main points:

1. *Their prevalence in indoor environments*: as reported in section 1.3. of Bibliographic review, these three species are known to be common contaminants of homes, their presence being especially marked in water damaged dwellings.
2. *Their toxinogenic potential (and therefore their possible implication in health of occupants)*: These three species are able to produce different mycotoxins:
    - *A.versicolor* is likely to produce the sterigmatocystin, which is carcinogenic in animals (and has been classified in 2B group (possible human carcinogen) by International Agency for Research on Cancer);
    - *P. brevicompactum* can produce mycophenolic acid, which is immunosuppressive;
    - *S. chartarum* is a producer of several toxins belonging to the family of macrocyclic trichothecenes. The presence of *Stachybotrys* and these mycotoxins has been suspected to be responsible for disorders including the appearance of cases of pulmonary hemorrhages in infants in the USA. More recently, it's being investigated for its possible relationship with sick building syndrome.
3. *Their structures' diversity*: Chosen species present very different conidial organizations and mycelial structures, which can suggest that they will have different behavior regarding aerosolization.

Sterigmatocystin

1. General information

Sterigmatocystin (STG) is a mycotoxin that can be produced by more than fifty fungal species, including many *Aspergillus* species, like *Aspergillus flavus, A. parasiticus, A. versicolor* and *A. nidulans*. Among them, *A. versicolor* is the most common source (Rank *et al.*, 2011). According to the authors, the frequency of toxinogenic *A. versicolor* strains is approximately 74% (EFSA, 2013). Other species belonging to other genera such as *Penicillium, Bipolaris, Chaetomium* and *Emiricella* are also able to produce this mycotoxin (Veršilovskis and de Saeger, 2010).

2. Conditions of production

STG is a polyketide mycotoxin structurally related to AFB1 (see Figure 14). The main STG producer, *A. versicolor*, is unable to biotransform STG into O-methylsterigmatocystin, the direct precursor of aflatoxin B1 (AFB1) and G1 (AFG1) (Böhm *et al.*, 2013).

A. versicolor is generally xerophilic, which means it can grow at low water activity (<0.8). The minimum and maximum growth temperatures for *A. versicolor* are 4 and 40 °C with an optimum at 30 °C. Its optimal water activity is 0.95 with a minimum at 0.75. Optimal conditions for STG production (by *A. versicolor*) are temperatures between 23 and 29 °C, water activity starting from 0.76 and a moisture content above 15% (Veršilovskis and de Saeger, 2010).

3. Toxicity of sterigmatocystin

The acute toxicity of STG is summarized in EFSA recent report (EFSA, 2013). Acute oral toxicity of STG is relatively low (LD50 values range 120–166 mg/kg body weight), with liver and kidneys as target organs (Veršilovskis and de Saeger, 2010). STG is hepatotoxic in poultry, rat, mouse, monkey and guinea pig. Incidence of hepatocellular necrosis and haemorrhages increase with dose and duration of exposure. In the kidney, hyaline degeneration, tubular necrosis and haemorrhages were described in rats and/or vervet monkeys exposed to STG. It is also nephrotoxic in poultry and toxic for several fish species.

STG is mutagenic in both bacterial and mammalian cells after metabolic activation and forms DNA adducts. They are related to the metabolisation of the molecule in the liver and lung by various cytochrome P450 enzymes into different hydroxymetabolites and reactive exo-epoxide that readily forms DNA adducts (Veršilovskis and de Saeger, 2010).

Carcinogenicity has been observed in different tested animal species (rat, mouse, Mongolian gerbils, monkey and fish) after oral, intraperitoneal, subcutaneous and dermal administration resulting in hepatocellular carcinomas, haemangiosarcomas in the liver, angiosarcomas in brown

fat and lung adenomas. The International Agency for Research on Cancer (IARC) has assessed the carcinogenic potential of STG (IARC, 1976, 1987) and concluded that oral administration of STG lead to the development of lung tumours in mice and liver tumours in rats. No case reports or epidemiological studies were available for evaluation of carcinogenicity in human by IARC and it was therefore classified in 2B group.

Mycophenolic acid

1. General information

Mycophenolic acid (MPA) is a weak organic acid, considered as an immunosuppressive pharmaceutical drug, but also as a postharvest mycotoxin due to its undesirable presence in various feedstuffs. It is lipid soluble and is well absorbed orally (Seguin *et al.*, 2014).

Mycophenolic acid (MPA) was first isolated from *Penicillium* cultures including *Penicillium brevicompactum, Penicillium roqueforti, Penicillium stoloniferum*, but it can be produced by some other species like *Byssochlamys nivea* and *Aspergillus unilateralis*. *Penicillium brevicompactum* has been identified as the major producer of mycophenolic acid (Ardestani *et al.*, 2010; Seguin *et al.*, 2014; Van Pamel *et al.*, 2011; Visagie *et al.*, 2014).

MPA has also been reported to possess antibacterial, antifungal, antiviral, as well as antitumor and antipsoriasis activities (Regueira *et al.*, 2011). Most importantly, it is being used as an immunosuppressant in kidney, heart, and liver transplantation patients (Tönshoff *et al.*, 2011) and is marketed under the two brands CellCept (mycophenolate mofetil; Roche) and Myfortic (mycophenolate sodium; Novartis) (Regueira *et al.*, 2011).

MPA is used as is an immunosuppressive drug as a mycophenolate mofetil (which is semisynthetic ethyl ester) in solid organ transplantation to prevent acute allograft rejection (van Hest, 2006). Moreover, MPA is used for the treatment of non-transplant, renal, autoimmune, rheumatological, dermatological, ophthalmological and neurological diseases (Qasim *et al.*, 2014).

2. Conditions of mycophenolic acid production

The minimum and maximum temperatures for growth of *Penicillium brevicompactum* are -2 and 30 °C, respectively, with an optimum near 23 °C. The minimum a_w for germination and growth is 0.78 at 25 °C (Frisvad *et al.*, 2004). Mycophenolic acid production processes have been carried out using fermentation modes, such as submerged and solid-state batch cultures (Ardestani *et al.*, 2010).

3. Toxicity of mycophenolic acid

Mycophenolic acid has a weak acute toxicity with an oral LD50 of 700 mg/kg in rats (Pitt and Hocking, 2009).

The most commonly reported side effect of MPA (and of MMF) is gastrointestinal disturbance. It is dose dependent and includes nausea, diarrhea, soft stools, anorexia, abdominal cramps, frequent stools, vomiting, and anal tenderness (Qasim *et al.*, 2014; Tönshoff *et al.*, 2011). MMF has no clinically significant hepatotoxicity (Kitchin *et al.*, 1997). Occasional neurologic side effects, including weakness, tiredness, headache, tinnitus, and difficulty sleeping, may occur with use of MPA. Some of these symptoms seem to decrease in incidence after the first several years of therapy. The adverse effects of MMF appear to be most prominent in young children younger than 6 years (Tönshoff *et al.*, 2011). These symptoms have not been severe enough to cause discontinuation of MPA therapy (Kitchin *et al.*, 1997).

Macrocyclic trichothecenes

1. General information

The trichothecene mycotoxins comprise more than 180 structurally related sesquiterpenoid metabolites (Islam *et al.*, 2007). All trichothecenes have a common 9, 10 double bond and a 12, 13 epoxide group (Pestka *et al.*, 2008) which is responsible for their toxicological activity (Zain, 2011).

Macrocyclic trichothecenes, which have a cyclic diester or triester ring linking C-4 to C-15 (Aleksic *et al.*, 2016; Gareis and Gottschalk, 2014), are produced by several common soil fungi, including primarily *Stachybotrys chartarum* (Hossain *et al.*, 2004) but also *Myrothecium verrucaria* (Abbas *et al.*, 2002).

In 1973, Eppley and Bailey isolated macrocyclic trichothecenes produced by *Stachybotrys chartarum*. This species produced several types of mycotoxins belonging to the following families: satratoxins, roridins, verrucarins and stachybocins (Sudakin, 2003).

Satatoxins are considered to be the most deleterious of toxins produced by mould even if their total quantity is often relatively low (Fremy, 2009; Jarvis *et al.*, 1986; Nelson, 2001).

2. Conditions of toxins' production

S. chartarum is saprophytic fungi that grows best on highly cellulosic substrates. Optimum growth in buildings for most molds occurs at 20 to 25°C. Water activity is the most important factor for

growth and an activity of at least 0.98 is considered as optimal for mycotoxin production by *S. chartarum*. At a value of 0.75, mold growth, as well as mycotoxin production, were inhibited. At 84-100% relative humidity, the fungus reproduces rapidly on wallpapers and plasters and produces satratoxins G and H (Piecková and Jesenská, 1999).

Although it does not grow as quickly as *Aspergillus, Penicillium* and *Cladosporium*, it usually becomes dominant within one to two weeks and may overgrow the other moulds (Integrated Laboratory Systems, 2004). Optimal pH is 5.6-6.0, but *S. chartarum* can grow at pH ranging from 3.0 to 9.8.

Biosynthesis pathway for *S. chartarum* mycotoxins is only partially known (Nielsen, 2002; Semeiks *et al.*, 2014).

3. Toxicity of macrocyclic trichothecenes

Since 1930 *Stachybotrys* has attracted attention as the causative agent of stachybotryotoxicosis, a severe necrotic and haemorrhagic disease, characterized by symptoms such as irritation of the mouth, throat, and nose, shock, dermal necrosis, hemorrhage, leukopenia, nervous disorder, and death, especially observed in horses after feeding mouldy straw (Gareis and Gottschalk, 2014). Later, its highly toxic metabolites, the macrocyclic trichothecenes, were found to be responsible for this disease (Bata *et al.*, 1985; Harrach *et al.*, 1981, 1983).

The first cases of human affliction were reported by Russian investigators; affected people included workers in cottonseed oil processing plants, grain processing plants, grain elevators, and textile mills. Common symptoms included rash, conjunctivitis, bloody rhinitis, fever, headache, fatigue, and a burning sensation of the eyes and nasal passage (Integrated Laboratory Systems, 2004).

In the 1990s, *S. chartarum* became the focus of attention following reports of its association with idiopathic pulmonary hemorrhage in infants in Cleveland, Ohio (Dearborn *et al.*, 1999) and identified as a serious contaminant in indoor environments. More recently, there has been increasing evidence of a possible relationship between the presence of *S. chartarum* in indoor environments and human illness such as sick building syndrome (Hossain *et al.*, 2004).

Epidemiologic investigations have suggested an association between exposure to *S. chartarum* and toxic inflammatory effects in infants, Sick building syndrome in courthouse workers, and in office workers, as well as between *S. chartarum* and extreme chronic fatigue syndrome in hospital workers. However, there is not sufficient evidence to substantiate this association. It is generally accepted that building-related asthma and an increased incidence of upper respiratory disease are associated with living or working in a mouldy environments (Integrated Laboratory Systems, 2004).

Mycotoxins produced by *S. chartarum* are known to be strong inhibitors of protein synthesis (Ulrich *et al.*, 2016) and their acute toxicity has been demonstrated following ingestion in horses (Aleksic *et al.*, 2016) contact with skin (Le Bars and Le Bars, 1986) and also after inhalation in rodents (Islam *et al.*, 2009). In addition, trichothecenes might affect cytokine production (Hossain *et al.*, 2004). These toxins are the most cytotoxic trichothecenes currently known (Nielsen *et al.*, 2009; Ulrich *et al.*, 2016). Numerous studies have demonstrated the toxicity of toxins *from S. chartarum* on animals and animal and human cells (Nelson, 2001). Yang et al. (2000) reported that satratoxin G was the most cytotoxic of eight trichothecenes tested on mammalian cells, even more toxic than the well known T-2 toxin associated with alimentary toxic aleukia. Other researchers have also reported the higher toxicity of satratoxins compared to other trichothecenes. The LD50 in mice for satratoxins is ~1 mg/kg (Nelson, 2001).

In a recent study, Carey et al. (2012) demonstrated that intranasal exposure to satratoxin G induces rhinitis, atrophy of the olfactory epithelium and apoptosis of olfactory sensory neurons in both mice and Rhesus monkeys.

It has been postulated that *S. chartarum* and its trichothecene mycotoxins are contributors to debilitating respiratory illnesses (Hossain *et al.*, 2004; Islam *et al.*, 2007; Jarvis *et al.*, 1986, 1998) as well as immune dysfunction (Johanning *et al.*, 1996) and cognitive impairment (Islam *et al.*, 2007).

S. chartarum was reported (Integrated Laboratory Systems, 2004) to induce sensory irritation, inflammatory, and/or pulmonary responses in mice and rats exposed via intranasal instillation, intratracheal instillation, and inhalation. In one reproductive toxicity study, *Stachybotrys* caused a decrease in the percentage of pregnant mice. Inflammatory responses (e.g., increases in BALF total protein immunoglobulin E [IgE] levels) were seen in mice when exposed to an extract composed of five isolates of *S. chartarum* over a four-week period.

In vitro cytotoxic effects of macrocyclic trichothecenes included induction of tumour necrosis factor-α and interleukin-6 production. Changes in total protein, albumin, pro-inflammatory cytokine, and lactate dehydrogenase in bronchoalveolar lavage fluid were observed in mice exposed to *S. chartarum* (Integrated Laboratory Systems, 2004).

For all three species, the strategy was to:

- ✓ Identify a couple made of a toxinogenic strain and a non-toxinogenic strain (further named pair TOX +/TOX-). This pair was needed since one of the aims was to analyse the possible presence of microbial volatile organic compounds (MVOCs) specific to toxinogenesis. The

best would have been to compare VOCs produced by TOX+ and TOX- strains growing in the same conditions (Chapter 2). However, in case no TOX- strain was identified, we selected the strain with the lowest toxinogenic potential. This first screening was done on culture medium.

- ✓ Evaluate the toxinogenesis of all pairs on fiberglass, in the experimental conditions used for the measurement of MVOCs. Indeed, to determine MVOCs that are specifically associated with toxinogenesis, it is necessary to cultivate strains on a neutral medium, not emitting VOCs. Fiberglass was previously identified as adequate material for such determination (Le Moullec and Squinazi, 1996).
- ✓ Evaluate the toxin production during development on different building materials. For that, a UPLC-MS/MS method was developed. The objective was to have a single method, sufficiently sensitive and specific for the determination of toxin production on materials but which can also easily be applied for monitoring of toxins in aerosols (Chapter 3). Moreover, our aim was characterization of the toxin profile of *Stachybotrys* strains and determination of the nature and the relative proportion of aerosolized macrocyclic trichothecenes.

1.2 Selection of strains of interest for this study

1.2.1 Materials and methods

1.2.1.1 Mycotoxin standards

Standards of sterigmatocystin (STG) and mycophenolic acid (MPA) were purchased from Sigma (Saint-Quentin Fallavier, France). Standards were dissolved in methanol (MeOH) to obtain stock solutions that were stored at -20 °C.

1.2.1.2 Solvents and reagents

All reagents including the solvents used for sample preparation were purchased from ICS (Lapeyrouse-Fossat, France) and were analytical grade.

1.2.1.3 Fungal strains

The tested fungal strains mostly originate from fungal collections of partner laboratories or international collections (Table 10).

All strains were stored at 4 °C on malt extract agar (MEA) (15 g agar, 30 g malt extract per litre) (Biokar, France) and regularly plated on new medium to check their viability and purity.

Table 10: Identification and origin of the different fungal strains tested in this study

Species	Strain N°	Identification N°	Laboratory of origin
Aspergillus versicolor	Av 1	18884	CSTB
	Av 2	RT 001-06	CSTB
	Av 3	RT 009-03	CSTB
	Av 4	RT 012-03	CSTB
	Av 5	RT 033-06	CSTB
	Av 6	BNF	CSTB
	Av 7	E17	ENVT
	Av 8	COB	ENVT
	Av 9	NCPT 54	INRA
	Av 10	02033	CSTB
	Av 11	RT 024-03	CSTB
	Av 12	E 26 b	ENVT
	Av 13	E 26 a	ENVT
	Av 14	E 64	ENVT
Penicillium brevicompactum	Pb 16	04891	CSTB
	Pb 17	NCPT 23	INRA
	Pb 18	RT 017-06	CSTB
	Pb 19	*mpa* CΔ (mutant)	TUD (Prof JB. Nielsen)
	Pb 20	NRRL 2012	ARS
	Pb 21	NRRL 28120	ARS
	Pb 22	NRRL 864	ARS
	Pb 23	NRRL 866	ARS
	Pb 24	M 362	ENVT
	Pb 25	IBT 23078 (parent)	TUD (Prof JB. Nielsen)
Stachybotrys chartarum	ST 81	ST 81	ENVT
	ST 82	ST 82	ENVT

1.2.1.4 Determination of toxinogenic potential

1.2.1.4.1 Determination of toxinogenic potential on culture medium

One week cultures on potato dextrose agar (PDA) (4 g potato extract, 20 g dextrose, 15 g agar per litre) (BioKar, France) at 25 °C were used to prepare spore suspensions by adding 10 mL of Tween 80 (0.05 %) to the Petri dish (PD). The number of spores was quantified by direct counting on a Malassez cell. Spore suspensions were then diluted to obtain the concentration of 10^6 spores/mL. One hundred µL of these solutions were plated on each PD with PDA and incubated at 25 °C for 10 days.

1.2.1.4.2 Determination of toxinogenic potential on fiberglass

For tests on fiberglass (FG), spore suspensions were prepared as previously described but the spores' concentrations were adjusted to 10^7 spores/mL.

Fiberglass (GF/B glass fiber filter, Whatman) was cut into 2 x 5 cm pieces and then sterilized (121 °C, 20 min) as described by Górny et al. (2001) and Peitzsch et al. (2012a). Then, they were soaked with 0.5 or 1 mL of a nutritional solution (NS) (30 g glucose, 3 g $NaNO_3$, 0.5 g $MgSO_4$, 10 mg $FeSO_4$ in 1 L Sigma Buffer pH 7.6).

Materials were placed in flasks, on a layer of 2 cm of glass beads (corresponding to volume of 50 mL of 6 mm glass beads). Eight mL of sterile distilled water was added to each flask, in order to maintain moisture at a saturation level throughout the test. Contamination was achieved by applying 100 µL of spore suspension on each sample. Flasks were then incubated for 10 days at 25 °C in darkness (Figure 6).

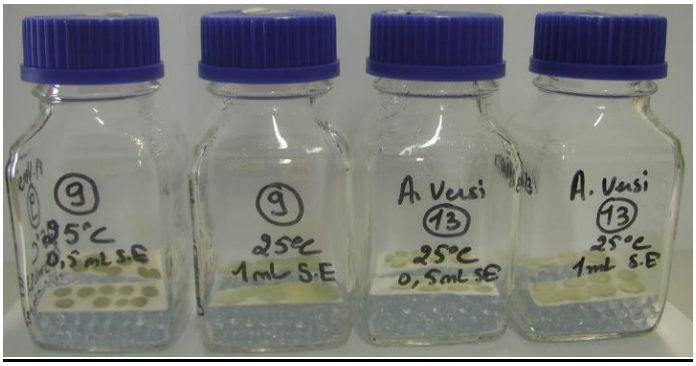

Figure 6: Fiberglass samples (supplemented with 0.5 mL or 1 mL of NS) contaminated with 2 different strains, NCPT 54 strain (TOX+) and E26a strain (TOX-) of *Aspergillus versicolor* after cultivation at 25 °C for 10 days

1.2.1.5 Extraction procedures

For mycotoxin determinations, culture medium samples and FG samples were extracted after 10 days of incubation.

1.2.1.5.1 Culture medium

Culture medium, taken from whole PD, was extracted by 50 mL of cold potassium phosphate buffer (50 mM; pH 7.4), homogenized with Turax. For AMP and STG, 10 mL were further extracted with the same volume of $CHCl_3$ by mechanical agitation on vortex. Extracts were centrifuged for 5 min at 3500 rpm and filtered through a phase separator filter (Whatman 1 PS).

1.2.1.5.2 Fiberglass

FG samples contaminated with *Aspergillus versicolor* and *Penicillium brevicompactum* were extracted in 2 mL of cold potassium phosphate buffer (50 mM; pH 7.4) and 5 mL of $CHCl_3$, with 5

glass beads (in order to facilitate mechanical agitation and recover the maximum of toxins). Extracts were filtered through a phase separator filter (Whatman 1 PS). For *Stachybotrys chartarum*, FG was extracted with 5 mL of phosphate buffer saline (PBS) with 5 glass beads. Supernatan was collected and centrifuged for 10 minutes at 7000 rpm before analysis.

1.2.1.6 Toxin quantification

1.2.1.6.1 Quantification of sterigmatocystin and mycophenolic acid by thin layer chromatography

STG and MPA were measured by fluorodensitometry (Shimadzu CS-930 fluorodensitometer, Shimadzu Corp., Kyoto, Japan) after separation on thin layer chromatography (TLC) plates (Merck n°5553, VWR, Fontenay sous-bois, France). Quantification was performed by comparisons with standards run on the same plate.

1.2.1.6.1.1 Quantification of sterigmatocystin

Extracts were evaporated to dryness and then suspended in toluene-acetonitrile (98:2, vol: vol). Quantification of STG was carried out according to the method of Anthnasios and Kuhn (1977). Three microliter of extracts were separated by toluene/acetic acid (90:10, vol: vol). After drying, the plate was revealed by spraying with 20 % aluminium chloride in ethanol: water (50:50) and heated for 10 minutes at 120 °C (Van Egmond *et al.*, 1980). Toxin was measured at excitation wavelength of 360 nm. The limit of quantitation was 3 ng/µL.

1.2.1.6.1.2 Quantification of mycophenolic acid

Extracts were evaporated to dryness and then suspended in MeOH. Three µL of sample were separated in toluene: ethyl acetate: formic acid (50: 40: 10, vol: vol: vol). After drying, the plate was sprayed with diethylamine. Toxin was measured at excitation wavelength of 365 nm. The limit of quantitation of this method was 2, 5 ng/µL.

1.2.1.6.2 Quantification of macrocyclic trichothecenes by ELISA

Macrocyclic trichothecenes (MCT) were quantified using an ELISA kit as recommended by manufacturer (QuantiTox Kit for Trichothecenes; EnviroLogix, Portland, USA). This kit allowed the determination of the global amount of 10 macrocyclic trichothecenes (satratoxins G and H, isosatratoxin F, verrucarol, verrucarins A and J, roridins A, L2, E and H) expressed as equivalent roridin A by using determined cross-reactivity ratio between all mycotoxins (Charpin-Kadouch *et al.*, 2006). The quantification range was 0.2-18 ng/mL of macrocyclic trichothecenes expressed in equivalent roridin A.

ELISA was performed with 50 µL of PBS extract. Each dosage was done in duplicate on the same plate. Lecture was done by Optical Density determination at 450 nm using a Stat-Fax 303 Plus

Reader (Neogen, Auchincruive, Scotland, UK) within 30 min of the addition of Stop solution to wells.

1.2.2 Results and discussion

1.2.2.1 Sterigmatocystin production by Aspergillus versicolor strains

1.2.2.1.1 On agar medium

Fourteen strains of *A. versicolor* were tested for STG after culturing on PDA at 25 °C for 10 days. Figure 7 summarizes the results of the screening and the amounts of STG [µg/PD] synthesized by each strain. From this assay, strains 9 (NCPT54) and 13 (E26a) were chosen as TOX+/TOX-, respectively.

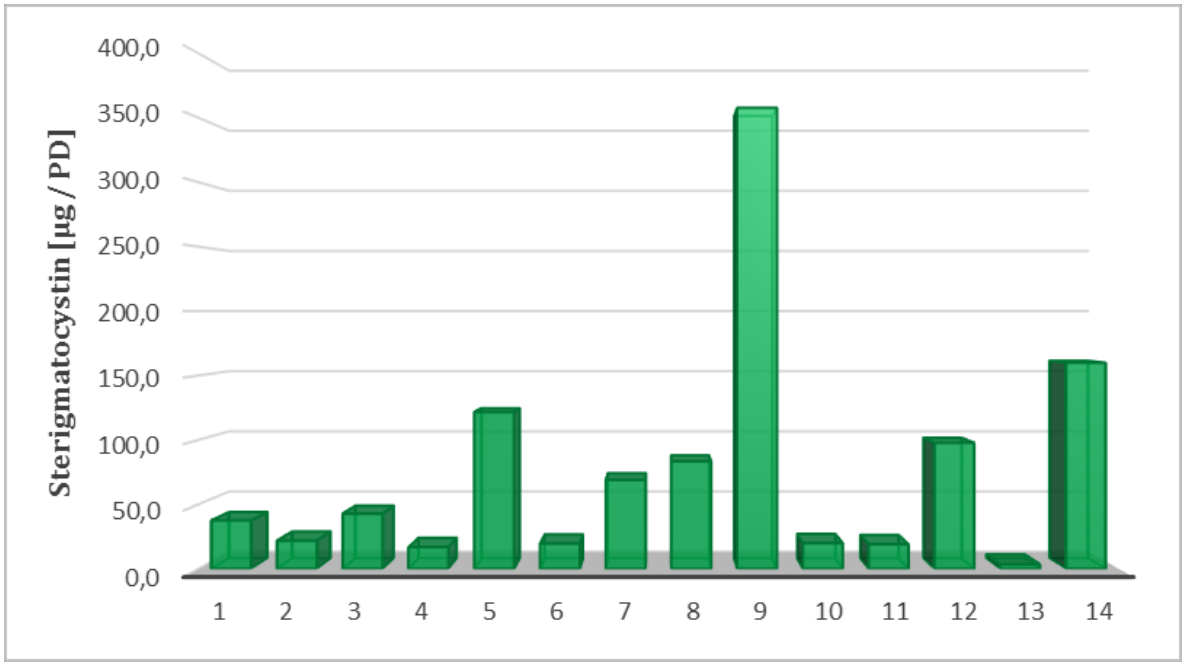

Figure 7: Sterigmatocystin production by 14 *Aspergillus versicolor* strains tested after 10 days of cultivation at 25 °C on PDA

1.2.2.1.2 On fiberglass

The pieces of FG were inoculated in order to determine fungal growth and associated toxin production. Tests for toxin production were carried out using several volumes of NS (0.5 and 1 mL). Indeed, preliminary tests reported that adding too much of NS tended to slow down fungal growth. The results are summarized in Table 11. The TOX+ strain (strain NCPT 54) produced relatively large amounts of STG after culturing on FG at temperature of 25 °C. The TOX- strain (strain E26a) produced only weak amounts of STG on FG. Tested volumes of NS only mildly

affected the amount of produced toxin. However, a volume of 0.5 mL appears to be slightly more favorable and was therefore used in experiments for measurement of VOC emissions.

Table 11: Sterigmatocystin production by *A. versicolor* strains NCPT54 (TOX+) and E26a (TOX-) after 10 days of incubation on FG [µg/FG]

Strains	**Nutritional solution [mL]**	
	0.5	**1**
***A. versicolor* NCPT 54 (Av9) TOX +**	15.4	7.3
***A. versicolor* E26a (Av13) TOX -**	0.4	1.8

1.2.2.2 Mycophenolic acid production by Penicillium brevicompactum strains

1.2.2.2.1 On agar medium

Initially we only had a limited number of tested strains. In fact, only 3 strains were available, Pb16, Pb17, Pb18 (2 from CSTB and 1 from INRA collections). In order to identify the pair TOX+/TOX- , MPA production of these strains was tested after growing on PDA at 25 °C for 10 days.

Figure 8 shows levels of MPA produced by each strain. It is interesting that all strains revealed to be toxinogenic. Strain Pb16 (04891) appeared slightly more toxinogenic than others, and it was retained as strain TOX+.

Then, to find a non-toxinogenic strain, 4 more strains (from NRRL) were tested, as well as one strain isolated from feed (strain M362). All these strains have proven to be producers of MPA (Figure 8).

To obtain a TOX- strain, we contacted Professor J. B. Nielsen in Denmark. This team tested over 500 *Penicillium brevicompactum* strains and all had proven toxinogenic potential (personnal communication). Therefore, to have a TOX- strain, he provided us a mutant obtained in his laboratory. As illustrated in the diagram below (Figure 8), this mutant (*mpaCΔ* or strain 19) did not produce MPA after culturing on PDA for 10 days and has been used as TOX- strain.

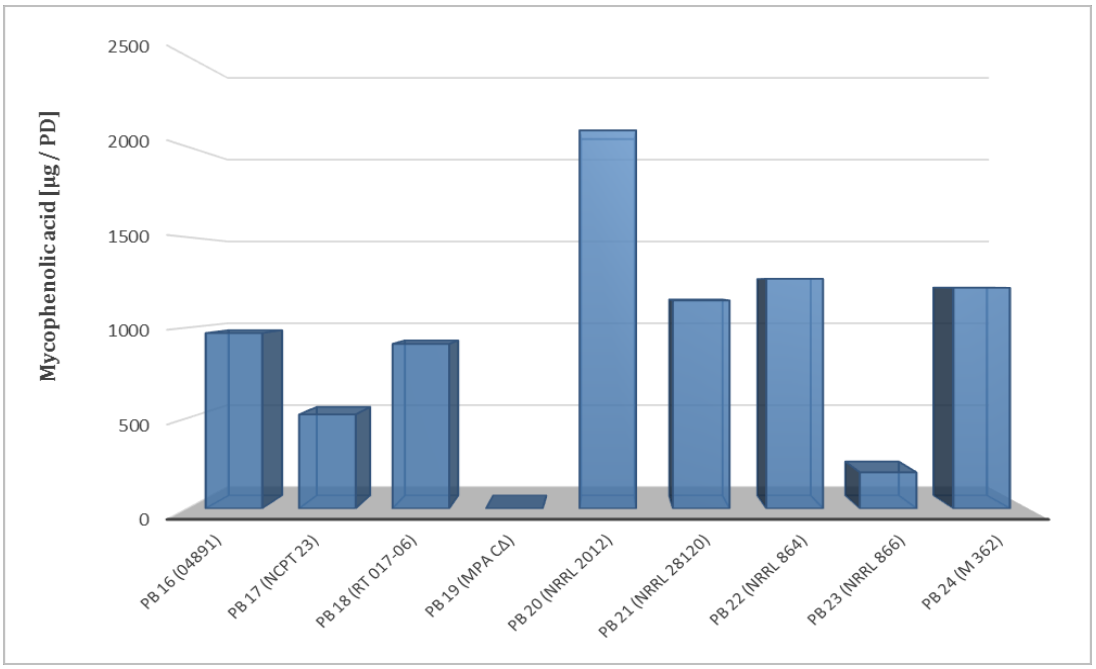

Figure 8: Production of MPA by 9 strains of *Penicillium brevicompactum* after 10 days of culture on PDA at 25 °C

After culturing on PDA, six latest tested strains (4 from NRRL, M362 and mutant) had green pigmentation and important sporulation, except NRRL 866, whose toxinogenic potential was low (Figure 9).

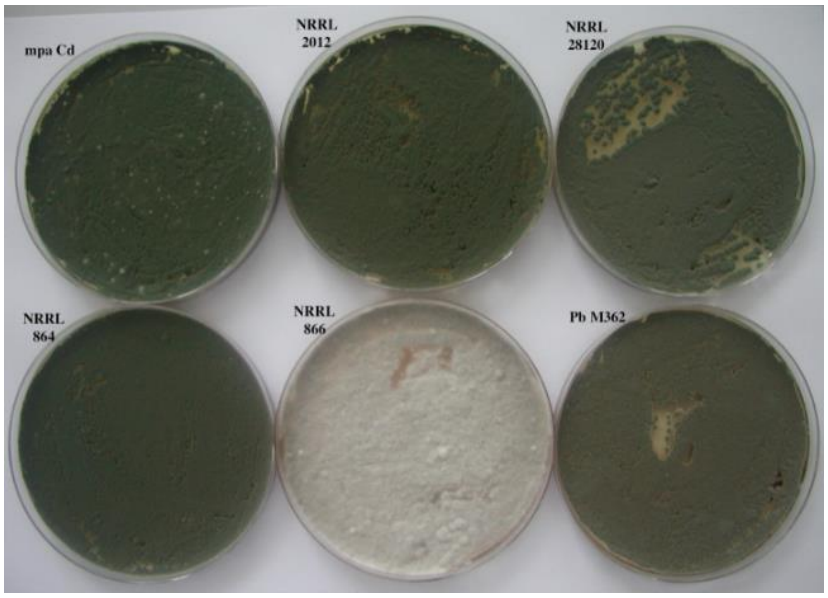

Figure 9: Appearance of 6 strains of *Penicillium brevicompactum* after 10 days of culture on PDA at 25 °C (Photo: S. Bailly)

Parental strain Pb25 (toxin-producing strain) was obtained later and its production of mycophenolic acid on PDA medium, in the same conditions as described above, was measured at 3.5 mg/PD.

1.2.2.2.2 On Fiberglass

Toxin production of Pb 16 (04891) strain (TOX+) and Pb 19 (*mpaCΔ*) strain (TOX-) was evaluated on FG. The TOX+ strain proved to be capable of producing MPA in high quantities after cultivation on this substrate (37.3 µg/FG with 0.5 mL of NS, and 43 µg/FG with 1 mL of NS). The mutant, meanwhile, did not produce detectable amount of toxin.

It may be noted that the volume of nutritional solution had no real impact on toxinogenesis despite very different aspect of fungi (Figure 10). The growth appeared to be greater with 1 mL of NS but this may be more related to the fact that there is a wider dissemination of spores when seeding. Examination under stereo-microscope showed no significant difference in development or sporulation in both cases.

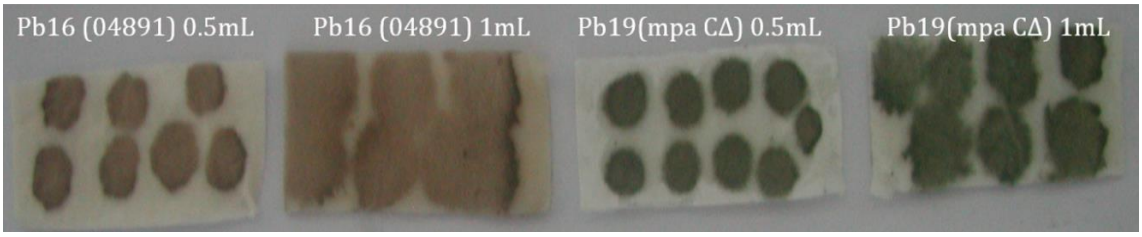

Figure 10: Appearance of TOX+ and TOX- strains of *Penicillium brevicompactum* after growing on fiberglass supplemented with 0.5 mL and 1 mL NS

1.2.2.3 Macrocyclic trichothecenes production by Stachybotrys chartarum strains

1.2.2.3.1 On agar medium

Previous work of our team already identified *Stachybotrys chartarum* ST82 as a highly toxinogenic strain whereas ST81 was characterized as a weakly toxinogenic strain.

The toxinogenic potential of strains has been checked after growing on PDA at 25 °C. As expected, the ST82 strain appeared highly toxinogenic (7500 ppb in the supernatant, which represents 300µg per PD). Strain ST81 at 25 °C produced very small quantity of MCT (2.1 ppb) and was therefore further considered as mildly toxinogenic (Table 12 below).

1.2.2.3.2 On Fiberglass

After culturing on FG, TOX+ strain ST82, produced relatively large amounts of MCT at 25 °C. Meanwhile, TOX- strain (ST81) showed to be weak toxin producer (table below). Figure 11 shows the appearance of the ST81 and ST82 strains after growing on FG at 25 °C.

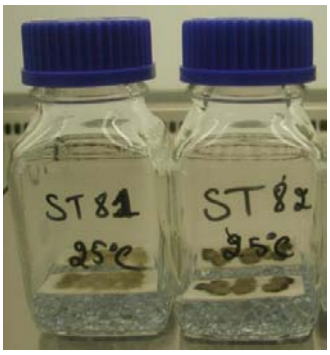

Figure 11: Aspect of the *S. chartarum* ST81 and ST82 strains after cultivation on fiberglass at 25 °C

Table 12: Toxin production by *S. chartarum* strains quantified by ELISA [ng/mL] (equivalent of Roridin A)

Support	**Strain**			
	ST81		**ST82**	
PDA medium	2.1		7500	
	Volume of nutritional suspension [25°C]			
	0.5	**1**	**0.5**	**1**
Fiberglass	9.8	0.2	1520	670

This time, volume of the NS seemed to have more significant influence on toxin synthesis. Indeed, the level of MCT produced by ST82 was more than two times higher when the FG is dampened with only 0.5 mL of NS. This condition was therefore chosen to be used for the analysis of VOCs.

1.2.3 Conclusion

Our project has the overall objective to assess the risk of inhalation of mycotoxins during growth of toxinogenic mould in indoor habitats and evaluate the possible use of specific VOCs emitted during toxinogenesis as environmental markers and tools for risk assessment.

To conduct this study, we have chosen to work on three very different fungal species *Aspergillus versicolor*, *Penicillium brevicompactum* and *Stachybotrys chartarum*. These three species were chosen because of their general predominance in homes, their very different structure suggesting the variable capacity of aerosolization and finally their potential to produce different toxins.

For each fungal species, we:

- Identified a pair made of one toxinogenic strain (see Annex 1) and one non- or weakly toxinogenic strain (to identify VOCs specifically emitted by the first during its development)

- Validated that specific experimental conditions (previously established for the measurement of VOCs) allow correct development of both strains and an active toxin production by toxinogenic strain.

Mycotoxin production is strain's specificity. All strains may not have this ability. In many fungal species the proportion of toxinogenic strains is often about 50 to 80% (El Mahgubi *et al.*, 2013). It appears that for two of the studied species, the proportion of toxinogenic strains was much more important. Thus, for *Aspergillus versicolor*, only one out of 14 tested strains was found as poorly toxinogenic. Similar results were observed for *Penicillium brevicompactum*. Even if the number of tested strains in this study is limited, they were all found to be toxinogenic, which is in the agreement with the work conducted by Prof. J. B. Nielsen on a much larger number of strains (> 500) (J. B. Nielsen, personal communication). In the latter case, we had to use a mutant strain as non-toxinogenic strain to be able to compare the emission of VOCs and define those that are specifically associated with toxin production. Such observation of the high proportion of toxinogenic strains in that two species is of interest when correlated to their high frequency in indoor environments.

For *P. brevicompactum*, it is possible that the mutation that allowed shutting down mycophenolic acid production (by deletion of the polyketide synthase *mpaC* gene) (Regueira *et al.*, 2011) may also have other metabolic consequences. Thus, it is possible that for the mutant, profile of VOCs emission will not be the same as one of a parental strain. That is why experiments were done in parallel using Pb 16 strain.

In order to be able to follow toxin production after development of chosen three species on different building materials and their possible subsequent aerosolization, the next step of our work was to develop an accurate and sensitive analytical method.

1.3 Development of an analytical method for mycotoxins' analysis by UPLC-MS/MS

The development of analytical method aimed to:

- Have a single method, sensitive and specific enough for the determination of all toxins of interest on different building materials but which can also be used for monitoring toxins in aerosols

- Characterize more precisely the toxin profile of *Stachybotrys* strains and determine the nature and the relative proportion of aerosolized toxins

- Compensate the end of commercialization of ELISA kits initially used for the determination of macrocyclic trichothecenes.

This was made possible thanks to Professor J. J. Pestka (Michigan State University, USA), who gave us standards of 4 major macrocyclic trichothecenes (roridin L2, verrucarin J, satratoxins G and H), not commercially available.

1.3.1 Materials and methods

1.3.1.1 Mycotoxin standards

Standards of sterigmatocystin (STG), mycophenolic acid (MPA), mycophenolic acid - d3 (MPA-d3) and verrucarin A (VerA) were purchased from Sigma (Saint-Quentin Fallavier, France) whereas o-methyl sterigmatocystin (o-mSTG) was purchased from Santacruz Biotech. Macrocyclic trichothecenes were generous gift from Professor J. J. Pestka and were prepared as follow: satratoxin G (SG) and roridin L2 (RL2) were purified as previously described by Islam et al. (2009). Satratoxin H (SH) was prepared from cultures of *Stachybotrys chartarum* (ATCC 62765) as described by Jarvis et al. (1995) and verrucarin J (VerJ) was isolated and characterized from *Myrothecium verrucaria* (ATCC 24571), another MCT producing species as previously reported by Jarvis et al. (1982). All standards were dissolved in methanol (MeOH) to obtain stock solutions that were stored at -20 °C.

1.3.1.2 Solvents and reagents

All reagents and solvents were purchased from ICS (Lapeyrouse-Fossat, France) and were analytical grade. Acetonitrile (AcN) used for mobile phase was LC/MS grade and purchased from

Thermo Fischer Scientific (Illkirch, France) and water was obtained from an ultrapure water (18.2 MU) system (Elga Labwater Veolia, Anthony, France).

1.3.1.3 Building materials

Fiberglass (FG) (GF/B glass fiber filter, Whatman) was used as a reference material. Four other commercially available materials, frequently encountered in indoor environments, were tested. These materials were purchased in a specialized store and were as follows: painted fiberglass wallpaper (FWP) (Toile de verre, maille chevron, BATCH N-S2009061492 þ Paint MS SAT LUXENS, Leroy Merlin, France), wallpaper (WP) (Papier Peint BLAN BLA 0 INSP, Leroy Merlin, France), vinyl wallpaper (VWP) (P.VINYL/INT BLANBLA 0 INSP, Leroy Merlin, France), and fir (Leroy Merlin, France). They were cut into 2 x 5 cm pieces and then sterilized (121 °C, 20 min) before use as described by Górny et al. (2001) and Peitzsch et al. (2012a).

1.3.1.4 Instrumental conditions

Toxin quantification was performed with an Acquity ultra performance liquid chromatography (UPLC) system coupled to a Xevo triple quadrupole mass spectrometer (Waters, Milford, MA, USA) and an electrospray ionization source (ESI) (Figure 12).

Figure 12: UPLC-MS/MS System used in this study

1.3.1.5 Optimization of analytical conditions

Toxins and the internal standards were separated on an Acquity BEH C18 column (2.1 x 100 mm; 1.7 µm; Waters) at 40 °C. Samples were ionized in positive electrospray ionization mode (ESI^+).

The source parameters were optimized from solutions of mycotoxin standards at 10 µg/mL injected in mode combined with the chromatographic system with a mixture AcN/H_2O (50/50) in positive mode and presented in Table 13.

Table 13: Parameters of the electrospray source in positive mode ESI^+ used for ionization of mycotoxins of interest

Parameter	Analytical condition
Capillary voltage	3.5 kV
Source temperature	150 °C
Desolvation temperature	650 °C
Nitrogen flow rate	800 L / h
Argon flow rate	0.12 mL/min

Mycotoxins (5 µL of samples) were eluted with an AcN/H_2O gradient detailed in the table 14 at a flow rate of 0.35 mL/min.

Table 14: AcN / H_2O gradient used in developed method

Time [min]	Flow rate [mL/min]	% H_2O	% AcN
0	0.35	90	10
0.5	0.35	90	10
4.00	0.35	10	90
4.10	0.35	90	10
5.00	0.35	90	10

Chemical structures of tested mycotoxins as well as their paired internal standards are presented in figures 13 and 14.

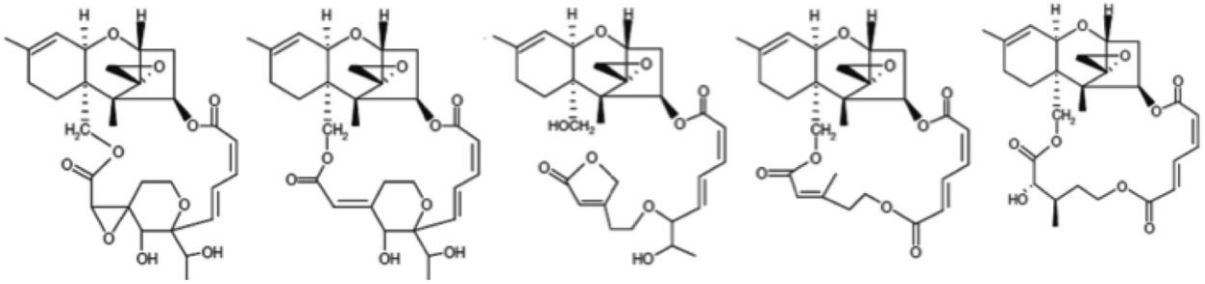

Figure 13: Structures of analysed macrocyclic trichothecenes

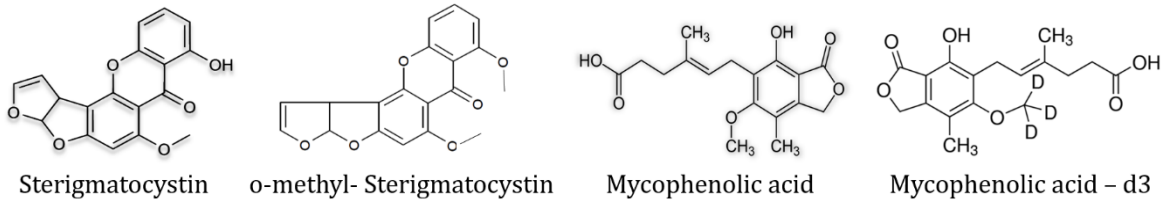

Figure 14: Structures of sterigmatocystin and mycophenolic acid, and their paired internal standards

Quantification was carried out by Multiple Reaction Monitoring (MRM) in positive electrospray ionization (ESI^+) and all the toxins gave the protonated parent $[M+H]^+$ or the sodium/potassium adduct $[M+Na]^+$ or $[M+K]^+$. The MRM transition with the highest signal to noise ratio and the highest intensity was selected for quantification and the second MRM transition was used for confirmation. MRM transitions, cone voltage and collision energies used for the different toxins are listed in Table 15. Chromatographic data were monitored by Masslynx 4.1 software (Waters, Milford, MA, USA).

Table 15 : MRM transitions, cone voltages and collision energies used for toxin detection

Mycotoxin	Molecular weight [g/mol]	Parent ions	MRM fragments	Cone voltage [V]	Collision energy [eV]
Mycophenolic acid	320	321 321	159 207	16 16	36 22
Sterigmatocystin	324	325 325	115 310	40 40	64 24
Roridin L2	530	553 553	249 305	42 42	16 26
Satratoxin G	544	545 545	81 231	20 20	34 16
Satratoxin H	528	529 551	249 303	24 48	16 28
Verrucarin J	484	523 523	151 293	46 46	32 34

1.3.1.6 Extraction procedure

Different solvents were tested for MCT: ethanol (EtOH), MeOH, PBS, chloroform ($CHCl_3$) and $CHCl_3$:MeOH (2:1), in order to determine the best extraction procedure.

Toxins (added in known concentration) were extracted from building materials by gentle mechanical agitation on a horizontal shaker (Reciprocating Shaker, IKA HS501 Digital, Grosseron, France) with 20 mL of chloroform: methanol ($CHCl_3$:MeOH) (2:1) or pure chloroform ($CHCl_3$). After 4 h, extracts were centrifuged for 5 min at 3500 rpm and filtered through a phase separator filter (Whatman 1 PS). Four milliliters of the filtered extract were evaporated to dryness and then suspended in 1 mL of MeOH or AcN/H_2O and analysed by UPLC-MS/MS.

1.3.1.7 Validation procedures

The method was validated for each toxin of interest according to the 2002/657/CE commission decision in terms of linearity, matrix effects, recovery, precision and accuracy, limit of quantitation and limit of detection (European Commission, 2002).

1.3.1.7.1 Internal standards

In order to overcome potential matrix effects and any possible losses during extraction procedure, mycotoxins were quantified using an appropriate internal standard (IS) added before sample extraction. This standard should have a structure similar to that of mycotoxin, in order to have same behavior during extraction and ionization, without potentially being generated by the fungal strains.

1.3.1.7.2 Extraction recoveries and matrix effects

Pure extraction recovery quantitatively described the yield of the extraction process. It was evaluated by comparing the peak areas of internal standard and paired toxin(s) standards spiked without matrix, obtained after the extraction procedure and the peak areas obtained after direct injection of compounds into the UPLC-MS/MS system.

Matrix effects were quantitatively estimated with the matrix factor (MF) defined as the percentage ratio of the analyte peak area extracted from the matrix to the analyte peak area extracted without matrix (Semeiks *et al.*, 2014). The matrix effect was investigated for each building material and each toxin of interest. Exception was done for MCT, where MF were evaluated with VerA (IS) because lacking of pure standards.

Total extraction recovery cover both extraction and matrix effect. It was evaluated by comparing the analyte peak area extracted from matrix to the analyte peak area directly injected in the system.

1.3.1.7.3 Linearity

Calibration curves were prepared with the IS (VerA, o-mSTG, MPA-d3) in MeOH using a minimum of five data points.

Calibration curve ranged from 0.01 µg/mL to 5 µg/mL for RL2 and VerJ and from 0.1 µg/mL to 5 µg/mL for SG and SH with a fixed IS concentration of 0.8 µg/mL, corresponding to the VerA concentration obtained after the building materials extraction procedure.

Calibration curve ranged from 0.01 µg/mL to 10 µg/mL for STG and 0.01 µg/mL to 10 µg/mL for MPA with fixed IS concentration of 0.35 µg/mL for o-mSTG and 0.2 µg/mL for MPA-d3, corresponding to the concentrations obtained after extraction procedure.

Each calibration standard was injected three times. Linear ($Y=aX+b$) and quadratic ($Y=aX^2+bX+c$) models were tested with weightings of 1, $1/X$ and $1/X^2$ (X=nominal concentration). Three approaches were used to assess the linearity of the calibration curve: 1) calculation of the relative standard deviation between the nominal concentration and the concentration obtained with the model (RSD %), which should be lower than ± 20 %, 2) visual inspection of the residual distribution, and 3) application of a lack of fit test to check the goodness of fit of the model (Jarvis *et al.*, 1986; Pasanen *et al.*, 1997).

1.3.1.7.4 Limits of detection and quantification

The lowest limit of detection (LOD) was defined as the lowest concentration that could be reliably differentiated from background noise (signal to noise > 3). LOD was determined from 3 injections of mycotoxins' standards at the lowest concentration that could be detected with a signal to noise ≥ 3. The limit of quantification (LOQ) was determined and validated for the lowest concentration of the calibration curve chosen for its relevance to mycotoxin investigation in building materials.

1.3.1.7.5 Precision and Accuracy

Intra-day (repeatability) and inter-day (reproducibility) precisions and accuracy of the method were assessed using quality control (QC) samples of RL2 at a single concentration level of 0.022 µg/mL for macrocyclic trichothecenes, 1 µg/mL for MPA and 2 µg/mL for STG. Toxins were spiked on FG and extracted with $CHCl_3$:MeOH. For RL2 and MPA, three replicates of each QC were injected at 3-day intervals. As all samples were assayed on the same day, only intra-day precision was evaluated for STG. Accuracy was calculated as the percentage of the ratio between the mean calculated concentration and the theoretical nominal value. Precisions were expressed by the coefficient of variation (CV% = (SD/mean)*100). Intra/inter-day standard deviation was obtained from an analysis of variance (ANOVA) with a single factor.

1.3.2 Results and discussion

LC-MS/MS conditions were first optimized in order to determine the best solvent of extraction and internal standard for each compound of interest. Matrix effects were evaluated on all five types of material. Then the method was validated in terms of selectivity, linearity, sensitivity, precision and accuracy.

1.3.2.1 Validation of the analytical procedure

1.3.2.1.1 UPLC – MS/MS optimization

The UPLC conditions were optimized in order to elute each toxin of interest in the shortest run time with reliable retention times and MS ionization closed to the IS ionization. Used chromatographic conditions allowed the separation of all 6 toxins of interest within 5 min with a relative standard deviation on the retention times of less than 1% for each analyte (Figure 15).

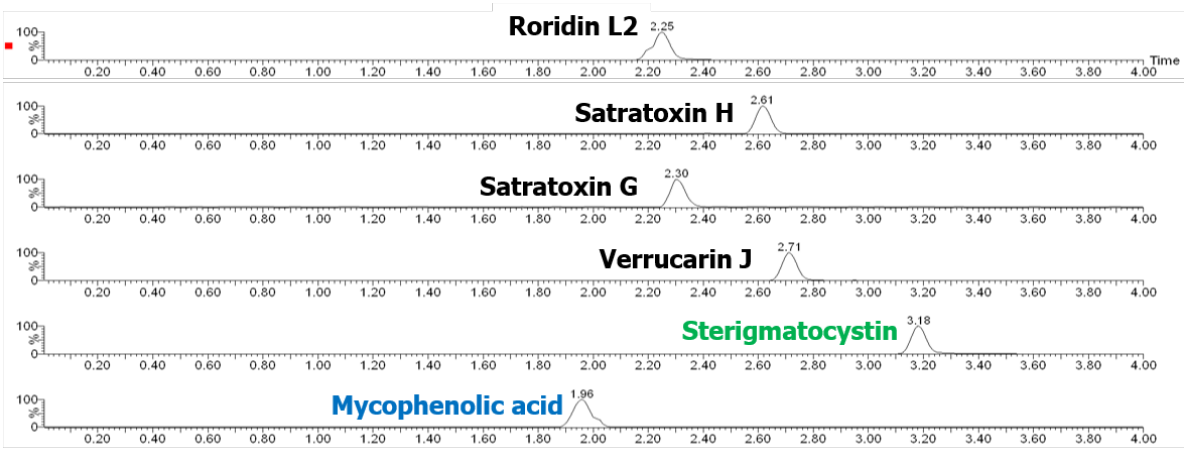

Figure 15: MRM chromatograms of all six mycotoxins of interest

1.3.2.1.2 Choice and validation of the solvent of extraction

To optimize extraction procedure and choose the best extraction solvent, agar cultures of toxigenic *Stachybotrys chartarum* ST82 strain were extracted by several solvent mixtures. The results are summarized in Table 16.

Table 16: UPLC-MS/MS analysis of toxins produced by *Stachybotrys chartarum* ST82 using different solvents for extraction. Results are expressed as means of MS peak areas with CV% (n=3)

Extraction solvent	Mean MS area (CV %)			
	Roridin L2	Verrucarin J	Satratoxin G	Satratoxin H
MeOH	200099 (13%)	470 (32%)	611 (23%)	2374 (19%)
AcN	152573 (48%)	203 (46%)	825 (53%)	2441 (32%)
PBS	455262 (22%)	823 (48%)	169 (54%)	993 (17%)
$CHCl_3$	165777 (1%)	105 (24%)	1613 (24%)	4627 (17%)
$CHCl_3$:MeOH (2:1)	130062 (7%)	134 (4%)	1418 (12%)	4527 (5%)

From these results, it was concluded that $CHCl_3$ and $CHCl_3$:MeOH (2:1) allowed the best extractions of SG and SH and satisfactory extractions of RL2 and VerJ. Moreover, $CHCl_3$:MeOH extraction procedure displayed better repeatability than $CHCl_3$, as illustrated by the CV% which was lower than 12%. In order to develop unique analytical method for all toxins of interest, the $CHCl_3$:MeOH (2:1) mixture was retained as solvent of extraction and applied to MPA and STG. The extraction recoveries were 87 ± 5 % and 104 ± 11 % for MPA and STG, respectively.

1.3.2.1.3 Choice and validation of the internal standard

In the EFSA report (2013), it is highlighted that development of analytical method for mycotoxin identification and quantification should include systematic use of IS (Böhm *et al.*, 2013). This should be especially case when high matrix effect can be expected, like in cereals or other foodstuff

(Spanjer *et al.*, 2008; Van Pamel *et al.*, 2011) as well as in analysis of building materials as studied here.

Internal standard was chosen according to its availability, chromatographic features as well as its similarity of behavior during extraction from contaminated materials. Theoretically, the use of a stable, isotopically labeled compound as internal standard, for every single mycotoxin included in the method is recommended for MS quantification, but not possible in practice. Lack of commercial availability of those compounds force the search for one or more structurally related compounds instead. In that case, such compound must be validated as IS, in order to demonstrate that both, its extraction behavior and its ionization, are the same as the analyte's (toxin of interest). Moreover, IS has to be discriminated from paired analyte of interest.

Use of MPA-d3 as IS for MPA in analytical methods used in medical purposes is already described and contributed to improve the analytical performances of the method (Klepacki *et al.*, 2012; Nguyen Thi *et al.*, 2013). Having those information in mind, MPA-d3 is assumed to be the best internal standard for MPA and no more investigations were carried out to check its relevance as IS of MPA.

For STG, we tested O-mSTG as IS: this molecule, not produced by *A. versicolor*, differs from STG just in one methyl group if comparing chemical structures.

For MCT toxins, situation was more complex. Indeed, those compounds are not commercially available, so the use of marked molecules was not an option. Therefore, we searched for molecule with similar structure and Verrucarin A (VerA) was tested as a possible IS. Indeed, this MCT is the only one that is commercially available as pure standard and displays a similar structure to other toxins from this family (presence of the typical multi-cyclic sesquiterpene structure) (Figure 13). Moreover, this compound is not produced by *Stachybotrys chartarum* (Andersen *et al.*, 2003; Jarvis *et al.*, 1982).

To ensure that VerA and O-mSTG could be suitable internal standards for MCT and STG, respectively, on the one side, their MRM transitions were optimized and they were eluted under the developed UPLC-MS/MS conditions. On the other side, 100 µL of RL2 (1.1 µg/mL), VerA (20 µg/mL), STG (10 µg/mL) and O-mSTG (35 µg/mL) diluted in MeOH were extracted with $CHCl_3$:MeOH (2:1) mixture to check their extraction behavior. The pure extraction recovery was calculated for each pair of molecules and reported in Table 17.

Table 17 : Extraction recovery of the pair Internal Standard/Analyte of interest

Pair Internal Standard/Analyte of interest	Extraction Recovery (± SD)	
	Internal standard	Analyte
VerA/RL2	107% (±20%)	119% (±20%)
O-mSTG/ STG	118% (±9%)	104% (±11%)

For both tested internal standards, pure extraction recoveries were consistent and closed to their respective analytes. VerA and O-mSTG seemed suitable to be use as internal standards. Nevertheless, matrix effects evaluation should be investigated to confirm these results.

1.3.2.1.4 Matrix effects

There may be interactions between the materials and the toxins of interest, altering the ionization and thus the detection of the compounds. The validation of the analytical method used to quantify toxins on materials therefore requires evaluating these interactions, which have to be taken into account when quantifying. For this, each material was contaminated with a known concentration of toxin and then extracted and analysed under the conditions described above (1.3.1.6). Matrix effects were tested for fiberglass (FG), wallpaper (WP), painted fiberglass wallpaper (FWP), vinyl wallpaper (VWP) and fir. These effects were evaluated with the matrix factor (MF %). Table 18 shows the results obtained for MPA, STG, O-mSTG, RL2 and VerA on various materials used in our study.

Table 18 : Matrix factors observed for STG, O-mSTG, MPA, VerA and RL2 (n=3)

Material	Matrix factor [%]				
	STG	O-mSTG	MPA	VerA	RL2
Fiberglass	112	104	109	97	116
Wallpaper	116	100	109	99	--
Painted fiberglass wallpaper	94	102	34	21	--
Vinyl wallpaper	108	109	16	35	--
Fir	111	94	140	52	--

The pair STG and O-mSTG was not affected by the matrices; MF were closed to 100 % whatever the building material. Conversely, matrix effects for MCT (evaluated with VerA) and MPA were broadly comparable to each other (except for fir where they were completely opposed). MF were closed to 100 % for FG and WP whereas they were lower than 50 % for FWP and VWP. Low MF in FWP and VWP could be explained either by a possible loss of analyte during the extraction from these materials or by an ion suppression observed in mass spectrometry due to the complex mixture of paints and vinyl polymers respectively presents in these materials. To verify these

interactions, the same extracted samples of MPA were assayed by TLC using a method routinely used in the laboratory. MF were calculated for TLC and compared with MF obtained with UPLC-MS/MS (Table 19).

Table 19: Comparison of MF between UPLC-MS/MS and TLC method for MPA

Material	Matrix factor %	
	UPLC-MS/MS	TLC
Fiberglass	109	108
Wallpaper	109	82
Painted fiberglass wallpaper	34	91
Vinyl wallpaper	16	90
Fir	140	70

When TLC method was used, MF of MPA were close to 100 % whatever the material, and particularly in cases of FWP and VWP. Therefore, the interactions observed in mass spectrometry certainly occur during ionization of compounds. In those cases, the use of internal standard is unavoidable to correct these matrix effects.

1.3.2.1.5 Validation of the method

Once the internal standards and the extraction conditions were optimized, the method was then validated in terms of sensitivity, linearity, recovery, precision and accuracy.

1.3.2.1.5.1 Linearity

All six mycotoxins were validated with a linear model weighted by $1/X^2$ (with X = concentration) with RSD lower than 20 % for all calibration standards (Table 20). The weighted residuals obtained with this calibration curve were randomly distributed around the mean and the linearity of the curve was confirmed by a lack of fit test.

Table 20: Results of mycotoxins calibration standards used to assess linearity. Each calibration curve was validated with a linear model weighted by $1/X^2$ (X= concentration)

(n=3)	RL2		SG		SH		VerJ		MPA		STG	
Concentration [µg/mL]	Measured mean [µg/mL]	RSD %	Measured mean [µg/mL]	RSD %	Measured mean [µg/mL]	RSD %	Measured mean [µg/mL]	RSD %	Measured mean [µg/mL]	RSD %	Measured mean [µg/mL]	RSD %
0.01	0.010	-2					0.010	-1	0.010	-2	0.010	-3
0.1	0.115	15	0.095	-5	0.103	3	0.107	7	0.054	-7	0.053	6
0.2	0.262	5	0.284	13	0.231	-7	0.239	-4	0.105	5	0.117	17
1	1.001	0	1.027	3	0.957	-4	1.018	2	0.495	-1	0.590	18
2	1.929	-4	1.956	-2	2.076	4	1.998	0	1.088	9	0.965	-4
5	4.250	-15	4.571	-9	5.239	5	4.824	-4	4.833	-3	4.322	-14
10									8.523	-15	7.921	-21

1.3.2.1.5.2 Sensitivity, precision and accuracy

The lowest concentrations that could be detected and differentiated from the baseline with S/N>3 were 0.2 ng/mL for RL2; 5 ng/mL for VerJ; 10 ng/mL for SH and SG; STG; MPA. These values were considered as the LODs of each mycotoxin.

The LOQs were set as the lowest standard point of the calibration curves at 10 ng/mL for RL2, VerJ, STG and MPA; at 100 ng/mL for SG and SH (Table 21).

Table 21: Validation results and chosen internal standards for each mycotoxin of interest

Mycotoxin	Internal Standard	LOD [ng/mL]	LOQ [ng/mL]	Precision		Accuracy %
				Intra-day (CV%)	Inter-day (CV%)	
MPA	MPA-d3	1	10	14%	14%	122%
STG	O-mSTG	0.1	10	20%	--	96%
RL2	VerA	0.2	10	12%	14%	105%
SG		5	100	--	--	--
SH		10	100	--	--	--
VerJ		5	10	--	--	--

1.3.2.1.5.3 Global extraction recovery

Global extraction recovery summing the matrix effect with the extraction process are presented in table 22 for MPA, the pair STG/O-mSTG and the pair VerA/RL2 (on FG).

Table 22: Global extraction recoveries observed for STG, O-mSTG, MPA, VerA and RL2 (n=3)

Material	Global extraction recovery [%]				
	STG	O-mSTG	MPA	VerA	RL2
Fiberglass	116	124	94	82	119
Wallpaper	120	119	94	84	--
Painted fiberglass wallpaper	97	121	29	18	--
Vinyl wallpaper	112	128	14	30	--
Fir	115	112	121	44	--

Global extraction recoveries were consistent with matrix effects evaluation and results were acceptable for quantifying mycotoxins in building materials.

1.3.3 Conclusion

A method for quantification of all six mycotoxins of interest by UPLC-MS/MS was developed and validated. This method, sensitive and highly specific, was then utilized to study toxinogenesis of our 3 species (strains) during their development on different materials as well as to quantify the toxic load aerosolized in various stress conditions.

The conditions of extraction and recovery of the various compounds were identical, as well as analytical conditions of this method, making possible the multi-detection of all mycotoxins.

1.4 Characterization of toxinogenesis of three fungal species after development on different indoor materials of interest

The next step of our work was to analyse ability of toxinogenic strains to effectively produce their toxins in case of development on different building materials.

The analysis of toxinogenesis of *Stachybotrys chartarum* after its development on different building material was published in Building & Environment and the article is presented below.

Results obtained for *A. versicolor* and STG and *P. brevicompactum* and MPA are subsequently presented as supplementary data.

1.4.1 Production of four macrocyclic trichothecenes by Stachybotrys chartarum during its development on different building materials as measured by UPLC-MS/MS. 2016. Building and Environment 106, 265–273

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Production of four macrocyclic trichothecenes by *Stachybotrys chartarum* during its development on different building materials as measured by UPLC-MS/MS

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ABSTRACT

Stachybotrys chartarum is a fungal contaminant of damp indoor environments that can produce several toxins belonging to the family of macrocyclic trichothecenes. These toxins are suspected to be involved in different pathologies among residents of moldy indoor environments. However there are only few data on the capacity of *S. chartarum* to produce its toxins (type and proportion) while growing on different building materials. This study aimed to quantify by UPLC-MS/MS the production of four major macrocyclic trichothecenes (Satratoxins G and H, Roridin L2 and Verrucarin J) during colonization of different building materials (fiberglass, painted fiberglass wallpaper, wallpaper, vinyl wallpaper, fir) by *S. chartarum*. It showed that the four molecules were produced upon development of a toxin-producing strain of *S. chartarum* on the material. The nature of building material strongly influenced the levels of macrocyclic trichothecenes produced. Wallpaper appeared to be the most favorable to both fungal development and production of the four toxins. By contrast, no toxin production was observed on vinyl wallpaper, in agreement with lack of fungal growth. Satratoxin H was always the main toxin produced, on all tested substrates, and its concentration reached 14.2, 3, 1.8 and 1.1 mg/m^2 on wallpaper, fir, fiberglass and fiberglass wallpaper, respectively. This knowledge is important to define monitoring strategies and assess risk related to those contaminants.

1. Introduction

In developed countries, people spend almost 80% of their time inside buildings (schools, offices, sports, homes …) and it is estimated that 20–40% of buildings in Northern Europe and North America, display macroscopically visible fungal development [1,2]. In France, more than 600,000 homes have moldy surfaces of more than 1 m^2 [3]. Fungal growth in damp or water-damaged buildings is an important public health issue worldwide since it can lead to several adverse effects on the occupants and especially allergic and respiratory troubles [4–6]. Futhermore, during their development, some fungal species might also produce secondary metabolites toxic for humans, called mycotoxins. These toxins could play a role in the development of such disorders [7,8].

Indeed, among fungal species frequently isolated from damp buildings, some are well known to be potent mycotoxins producers [9]. Thus, *Stachybotrys chartarum* is a frequent contaminant of indoor environments worldwide [9,10] and may be isolated in about 50% of building material samples in USA [11,12] and in 10–30% of samples in Europe depending on the surveys and history of buildings [13,14]. Indeed, *S. chartarum* is mostly found in dwellings following water damage [11,13,15].

S. chartarum became the focus of attention following reports of its association with idiopathic pulmonary hemorrhage in infants in Cleveland, Ohio [16]. More recently, there has been increasing evidence of a possible relationship between the presence of

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0360-1323/

S. chartarum in indoor environments and human illness such as sick building syndrome [17]. These deleterious effects could be related to either the allergic potential of spores [18] or mycotoxin production [8,19].

This fungal species is able to produce several toxic compounds that belong to the family of macrocyclic trichothecenes such as satratoxins G and H, roridin L2, verrucarin J. These mycotoxins are known to be strong inhibitors of protein synthesis and their acute toxicity has been demonstrated following ingestion in horses [20], contact with skin [21] and also after inhalation in rodents [22]. In a recent study, Carey et al. [23] demonstrated that intranasal exposure to satratoxin G induces rhinitis, atrophy of the olfactory epithelium and apoptosis of olfactory sensory neurons in both mice and Rhesus monkeys.

Thus, the frequent isolation of *S. chartarum* in buildings where inhabitants display respiratory problems raises the question of a possible involvement of macrocyclic trichothecenes in these pathologies.

The demonstration of such a relationship and subsequent risk assessment first require the quantification of the different toxins that may be produced during *S. chartarum*'s development on indoor materials. A few analytical methods, based on LC-MS or ELISA, have been developed to evaluate mycotoxins production by this contaminant but most of these were devoted to the characterization of the toxigenic potential of *Stachybotrys* strains on culture medium [24] or were more qualitative than quantitative [15,25,26]. The few quantitative surveys carried out focused only on satratoxins [27]. Moreover, in these studies, no relationship was drawn between fungal development and toxinogenesis or between material and the amount of toxin produced.

Within this context, the aim of this study was to measure the production of four major macrocyclic trichothecenes (satratoxins G and H, roridin L2 and verrucarin J) by *S. chartarum* when colonizing different indoor building materials using an UPLC-MS/MS method.

2. Materials and methods

2.1. Mycotoxin standards

Among used, only Verrucarin A (VerA) is commercially available and was purchased from Sigma (Saint-Quentin Fallavier, France). Satratoxin G (SG) and roridin L2 (RL2) were purified as previously described by Islam et al. [22]. Satratoxin H (SH) was prepared from cultures of *S. chartarum* (ATCC 62765) as described by Jarvis et al. [28] and verrucarin J (VerJ) was isolated and characterized from *Myrothecium verrucaria*, another macrocyclic trichothecene producing species (ATCC 24571), as previously reported by Jarvis [29]. Standards were dissolved in methanol (MeOH) to obtain stock solutions that were stored at −20 °C.

2.2. Solvents and reagents

All reagents including the solvents used for sample preparation were purchased from ICS (Lapeyrouse-Fossat, France) and were analytical grade. Acetonitrile (AcN) used for mobile phase was LC/MS grade and purchased from Thermo Fischer Scientific (Illkirch, France) and water was obtained from an ultrapure water (18.2 MΩ) system (Elga Labwater Veolia, Anthony, France).

2.3. Fungal strains

The study was conducted using a strain of *S. chartarum*, previously isolated from contaminated straw by direct plating on Malt Extract Agar (MEA, BioKar, France), shown to produce SG, SH, RL2 and VerJ on MEA (ST82 strain). It was stored at 4 °C and its viability checked regularly by culture on MEA.

2.4. Materials for toxinogenesis tests

Fiberglass (FG) (GF/B glass fiber filter, Whatman) was used as a reference material. Four other commercially available materials frequently encountered in indoor environments were tested. These materials were purchased in a specialized store and were as follows: painted fiberglass wallpaper (FWP) (Toile de verre, maille chevron, BATCH N°S2009061492 + Paint MS SAT LUXENS, Leroy Merlin, France), wallpaper (WP) (Papier Peint BLAN BLA 0 INSP, Leroy Merlin, France), vinyl wallpaper (VWP) (P.VINYL/INT BLAN BLA 0 INSP, Leroy Merlin, France), and fir (Leroy Merlin, France). They were cut into 2 × 5 cm pieces and then sterilized (121 °C, 20 min) before use as described by Gorny et al. [30] and Peitzsch et al. [31].

2.5. Characterization of macrocyclic trichothecenes production on materials

The ST82 strain of *S. chartarum* was grown on potato dextrose agar (PDA, BioKar, France) for 14 days at 25 °C to obtain highly sporulating cultures. Spore suspension was prepared from these fungal cultures by adding 10 mL of Tween 80 (0.05%) to the Petri dish. The number of spores was quantified by direct counting on a Malassez cell. Spore suspension was then diluted to obtain the required concentration. Contamination was achieved by applying 100 μL of this suspension (10^7 spores/mL) to each material. Contaminated materials were placed in flasks, on a layer of 2 cm of glass beads with sterile water in order to maintain moisture level at a saturation level throughout the test. Samples were then cultured for 10 days at 25 °C in total darkness. After incubation, fungal development was assessed by examination of samples under stereo-microscope (magnification from 12 to 120) (Olympus SZX9) and evaluation of both hyphae development (density and colonized surface) and density of sporulated conidial heads on the whole sample (10 cm^2). In order to determine mycotoxin concentration in the initial inoculum (T0 value), fiberglass material was frozen immediately after spore deposition and without incubation to avoid fungal development. It was further analyzed similarly to other samples.

All analyses were done in triplicate and three independent experiments were carried out. Results are expressed as the mean with CV %.

2.6. Extraction procedure

Macrocyclic trichothecenes were extracted from building materials by gentle mechanical agitation on a horizontal shaker (Reciprocating Shaker, IKA HS501 Digital, Grosseron, France) in 20 mL of chloroform/methanol (2:1). After 4 h, extracts were centrifuged for 5 min at 3500 rpm and filtered through a phase separator filter (Whatman 1 PS). Four milliliters of the filtered extract were evaporated to dryness and then suspended in 1 mL of MeOH. For each of the macrocyclic trichothecene quantification assays on building material, VerA was added (100 μL of a 40 μg/mL solution in MeOH) as an Internal Standard (IS) before starting the extraction procedure. Indeed, this macrocyclic trichothecene is the only one that is commercially available as a pure standard, it displays a similar structure to other toxins of this family (presence of the typical multi-cyclic sesquiterpene structure) and is not produced by *S. chartarum* [24,29].

2.7. Instrumental and analytical conditions

Macrocyclic trichothecenes quantification was performed with an Acquity ultra performance liquid chromatography (UPLC) system coupled to a Xevo triple quadrupole mass spectrometer (Waters, Milford, MA, USA). Macrocyclic trichothecenes and the internal standard (VerA) were separated on an Acquity BEH C18 column (2.1 × 100 mm; 1.7 μm; Waters). Samples were ionized in positive electrospray ionization mode (ESI^+). The capillary voltage and source temperature were set at 3.5 kV and 150 °C, respectively. The desolvation temperature and nitrogen flow rate were set at 650 °C and 800 L/h, respectively. Argon was used as the collision gas at a flow rate of 0.12 mL/min. Mycotoxins (5 μL of samples) were eluted on an Acquity BEH C18 column with an AcN/H_2O gradient (t(0–0.5 min): 10% AcN; t(0.5–4 min): 90% AcN) at a flow rate of 0.35 mL/min. Quantification was carried out by Multiple Reaction Monitoring (MRM) mode in positive electrospray ionization (ESI^+) and all the toxins gave the protonated parent $[M+H]^+$ or the sodium/potassium adduct $[M+Na]^+$ or $[M+K]^+$. The two MRM transitions generated for each macrocyclic trichothecene gave MS/MS fragments corresponding to the cleavage at the ester C4 and C15 (except for RL2) bonds between the 12,13-epoxy-trichothec-9-ene cycle and the macrocycle (or the aliphatic chain for RL2) (Fig. 1). The MRM transition with the highest signal to noise ratio and the highest intensity was selected for quantification and the second MRM transition was used for confirmation.

MRM transitions, cone voltage and collision energies used for the different toxins are listed in Table 1. Chromatographic data were monitored by Masslynx 4.1 software (Waters, Milford, MA, USA).

The chromatographic conditions used allowed the separation of the macrocyclic trichothecenes within 5 min with a relative standard deviation on the retention times of less than 1% for each analyte (Fig. 2).

2.8. Validation procedures

The method was validated for each macrocyclic trichothecene according to the 2002/657/CE commission decision in terms of specificity, linearity, matrix effects, recovery, precision and accuracy, limit of quantitation and limit of detection [32].

2.8.1. Selectivity

The selectivity of the method was assessed by contaminating building materials with an *Aspergillus versicolor* strain, which does not produce macrocyclic trichothecenes. The chromatograms of contaminated samples were compared to those of macrocyclic trichothecenes at the lowest limit of quantitation. Retention times (RT) were checked for each toxin with a tolerable deviation of 1% from the expected RT value. This was done in triplicate.

2.8.2. Linearity

Calibration curves were prepared with the IS (VerA) in MeOH using a minimum of five data points. The calibration curve ranged from 0.01 μg/mL to 5 μg/mL for RL2 and VerJ and from 0.1 μg/mL to 5 μg/mL for SG and SH with a fixed IS concentration of 0.8 μg/mL, corresponding to the IS concentration obtained after the building materials extraction procedure. Each calibration standard was injected three times. Linear model ($Y = aX + b$) was tested with weightings of 1, 1/X and $1/X^2$ (X = nominal concentration) (Supplementary figure). Three approaches were used to assess the linearity of the calibration curve: 1) calculation of the relative error between the nominal concentration and the concentration obtained with the model (RE%), which should be lower than ± 20%, 2) visual inspection of the residual distribution, and 3) application of a lack of fit test to check the goodness of fit of the model [33,34]. All macrocyclic trichothecenes were validated with a linear model weighted by $1/X^2$ (with X = concentration) with RE lower than 20% for all calibration standards (Table 2). The weighted residuals obtained with this calibration curve were randomly distributed around the mean and the linearity of the curve was confirmed by a lack of fit test (Supplementary table).

2.8.3. Limits of detection and quantification

The lowest limit of detection (LOD) was defined as the lowest concentration that could be reliably differentiated from background noise (signal to noise > 3). LOD was determined from 3 injections of macrocyclic trichothecenes standards at the lowest concentration that could be detected with a signal to noise ≥3. The limit of quantification (LOQ) was determined and validated for the lowest concentration of the calibration curve chosen for its relevance to macrocyclic trichothecenes investigation in building materials.

The lowest concentrations of calibration standard that could be detected and differentiated from the baseline with S/N > 3 were 0.2 ng/mL for RL2, 5 ng/mL for VerJ and 10 ng/mL for SH and SG: these values were considered as the LODs of each macrocyclic trichothecenes. The LOQs were set at 10 ng/mL for RL2 and VerJ and at 100 ng/mL for SG and SH which correspond to 0.05 mg/m^2 for RL2 and VerJ, and 0.5 mg/m^2 for SG and SH.

2.8.4. Precision and accuracy

In order to preserve the macrocyclic trichothecenes standards, intra-day (repeatability) and inter-day (reproducibility) precisions and accuracy of the method were assessed using quality control (QC) samples of RL2 at a single concentration level of 0.022 μg/mL. Three replicates of this QC were injected three times at 4-day

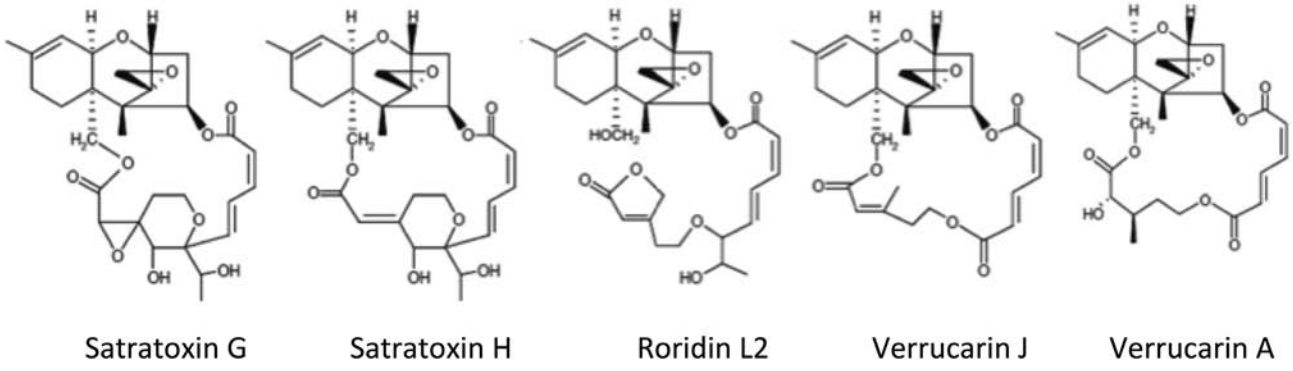

Fig. 1. Structure of analyzed macrocyclic trichothecenes.

Table 1
MRM transitions, cone voltages and collision energies used for macrocyclic trichothecenes detection.

TCT	Molecular weight	Parent ions	MRM fragments	Cone voltage (V)	Collision energy (eV)
RL2	530	553	249	42	16
		553	305	42	26
SG	544	545	81	20	34
		545	231	20	16
SH	528	529	249	24	16
		551	303	48	28
VerJ	484	523	151	46	32
		523	293	46	34
VerA	502	503	249	22	14
		525	295	44	30

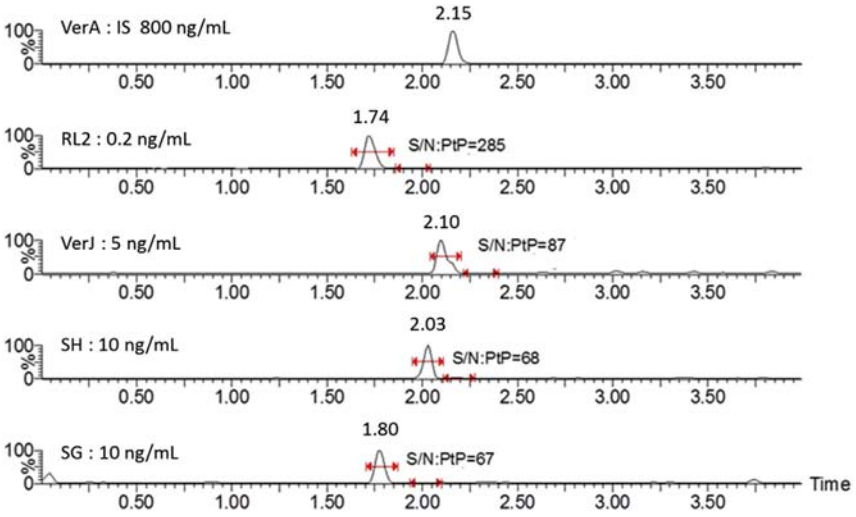

Fig. 2. MRM chromatogram of each macrocyclic trichothecene at LOD with its respective signal to noise ratio and retention times (RT).

Table 2
Validation results of macrocyclic trichothecenes calibration standards used to assess linearity. Each calibration curve was validated with a linear model weighted by $1/X^2$ (X = concentration).

(n = 3)	RL2		SG		SH		VerJ	
Concentration (μg/mL)	Measured mean (μg/mL)	RE %	Measured mean (μg/mL)	RE %	Measured mean (μg/mL)	RE %	Measured mean (μg/mL)	RE %
0.01	0.010	−2					0.010	−1
0.1	0.115	15	0.095	−5	0.103	3	0.107	7
0.2	0.262	5	0.284	13	0.231	−7	0.239	−4
1	1.001	0	1.027	3	0.957	−4	1.018	2
2	1.929	−4	1.956	−2	2.076	4	1.998	0
5	4.250	−15	4.571	−9	5.239	5	4.824	−4

intervals. Accuracy was calculated as the percentage of the ratio between the mean calculated concentration and the theoretical nominal value. Precisions were expressed by the coefficient of variation (CV% = SD/mean*100). Intra/inter-day standard deviation was obtained from an analysis of variance (ANOVA) with a single factor.

2.8.5. Percent recovery and matrix effects

VerA was used as an internal standard for quantification experiments. MRM transitions were optimized for VerA to ensure its suitability. The two major MRM transitions were obtained in ESI+ with both the protonated parent $[M+H]^+$ and the sodium adduct $[M+Na]^+$. Furthermore, the highest MRM transitions gave the product ion at m/z 249 which was directly observed for RL2 and SH and corresponded to the sesquiterpenic cycle.

Percent recovery was then evaluated by comparing the peak areas of VerA (100 μL at 20 μg/mL in MeOH) and RL2 (100 μL at 1.1 μg/mL in MeOH) standards spiked on fiberglass before extraction and obtained after the extraction procedure with the peak area of VerA and RL2 obtained after direct injection into the UPLC/MS system. Results were 107 ± 20% and 119 ± 20% for VerA and RL2 respectively. Matrix effects were quantitatively estimated with the matrix factor (MF) defined as the percentage ratio of the analyte peak area extracted from the matrix to the analyte peak area extracted without matrix [35]. The matrix effect was investigated for each building material with VerA (IS). Respective MFs for fiberglass, wallpaper, fir, vinyl wallpaper and painted fiberglass wallpaper were at 97%, 99%, 52%, 35% and 21%.

2.9. Statistical analysis

Data were analyzed by applying the GLM option of the ANOVA

program in the MiniTab software version v13.0. Significant differences between levels of toxins observed on the different materials tested in this study were evaluated by Tukey's test for each individual macrocyclic trichothecene. P value was set at 0.05.

3. Results

3.1. Development of S. chartarum ST82 strain on different building materials

The development of *S. chartarum* ST82 strain on different building materials was estimated by macro- and microscopic examination of hyphae development and density of sporulated conidial heads before mycotoxins extraction and analysis. *S. chartarum* growth varied according to the substrate (Fig. 3). The vinyl wallpaper did not allow the development of *S. chartarum* ST82 strain and thus no active sporulation was observed on this material, with the exception of the edge of the sample, where paper was no longer protected by the polyvinyl chloride layer (data not shown). On fiberglass, hyphae development was located on circular zones of 3–5 mm diameter surrounding the inoculation site. Density of conidial heads and sporulation were moderate. The dark color on the material resulted both from *S. chartarum*'s development and the initial color of the inoculum. On fir and wallpaper, development was intense and regular with abundant hyphae colonizing the whole sample's surface with many sporulated heads. On the painted fiberglass wallpaper, development was moderate and irregular, and sporulation was weak. Examination under stereomicroscope showed that development mostly occurred in zones that were unequally covered by paint due to the irregular surface of the material.

3.2. Toxinogenesis of S. chartarum ST82 strain on different materials

3.2.1. Levels of macrocyclic trichothecenes produced on building materials

Macrocyclic trichothecenes production during the development of *S. chartarum* ST82 strain on the different building materials was measured by UPLC-MS/MS (Fig. 4A). The four toxins were detected at different levels on tested materials. Initial levels of contamination (T0 value corresponding to inoculum deposit on the substrate) were 0.3 ± 0.01; 0.08 ± 0.02 and 1.3 ± 0.33 mg/m^2 of substrate for RL2, VerJ and SH, respectively. No SG was detected before incubation. It has to be noted that while fiberglass and wallpaper displayed only a mild matrix effect (MF near 100%), the interaction was more pronounced for the other substrates, thus demonstrating the importance of using an internal standard to quantify mycotoxins by UPLC-MS/MS on building materials.

All four quantified mycotoxins were produced at their highest level on wallpaper ($p < 0.05$) (Fig. 4A). By contrast, the levels of toxins found on vinyl wallpaper were not significantly different from the T0 value, indicating no active production of macrocyclic trichothecenes in agreement with the lack of fungal development observed on that substrate. Levels of SG, SH and RL2 measured on fiberglass, fir and painted fiberglass wallpaper were not significantly different despite the differences in growth and sporulation observed on these materials. Level of VerJ was significantly higher on fiberglass than on vinyl wallpaper (Fig. 4A).

3.2.2. Comparison of toxinogenesis profiles

Since *S. chartarum* can produce several toxins simultaneously, an analysis of their relative proportions on different building material is of importance for risk evaluations. Fig. 4B shows the relative proportion of each toxin as a function of the material used. SH was the predominant toxin, whatever the substrate, representing 51–64% of the total amount of macrocyclic trichothecenes produced. The proportions of the three other toxins varied with the substrate. Satratoxin G represented 26% of the total macrocyclic trichothecenes produced on wallpaper and fir, 16% on fiberglass and 10% on painted fiberglass wallpaper. RL2 was present on all substrates, and represented 11–21% of the total macrocyclic trichothecenes. VerJ was also found, on all substrates with the exception of vinyl wallpaper. However, it represented a lower percentage of total toxin load (2–7%) compared to SG, SH and RL2.

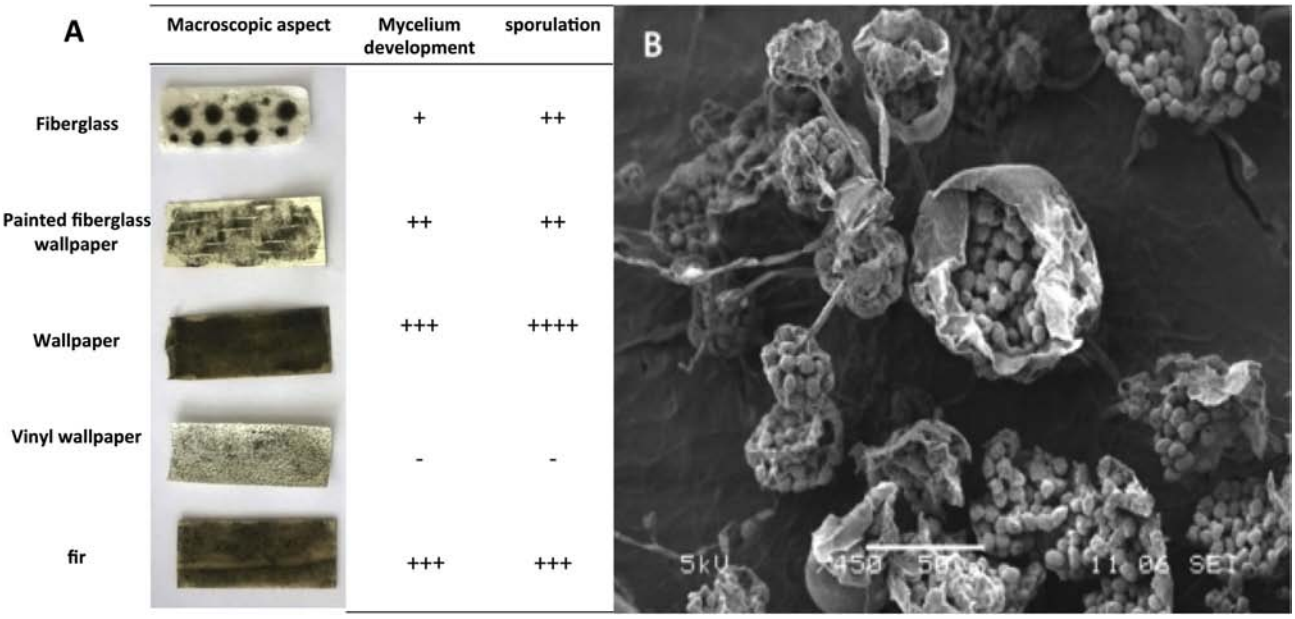

Fig. 3. Development of *S. chartarum* ST82 strain on different building materials. A: Macroscopic aspect of the different materials after development of ST 82 strain and evaluation of mycelium development and sporulation under stereomicroscope. B: Observation by Scanning Electron Microscopy of ST82 growth on wallpaper.

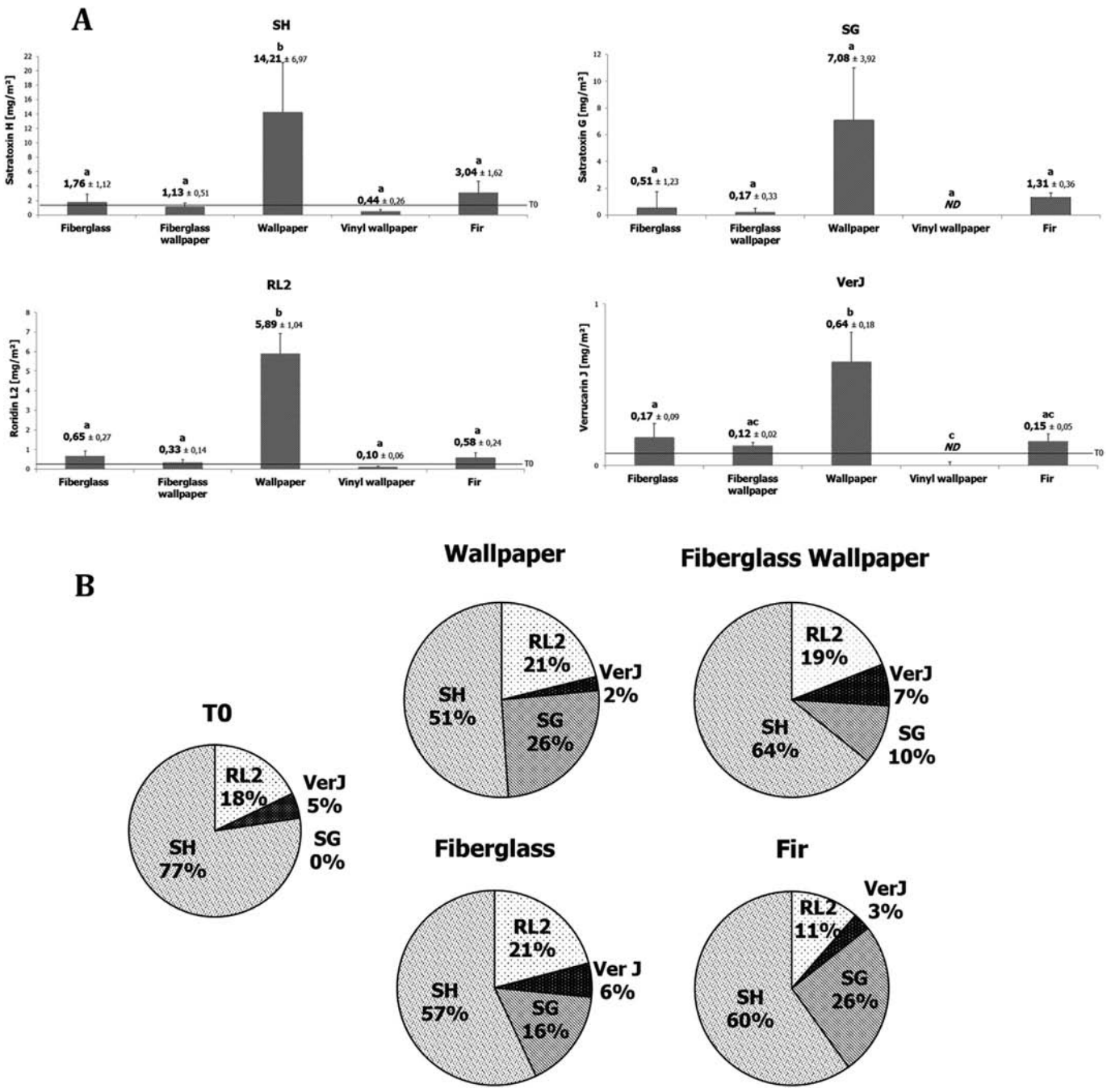

Fig. 4. Macrocyclic trichothecenes productions with ST82 strain on the different materials measured by UPLC-MS/MS. A: Results in mg/m^2 are expressed as mean ± SD of three distinct experiments (each in triplicate). Different letters indicate significant differences for one toxin as a function of the substrate. Horizontal line indicates T0 value for each toxin (no SG was detected at T0). B: Relative proportion (%) of each macrocyclic trichothecene detected in the initial inoculum (T0) and produced on the different materials.

4. Discussion

S. chartarum is a frequent contaminant of damp indoor environments where cellulosic substrates may allow its development. It occurs especially following a water damage that increases water activity to the levels required for *S. chartarum* development [17,36,37]. Despite the numerous data on its prevalence in indoor environments, the differential tropism of this fungal genus for the materials commonly used in habitations is poorly documented. Indeed most studies focused on airborne contamination. Nevertheless, Andersen et al. [14] highlighted the frequent presence of *S. chartarum* on both fiberglass and wallpaper. These materials are good substrates for the development of this fungus. This is not the case for other building materials such as linoleum or chipboard where other species were observed [14]. This is also in agreement with a previous study where *S. chartarum* was isolated on several wallpaper samples [27]. Our study demonstrated that wallpaper is the best substrate for both hyphal development and sporulation. Yet, we observed only a mild fungal development on painted fiberglass wallpaper. Although the paint used in the study

had no antimicrobial activity, the paint layer might limit access to nutrients and slow down *S. chartarum*'s growth [38,39]. The development on fiberglass was different with a limited development of mycelium and a sporulation comparable to that observed on painted fiberglass wallpaper. Once again, this observation could be related to a lack of nutrients accessibility that is essential for hyphal growth. Finally, the vinyl wallpaper appeared to be resistant to *S. chartarum*'s development for it did not allow its development or its sporulation. However, the observed development of *S. chartarum* on the edge of the sample, where the paper is no longer protected by the polyvinyl chloride layer, suggests that, despite an initial resistance to colonization, such material could become sensitive with time and aging, after protective layer becomes porous. All samples in this study were analyzed after a common incubation period of 10 days in order to compare them. It is then possible that the extension of incubation would have allowed larger colonization of this material.

In the present study, we quantified four major macrocyclic trichothecenes produced during the development of a toxigenic strain of *S. chartarum* on wallpaper, fir, fiberglass and painted fiberglass wallpaper. Toxin production appeared to be higher on wallpaper, which is a substrate that displays the highest and easiest access to cellulose. Whereas, for other materials, the limited access to nutrients (protected by PVC or paint) could explain the differences in toxin production. As an illustration, toxin production was lower on painted fiberglass wallpaper than on fiberglass. Moreover, our results demonstrate that there is no strict relationship between toxin production and fungal development. Indeed, on fir, development and sporulation of the *S. chartarum* strain were important and comparable to what was observed on wallpaper. However, the levels of toxins produced on fir were significantly lower than on wallpaper. The presence in fir of several terpenoid compounds, which were previously identified as possible inhibitors of mycotoxin production [40], could explain that observation.

Only few studies have investigated the production of macrocyclic trichothecenes on materials following *S. chartarum*'s development. Most of them were not quantitative but demonstrated the possible co-occurrence of several macrocyclic trichothecenes [41] and especially SG and SH [25]. In a study by Gottschalk et al. [27], SG and SH were quantified and the levels observed on wallpaper samples were in agreement with the present study. RL2 and VerJ were also previously detected but not quantified [27].

When related to an equivalent surface of 1 square meter of material, the concentrations of the different macrocyclic trichothecenes produced after *S. chartarum*'s development could reach several mg/m^2. For example, on wallpaper, mean concentrations of RL2, SG and SH reached 5.9, 7 and 14.2 mg/m^2, respectively. Such contamination levels of materials could be responsible for the reported detection of these mycotoxins in the air of water-damaged buildings with *Stachybotrys* contamination [15,26]. Although no clear dose-effect relationship has been established for these mycotoxins, it has been recently demonstrated that an intranasal exposure of Rhesus monkeys to 5 μg SG for 4 days led to a widespread apoptosis of olfactory sensory neurons and to epithelial and olfactory nerve atrophy as well as an acute neutrophilic rhinitis [23]. This dose corresponds to the quantity of SG that was measured on only 10 cm^2 of contaminated wallpaper in our study. However, for such an exposure, toxins have to be aerosolized from contaminated building materials. Due to their microscopic structure (production in clusters and covered with dry slime) (Fig. 3B), the spores of *Stachybotrys* are not easily aerosolized and are consequently found at a lower frequency in the air than the xerophilic spores of *Aspergillus* or *Penicillium* [17]. Nevertheless, a recent publication reported the possible aerosolization of *S. chartarum* spores from contaminated gypsum board [42]. Moreover, macrocyclic trichothecenes have also been observed in particles smaller than conidia [43]. This could be due to the excretion of the toxins in droplets and their subsequent adsorption on small dust particles that can then be aerosolized [44] or to the unhooking of small-contaminated particles of support following fungal degradation.

The presence of large quantities of macrocyclic trichothecenes on contaminated materials also raises the question on the impact of an occupational exposure to these mycotoxins during building remediation. Indeed both cutaneous toxicity and toxicity after inhalation have been clearly demonstrated for these compounds in animals [21,23]. Moreover, no current remediation treatment is able to completely eliminate toxins from supports [31,45]. Our study was conducted using a highly toxigenic strain of *S. chartarum* placed in environmental conditions favorable for toxin production. Although the conditions used in this study are not far from those that can be observed in homes with *S. chartarum* contamination following water damages (25 °C, high humidity, darkness since *S. chartarum* can develop behind furniture), results shall now be confirmed by testing samples from naturally contaminated indoor environments.

The substrate appeared to have a strong influence on the total level of toxins produced and modulates the proportions of different toxins. SH appeared to be the main toxin produced on building materials. This predominance was also previously reported on rice [46]. It has to be noted that SG was not detected in the initial inoculum but was present, at different proportions, on all materials after incubation and fungal development. Our results are consistent with the recent genome sequencing of *Stachybotrys* that revealed the presence of several gene clusters involved in macrocyclic trichothecenes synthesis with locations that are compatible with a co-regulation [47]. However, since levels of toxin production observed on fir, fiberglass and painted fiberglass wallpaper were moderate, it would be now interesting to analyze the proportion of the toxins produced on other materials or substrates (gypsum board, straw) favorable for *S. chartarum* development and toxin production to see if toxins are produced in the same relative proportions.

Thus, such data are of interest for risk assessment and the monitoring of the exposure of inhabitants to these toxins. Indeed, it suggests that SH alone, the most toxic macrocyclic trichothecene [22,48], could be used as a marker for contamination by the whole family. For that, rapid tests could probably be developed to allow direct monitoring in contaminated houses. Nevertheless, it would be interesting to analyze the toxigenic profile of other mycotoxin-producing strains of *S. chartarum* to assess possible inter-strain differences.

5. Conclusion

Characterization of the growth and toxinogenesis of a *S. chartarum* strain on different building materials showed that the nature of the material influenced the levels of macrocyclic trichothecenes produced by *S. chartarum*. The observed differences mainly concerned the total level of toxins produced, with no strict relation to fungal growth. Satratoxin H, the most toxic macrocyclic trichothecenes, was the main mycotoxin produced on all tested substrates. Such results are of importance for both risk assessment and monitoring of indoor exposure to those toxic compounds.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.buildenv.2016.07.002.

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1.4.2 Supplementary data

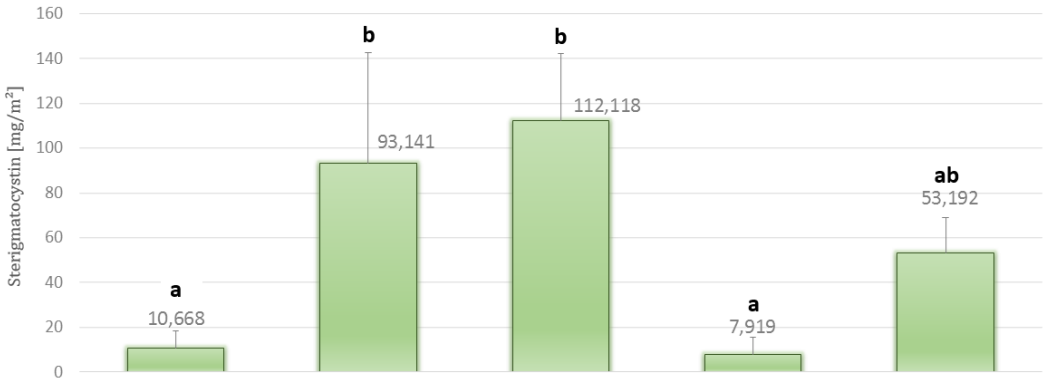
Development and toxinogenesis of *A. versicolor* and *P. brevicompactum* on the different building materials were assessed according to the same experimental procedures described before for *S. chartarum*. All experiments were done in triplicate and repeated three times.

1.4.2.1 Toxinogenesis of Aspergillus versicolor NCPT 54 on different building materials

The development of *Aspergillus versicolor* NCPT 54 on different building materials was estimated by macro- and microscopic examination of hyphae development and density of sporulated conidial heads before mycotoxins extraction and analysis. As previously observed for *S. chartarum*, development on VWP was very limited and no active sporulation was observed on this material.

On FG, hyphae development was located in circular zones of 3-5 mm diameter surrounding the inoculation site. Density of conidial heads and sporulation were moderate. The green color on the material resulted both from *A.versicolor* development and the initial color of the inoculum. On fir and WP, development was intense and regular with abundant hyphae colonizing the whole sample's surface with many sporulated heads. On the FWP, development was moderate and irregular, and sporulation was weak. Examination under stereomicroscope showed that development mostly occurred in zones that were unequally covered by paint due to the irregular surface of the material.

After 10 days of development of *A.versicolor* NCPT54 strain at 25 °C on the different building materials, STG production was measured by UPLC-MS/MS. Toxin was detected at different levels on tested materials. Initial level of contamination (T0 value corresponding to inoculum deposit on the substrate) was 0.13 ± 0.04 mg/m^2. These values are much lower than those found on different materials after incubation with the toxin-producing strain, demonstrating active toxin production during fungal growth.
Observed levels of contamination are highly variable depending on the material, nevertheless toxin was found on all contaminated materials (Figure 16).



Mycelial development	++	++	++ / +++	(+)	+++
Sporulation	++	++	++++	(+)	+++

Figure 16: STG production by toxinogenic strain of *Aspergillus versicolor* NCPT 54 after 10 days of culture on different materials at 25 °C [mg/m^2] followed by observation about fungal growth

WP and FWP were the most favorable materials for toxinogenesis. For these two materials, the quantities of produced toxin reported to square meter were about 110 and 90 mg of STG, respectively.

By contrast, VWP appears to be the least favorable medium for toxin production, in relation with weak fungal growth on this material.

1.4.2.2 Toxinogenesis of Penicillium brevicompactum Pb25 on different building materials

Development of *Penicillium brevicompactum* Pb25 on different building materials was also estimated by macro- and microscopic examination of hyphae development and density of sporulated heads before mycotoxins extraction and analysis. As for *A. versicolor*, growth varied according to the substrate (Figure 17). As for the two previously tested species, *A. versicolor* and *S. chartarum*, the VWP did not allow the development and thus no active sporulation was observed on this material, with the exception of the edge of the sample, where paper was no longer protected by the polyvinyl chloride layer (Figure 17A).

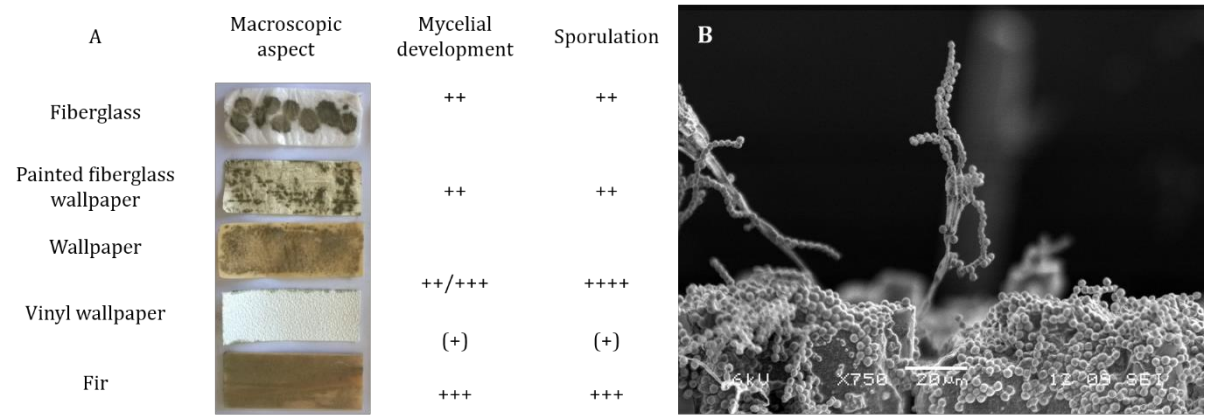


Figure 17: Development of *Penicillium brevicompactum* Pb25 strain on different building materials. A: Macroscopic aspect of the different materials after development of Pb25 strain and evaluation of mycelial development and sporulation under stereomicroscope. B: SEM observation of Pb25 growth on wallpaper

On FG, hyphae development was in circular zones of 3-5 mm diameter following the inoculation position. Density of sporulated *penicilli* were very strong. Pigmentation common for *P. brevicompactum* was present. On fir and WP, development was intense and regular with abundant hyphae colonizing the whole sample's surface with numerous large compact sporulated *penicilli*. On the FWP, development was irregular, in zones, but sporulation (as well as development) intensity was important. Examination under stereomicroscope showed that development in zones occurred (as previously noted) where paint was poorly applied to some regions due to the irregular surface of the material.

Production of MPA by *P. brevicompactum* Pb25 strain on different building materials was evaluated after 10 days of incubation at 25 °C (Figure 18). Initial level of contamination (T0 value corresponding to inoculum deposit on the substrate) was 0.02 ± 0.009 mg/m^2. After incubation, toxin was detected at different levels on tested materials. On FG, FWP, WP and fir, amounts of toxin were significantly higher than T0 value, demonstrating an active toxinogenesis from toxinogenic strain. Contrary, on VWP, detected quantity of MPA was still very low or undetectable, in agreement with the difficulty of the fungus to grow on this material.

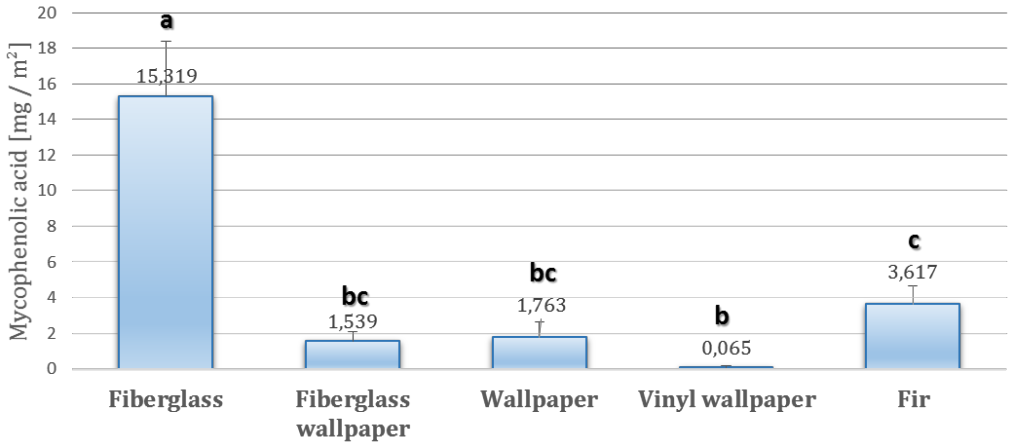


Figure 18 : Production of MPA by the toxinogenic strain of *Penicillium brevicompactum* Pb25, 10 days of incubation on different materials at 25 °C [mg/m²]

Unlike results obtained for other fungal species where toxin production was the greatest on WP, for *Penicillium brevicompactum,* FG appeared to be the most favorable material for the toxinogenesis. Amount of produced toxin was about 16 mg of MPA per square meter of contaminated FG. Among the three other materials, fir seems to be the most favorable for toxinogenesis with an amount of produced toxin 2 to 3 times greater than that observed on FWP and WP.

1.4.3 Conclusion

We demonstrated that all studied toxins can be produced on selected indoor materials in case of development of the corresponding toxinogenic fungi. Produced quantities vary depending on materials and were not directly related to fungal development. In all cases, wallpaper was very favorable for growth and sporulation of the three species tested in this study. With the exception of *P. brevicompactum,* which preferred fiberglass, this material also showed to be a very good support for toxinogenesis of *A. versicolor* and *S. chartarum*. Furthermore, quantities of produced toxins, reported to one square meter of contaminated material, appeared to be important, at least for sterigmatocystin and satratoxin H. Accordingly, wallpaper was further used as a model material for testing aerosolization of toxins.

Meanwhile, vinyl wallpaper revealed to be resistant support for both fungal growth and mycotoxins' production. However, since some development was noted on the edges, where vinyl layer was damaged, it raises the question of eventual fungal development and finally toxin production on this material after time and aging or water damage that could alter the protective layer.

CHAPTER TWO.

Development of a monitoring tool for indoor environments by VOCs analysis related to mycotoxin production

2.1 Introduction

The first part of our work allowed the demonstration of a possible active toxinogenesis in case of development of different toxinogenic moulds on diverse building materials. Some of analysed toxins were produced in high quantities, raising the question of their possible consequence on health of inhabitants in case of inhalation or contact with contaminated materials.

Since mycotoxins are secondary metabolites and their production directly depend on the toxinogenic potential of strains but also environmental conditions in which moulds grow, the risk assessment of fungal toxinogenesis in indoor environments also requires the setup of tools able to estimate the presence of an active toxin production associated with fungal development.

The works of Moularat et al. have shown that, as soon as they begin to grow, moulds can produce volatile organic compounds (VOCs) or more precisely microbial volatile organic compounds (MVOCs). MVOCs are VOCs emitted as products of mould's secondary metabolism. This study allowed the determination of a specific global footprint for fungal contamination of indoor environments (Moularat *et al.*, 2008b, 2008c, 2008d), from which was developed an index of fungal contamination. This qualitative index (presence/absence) overcomes the limitations associated with low concentrations of MVOCs and allows determination of an active fungal growth. Moreover, hidden mould contamination can be detected with this method (Betancourt *et al.*, 2013) as well as early fungal development (Hulin *et al.*, 2013).

With this index (Moularat *et al.*, 2008a, 2011) Moularat and co-workers have shown that around 70% of dwellings which have mould development according to this fungal index was not considered as contaminated when visual detection was used (Hulin *et al.*, 2013).

The developed tool has already been used in several studies for epidemiological purposes:

- ISAAC-FERMA conducted by the University Hospital of Clermont-Ferrand aiming to correlate exposure to fungal sprays and asthma in children (Moularat *et al.*, 2011)

- ESMHA, conducted by the Regional Health Observatory of Ile de France, concerning the health effects of mould in the housing.

These results indicated that the detection of target compounds is relevant to detect active fungal developments. Moreover, some literature's data suggest that some VOCs could be specific for mycotoxinogenesis. For instance, Zeringue et al. found the differences of VOCs emissions between toxinogenic strains of *Aspergillus flavus* (producing aflatoxin) and non-toxinogenic strains (Zeringue *et al.*, 1993). The same year, Desjardins demonstrated that the volatile trichodiene was

the first metabolite in the biosynthetic pathway of trichothecenes (Desjardins *et al.*, 1993). This result was confirmed by Jelen et al. who established a correlation between the synthesis of trichothecenes and production of trichodiene and other sesquiterpenes by *Fusarium sambucinum, F. sporotrichioides, F. poae* and *F. graminearum* (Jelen *et al.*, 1997a, 1997b, 1995). Pasanen et al. showed that the production of trichothecenes by *Fusarium* strains was accompanied by the emission of volatiles terpenes and sesquiterpenes (Pasanen *et al.*, 1996).

In that context, the second aim of our work was to study the VOCs emitted by the pairs of toxinogenic (TOX+) and non-toxinogenic (TOX-) strains, previously identified, in order to evaluate the possible presence of compounds that could be specific of an active toxinogenesis and that could serve as biomarkers of active mycotoxin synthesis in indoor environments.

The practical application of such findings could be the development of a biochemical fingerprint of mycotoxins' production, which could be used as a monitoring tool.

2.2 Materials and methods – Development of method

2.2.1 Samples

Five types of materials were cut into 2 x 5 cm pieces and then sterilized (121 °C, 20 min) before use as described earlier (Experimental work: 1.3.1.3). Each material was supplemented with nutrients by addition of 500 µL of NS. Fungal strains of interest were grown on MEA (BioKar, France) for 14 days at 25 °C to obtain highly sporulating cultures. Spore suspension was prepared from these fungal cultures by adding 10 mL of Tween 80 (0.05 %) to each PD. The number of spores was quantified by direct counting on a Malassez cell. Spore suspension was then diluted to obtain the required concentration. Contamination was achieved for each strain independently, by applying 100 µL of suspension (10^7 spores/mL) to each material. Pairs of TOX+ and TOX- strains are presented in table below (Table 23). Contaminated materials were placed in chambers.

Table 23: Pairs TOX+ and TOX- strains used for VOCs analysis for three species of interest

Species	**N° strain**	**Toxinogenic potential**
Aspergillus versicolor	Av 9 (NCPT 54) Av 13 (E26a)	TOX+ TOX-
Penicillium brevicompactum	Pb 25 (IBT 23078) parent Pb 19 (*mpa* CΔ) mutant	TOX+ TOX-
Stachybotrys chartarum	ST 81 ST 82	TOX+ TOX-

2.2.2 Experimental assembly and VOC sampling

The experimental setup was developed in previous studies of CSTB and presented on Figure 19. Its concept is schematically presented on Figure 20.

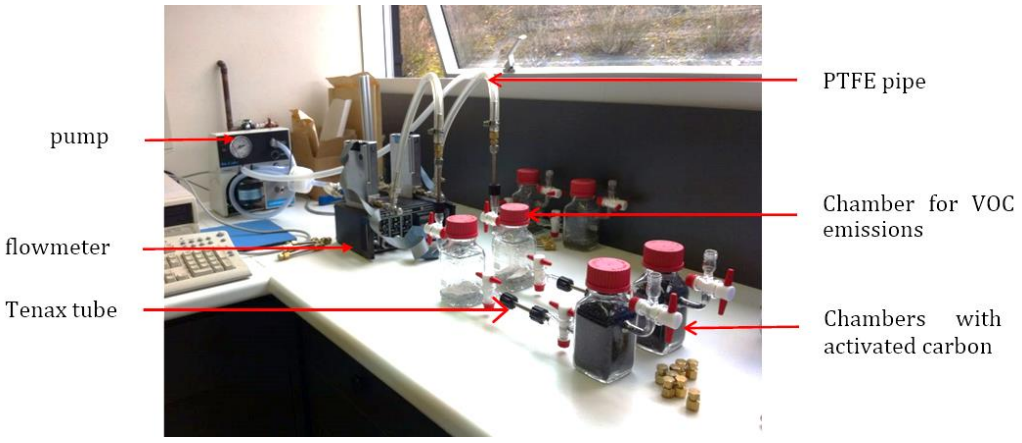


Figure 19: Experimental setup dedicated to collect the VOCs emitted during fungal development

System consists in two parallel branches, each comprising principally:

- An air-filtration chain containing chambers with active charcoal and Tenax tubes for supplying the chamber with air free of VOCs during the experiment,

- A chamber for sample incubation and VOC emissions,

- A collecting tube.

All pipes are made of PTFE. This material was chosen because it's non-emissive VOC and therefore it is non-contaminating towards VOC samples.

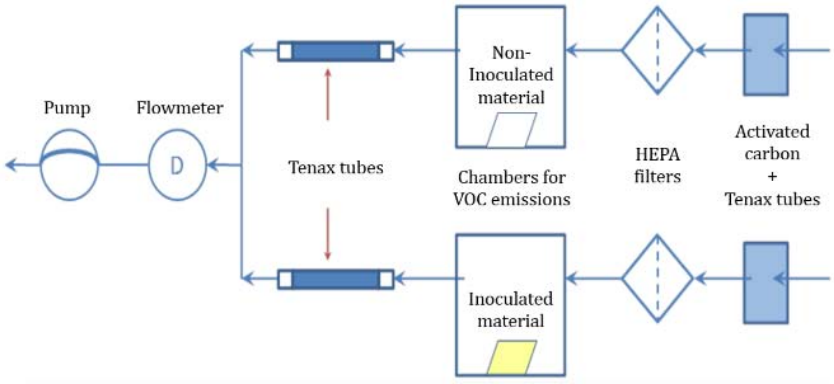


Figure 20: Schema of the experimental assembly (sampling chain)

Before setting up inoculated materials, chambers were sterilized by moist heat and filled with a layer of 2 cm of glass beads with sterile water in order to maintain moisture level at saturation

throughout the incubation period. In each chamber, 2 samples of material were then placed on glass bead layer. Some chambers hosted coupons inoculated with TOX+ strain and others chambers were filled with coupons inoculated with TOX- strain. Chambers containing coupons supplemented in nutrients but not inoculated served to measure the VOCs emissions of the support itself, regardless of any microbial growth. After placing coupons, chamber's air was replaced with VOC-free air produced by passage through the air-filtration chain of the experimental setup (Figure 20).

All chambers were then placed in incubators at 25 °C. After 10 days of incubation, regardless of the fungal species, the gaseous emissions in each chamber were collected on TENAX tube, at a rate of 100 mL/min for 30 min.

These sampling conditions allowed the aspiration of 10 times of the chamber volume, and therefore the collection of all emitted VOCs.

2.2.3 Chemical analysis of samples by GC-MS

An analytical chain composed of a gas chromatography (GC-Varian 3400), coupled to a mass spectrometer (MS-Saturn 2000) was used (Figure 21). The analysis parameters are reported in Table 24, and allow the detection of C4 - C20 compounds. The detection limit of analytical chain was 0.67 ng equivalent of Toluene.

Desorption of sampling tubes was done with the ATD 400 (Automatic Thermal Desorption System), data was retrieved through the acquisition system.

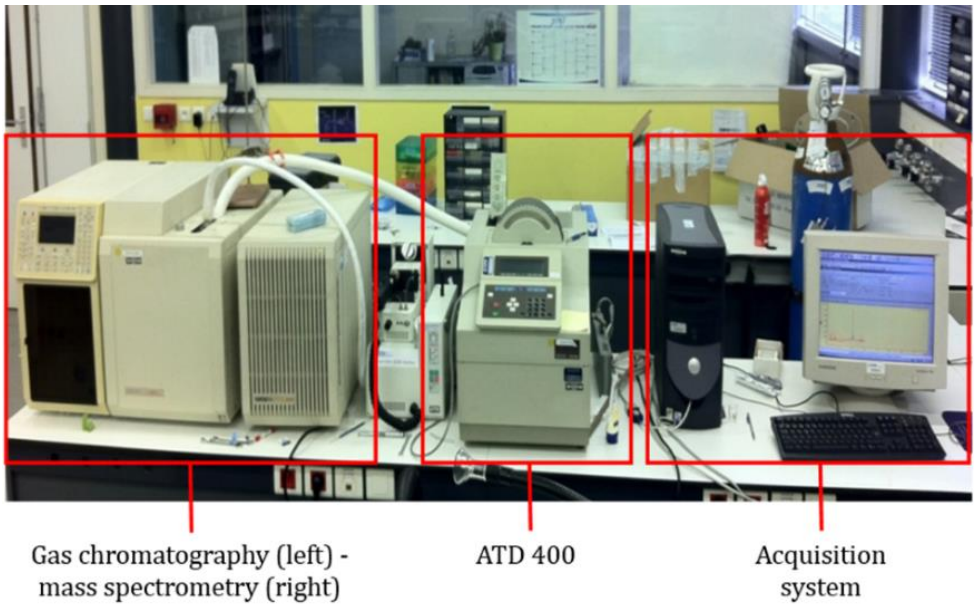


Figure 21: The GC–MS analytical chain

Table 24: Characteristics of the analytical chain and analysis parameters

Analytical parameters	Analytical conditions
Thermal desorber	ATD 400 (Perkin Elmer)
Desorption temperature	260 °C
Desorption flow	50 ± 2 mL/min, Nitrogen N50
Duration of desorption	15 min
Temperature of the cold setup (Tenax TA)	-30 °C
Injection temperature (40°C/s)	280 °C
Temperature of the transfer line	220 °C
Gas chromatography/Mass spectrometer	3800 gas chromatography / Saturn 2000 (Varian)
Column	SGE BPX5
Carrier gas	Helium N60
Constant pressure	30 psi
Temperature cycle	40 °C during 1 min 3 °C/min up to 200 °C 250 °C during 10 min
Temperature FID	270 °C
Mass spectrometer parameters	Quadrupole mode EI, Scanning (35 - 425)

2.2.4 Analysis of chromatograms

Identification of compounds was performed by comparison of mass spectra, associated with each peak, with a spectral library NIST (NIST, 2008).

Each obtained chromatogram (example illustrated on Figure 22) presents emissions during 80 minutes of sampling.

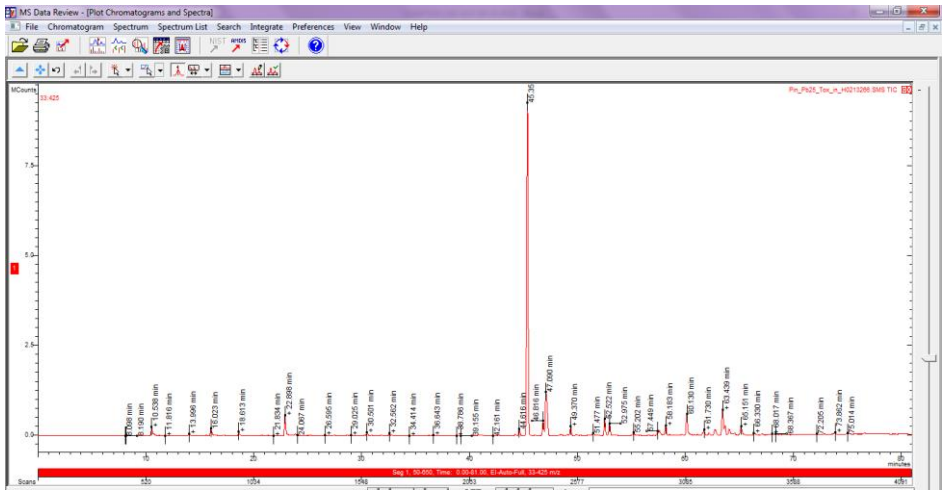


Figure 22: Obtained chromatogram for TOX+ strain of *P. brevicompactum* (Pb25 parent) emissions' while growing on WP

It should be noted that since the aim of this study was to find target VOCs indicating toxinogenesis, every peak, even if weak, needed to be considered. Therefore, each of the about 200 peaks of each chromatogram was investigated (illustration of the number of peaks observable on the same chromatogram as on figure 22 but zoomed between 60 and 65 minutes) (Figure 23).

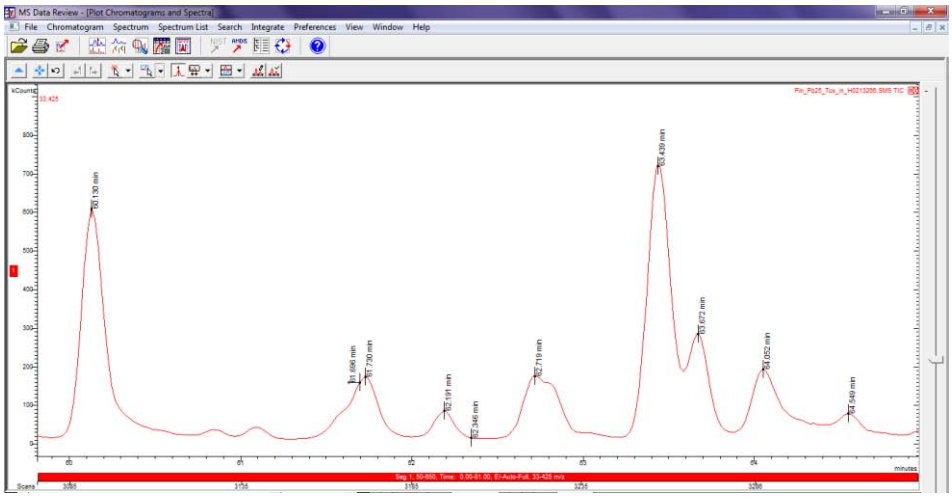


Figure 23: Chromatogram for TOX+ strain of *P. brevicompactum* (Pb25 parent) emissions' while growing on WP, between 60 and 65 minute of sampling

Moreover, parallel analysis needs to be simultaneously performed by comparing each peak from "fungal" chromatogram with peaks observed on control chromatogram (same material, treated in the same manner but without contamination with fungal strain), in order to discriminate VOCs emitted by material. All peaks having the same retention time on both chromatograms (illustrated on figure 24 for fir) and same spectra were then eliminated from consideration. Concerning retention time, it can, and sometimes often, be slightly different. The reason is that one chromatogram can be shifted in comparison to another. In that case, chromatograms are overlaid and fixed using the position of well-known compounds as toluene (Figure 24).

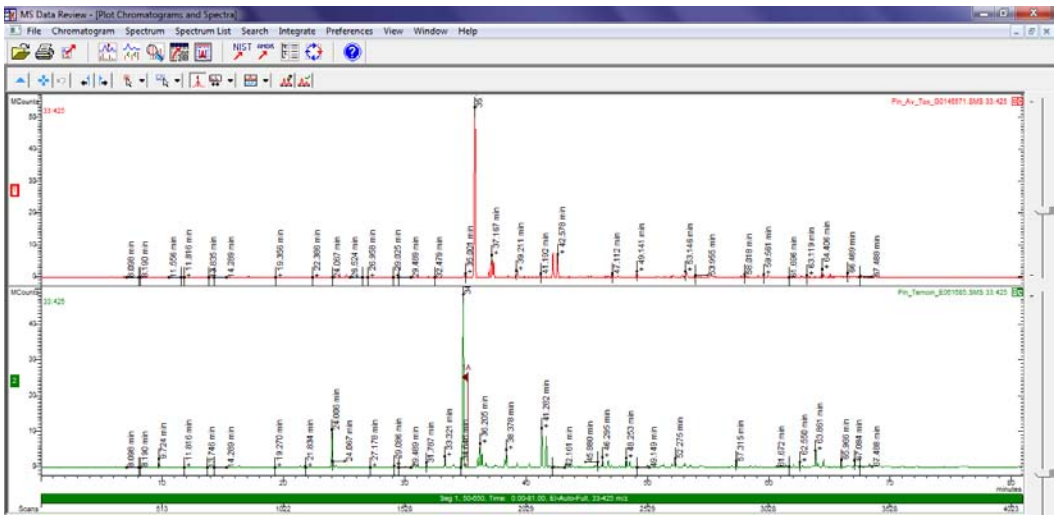


Figure 24: Chromatogram for TOX+ strain of *A. versicolor* (NCPT54) emissions' while growing on Fir (red), compared to paired control (fir without fungal inoculum) (green)

Each compound, represented by one peak, was identified thanks to comparison of its mass spectra (Figure 25A) with propositions given by spectral library (NIST, 2008). As showed on Figure 25C, list of proposed molecules (from the most probable to least probable) was obtained. It is up to analyst to choose which molecule really fits the mass spectra, since sometimes it is evident that proposed molecule is not adequate (Figure 25B). Example of correct pairing between spectra of interest (molecule to be identified) and proposed spectra is shown on Figure 26.

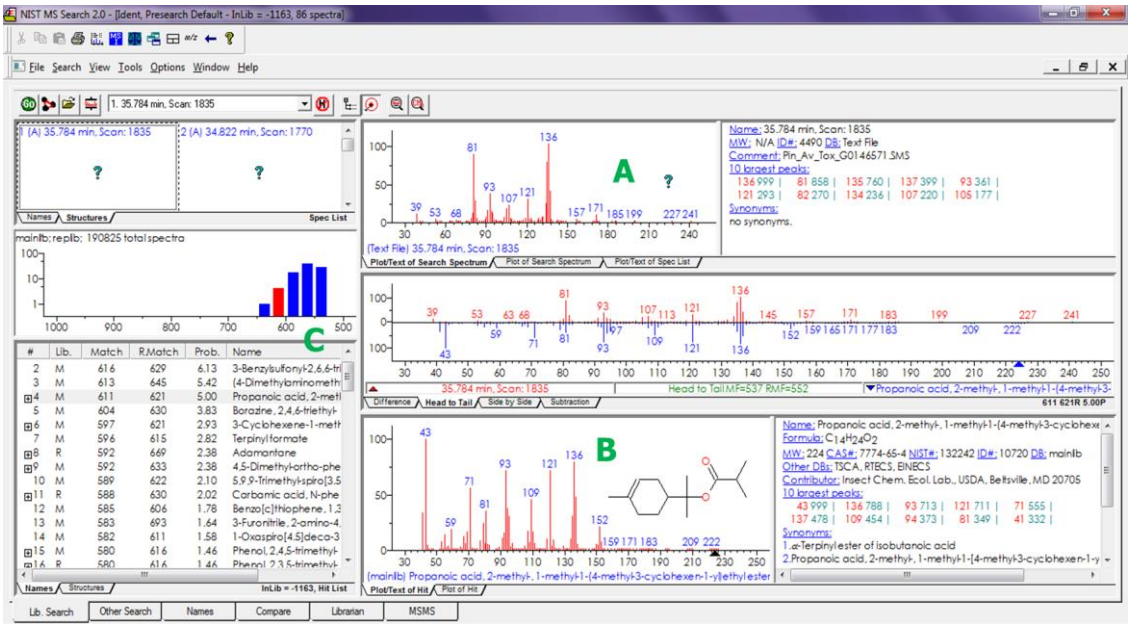


Figure 25: Analysis of mass spectra of chosen peak, thanks to NIST spectral library. A: spectra of peak to be identified, B: spectra of proposed molecule, C: list of proposed molecules (from the highest probably to least probable)

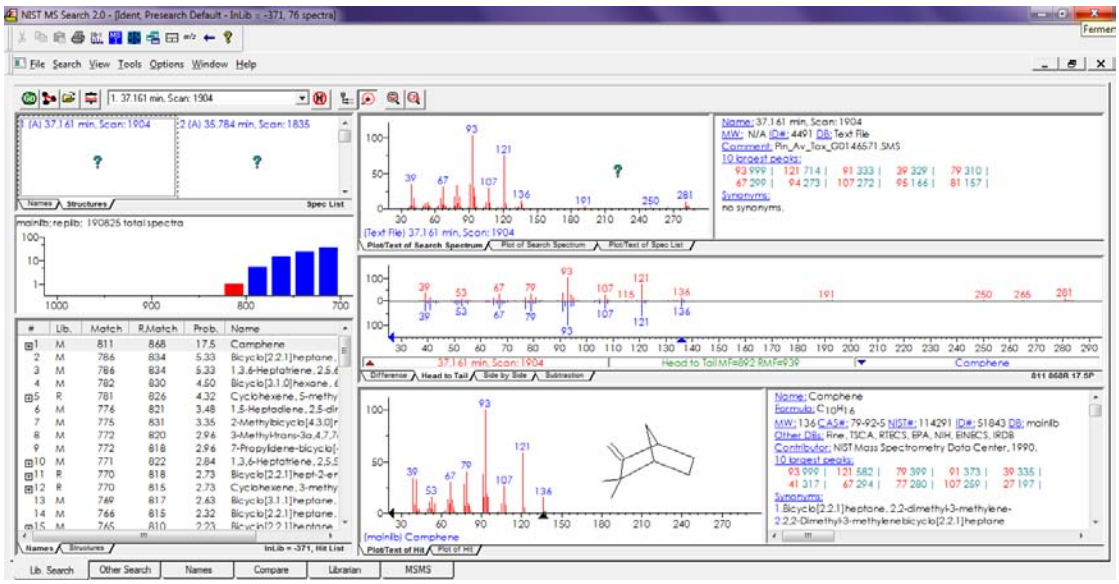


Figure 26: Analysis of mass spectra of chosen peak, thanks to NIST spectral library

One chromatogram represents one sample. We tested three species, *A. versicolor, P. brevicompactum* and *S. chartarum.* For each of them, we tested two strains (TOX+/TOX-), except for *P. brevicompactum* where 2 TOX+ and one TOX- strains were tested. Each of 7 strains was grown on 5 different materials (FG, FWP, WP, VWP and Fir). Finally, each condition was tested in triplicate. Combining conditions and number of samples, 105 chromatograms were obtained and analysed.

2.3 Results

The identification of chemical compounds that would sign mycotoxin production consisted in different stages:

- The systematic screening of VOCs emitted during fungal growth on various media for two fungal strains of *Penicillium brevicompactum*: a TOX+ strain (Pb25 - parent) and TOX- strain (Pb19 - mutant).

- Comparison of the chemical targets of interest identified in *P. brevicompactum* to MVOCs emissions by *Aspergillus versicolor* and *Stachybotrys chartarum* strains.

2.3.1 Comparative analysis of chemical emissions of *P. brevicompactum* (parent/mutant)

Emissions linked to the development of two strains of *P. brevicompactum*, Pb25-parent (TOX+) and Pb19-mutant (TOX-), on fiberglass (FG) support were analysed. FG was used as reference support since it does not emit the VOC, as previously described.
Table 25 presents the MVOCs emitted by each strain, VOCs emitted from the contamination-free media have been excluded from the table.
Fungal Contamination Index was applied to all of samples and concluded, as expected, an active fungal growth.

Table 25: MVOCs emitted during development of *P. brevicompactum* strains on FG (not identified components are labelled « unknown ») (presence of one component is noted with « 1 » and its absence with « 0 »)

Emitted MVOCs	Pb19 Mutant (TOX-)	Pb25 Parent (TOX+)
2,4-dimethyl-Hexane	0	1
Undecane	0	1
2-methyl-1-Pentene	0	1
Limonene	0	1

1-butoxy-2-Propanol	0	1
3-methyl-2-Pentanone	0	1
3-methyl-Decane	1	0
pentyl-cyclopropane	1	0
2,3-Dimethyldecane	1	0
2,4-dimethyl-Furan	1	0
2,6,7-trimethyl-Decane	1	0
2-pentyl-Furan	1	0
3-ethyl-hexane	1	0
butyl-Cyclopropane	1	0
ethyl-cyclobutane	1	0
nitroso-Methane	1	0
Phthalan	1	0
1,3-dimethyl-Benzene	1	0
1,2,3-Trifluorobenzene	1	0
1,2,3-trimethyl-Benzene	1	0
1,3,5,7-Cyclooctatetraene	1	0
4-methyl-1-Undecene	1	0
Cyclohexene, 3-methyl-6-(1-methylethyl)	1	0
Ethylbenzene	1	0
Xylene	1	0
3,4-dimethyl-1-Pentanol	1	0
1,9-Nonanediol	1	0
2,2'-oxybis-, diacétate Ethanol	1	0
2,6-dimethyl-7-Octen-2-ol	1	0
2-Hexyn-1-ol	1	0
2-methyl-2-Propanol	1	0
2-Octyn-1-ol	1	0
2-propyl-1-Heptanol	1	0
3-Hexanol	1	0
3-Octen-1-ol	1	0
Isotridecanol	1	0
Phenol	1	0
(1α,2β,5α)-2,6,6-trimethyl-Bicyclo[3.1.1]heptan-3-one	1	0
2,2-dihydroxy-1-phenyl-Ethanone	1	0
2-Butanone	1	0
2-methyl-Cyclopentanone	1	0
6-methyl-5-Hepten-2-one	1	0
2-Heptenal	1	0
Hexanal	1	0
2-Propylnonanoic acid	1	0
4-Amino-1,5-pentandioic acid	1	0
Butanoic acid, 2-ethyl-2,3,3-trimethyl-, methyl ester	1	0
Butanoic acid, 3,3-dimethyl-2-(1-methylethyl)-, methyl ester	1	0
Propanoic acid, 2-hydroxy-2-methyl-,	1	0
Benzeneacetic acid, octyl ester	1	0
Heptanoic acid, 2,2-dimethyl-6-oxo-, methyl ester	1	0
Methyl 2-methylhexanoate	1	0
O-decyl-Hydroxylamine	1	0
3,4-dihydro-2H-Pyran	1	0
1-Pentadecyne	1	0
2-n-Butyl furan	1	0
2-Octen-1-ol, 3,7-dimethyl-, isobutyrate	1	0
2-propyl-Tetrahydrofuran	1	0
Acetic anhydride	1	0
Benzonitrile	1	0
methoxy-phenyl-Oxime	1	0
Methyl 2,2-dimethyl-3-hydroxypropionate	1	0

N-methyl-2-amino-Propanamide	1	0
Phenylethyne	1	0
Unknown 1	1	0
Unknown 2	1	0
Unknown 3	1	0
Unknown 4	1	0
Unknown 5	1	0
Unknown 6	1	0

Among the listed 70 MVOCs:

- 64 were exclusively issued by the TOX- strain Pb19 (mutant) (highlighted in pink)

- 6 were exclusively produced by TOX+ strain Pb25 (parent) (highlighted in blue), and could represent potential tracers of MPA production.

It can be noted that genetic modifications done on the TOX+ strain (parent) to block the production of MPA impacted the chemical emissions. As an example, with 73 produced MVOCs, mutant strain produced much more compounds than its parent, which emitted only 15 MVOCs on same support.

To verify the robustness of this footprint, chemical emissions from these two strains were studied on three supports commonly used in indoor environments: wallpaper (WP), painted fiberglass wallpaper (FWP) and fir. Table 26 reports all the MVOCs identified for both strains. Application of Fungal Contamination Index on chromatograms revealed again presence of fungal activity.

Table 26: MVOCs emitted during development of *P. brevicompactum* strains according to the growth material (unidentified components are labelled « unknown ») (presence of one component is noted with « 1 » and its absence with « 0 »)

Emitted MVOCs	**Pb25 Parent (TOX+)**				**Pb19 Mutant (TOX-)**			
	FG	**WP**	**FWP**	**Fir**	**FG**	**WP**	**FWP**	**Fir**
3-methyl-Decane	0	0	0	0	1	1	1	1
pentyl-cyclopropane	0	0	0	0	1	1	1	1
2,4-dimethyl-Furan	0	0	0	0	1	1	1	1
butyl-Cyclopropane	0	0	0	0	1	1	1	1
nitroso-Methane	0	0	0	0	1	1	1	1
1,3,5,7-Cyclooctatetraene	0	0	0	0	1	1	1	1
4-methyl-1-Undecene	0	0	0	0	1	1	1	1
1,9-Nonanediol	0	0	0	0	1	1	1	1
2-Hexyn-1-ol	0	0	0	0	1	1	1	1
3-Hexanol	0	0	0	0	1	1	1	1
3-Octen-1-ol	0	0	0	0	1	1	1	1
Phenol	0	0	0	0	1	1	1	1
2,2-dihydroxy-1-phenyl-Ethanone	0	0	0	0	1	1	1	1
2-methyl-Cyclopentanone	0	0	0	0	1	1	1	1
2-Heptenal	0	0	0	0	1	1	1	1
Hexanal	0	0	0	0	1	1	1	1
Propanoic acid, 2-hydroxy-2-methyl-,	0	0	0	0	1	1	1	1

O-decyl-Hydroxylamine	0	0	0	0	1	1	1	1
2-n-Butyl furan	0	0	0	0	1	1	1	1
2-propyl-Tetrahydrofuran	0	0	0	0	1	1	1	1
Acetic anhydride	0	0	0	0	1	1	1	1
Benzonitrile	0	0	0	0	1	1	1	1
N-methyl-2-amino-Propanamide	0	0	0	0	1	1	1	1
2,4-dimethyl-Hexane	1	1	0	0	0	0	0	0
2,6,7-trimethyl-Decane	0	0	0	0	1	1	0	0
2-pentyl-Furan	0	0	0	1	1	1	1	1
3-ethyl-hexane	0	0	1	1	1	1	1	1
ethyl-cyclobutane	0	0	0	0	1	1	0	0
Phthalan	0	0	0	0	1	1	1	0
Undecane	1	1	0	0	0	0	0	0
1,3-dimethyl-Benzene	0	0	0	0	1	1	1	0
1,2,3-Trifluorobenzene	0	0	0	0	1	1	1	0
1,2,3-trimethyl-Benzene	0	0	0	0	1	1	1	0
2-methyl-1-Pentene	1	1	0	1	0	0	0	0
Cyclohexene, 3-methyl-6-(1-methylethyl)	0	0	0	0	1	1	0	1
Ethylbenzene	0	1	0	0	1	1	1	1
Limonene	1	1	0	1	0	0	1	1
Xylene	0	1	0	0	1	1	1	1
1-butoxy-2-Propanol	1	1	0	0	0	0	0	0
3,4-dimethyl-1-Pentanol	0	0	0	0	1	0	0	0
2,2'-oxybis-, diacétate Ethanol	0	0	0	0	1	1	0	0
2,6-dimethyl-7-Octen-2-ol	0	0	0	0	1	1	0	0
2-methyl-2-Propanol	0	0	1	1	1	1	1	1
2-Octyn-1-ol	0	0	0	0	1	1	0	0
2-propyl-1-Heptanol	0	0	0	0	1	1	1	0
Isotridecanol	0	0	0	0	1	1	0	0
(1α,2β,5α)-2,6,6-trimethyl-Bicyclo[3.1.1]heptan-3-one	0	0	0	0	1	1	0	1
2-Butanone	0	0	1	1	1	1	1	1
3-methyl-2-Pentanone	1	1	0	0	0	0	0	0
6-methyl-5-Hepten-2-one	0	0	0	0	1	1	0	1
2-Propylnonanoic acid	0	0	0	0	1	1	0	0
4-Amino-1,5-pentandioic acid	0	0	0	0	1	0	0	0
Butanoic acid, 2-ethyl-2,3,3-trimethyl-, methyl ester	0	0	0	0	1	0	0	0
Butanoic acid, 3,3-dimethyl-2-(1-methylethyl)-, methyl ester	0	0	0	0	1	1	0	0
Benzeneacetic acid, octyl ester	0	0	0	0	1	1	0	0
Heptanoic acid, 2,2-dimethyl-6-oxo-, methyl ester	0	0	0	0	1	0	0	0
Methyl 2-methylhexanoate	0	0	0	0	1	1	0	1
3,4-dihydro-2H-Pyran	0	0	0	0	1	1	0	1
1-Pentadecyne	0	0	0	0	1	1	0	0
2-Octen-1-ol, 3,7-dimethyl-, isobutyrate	0	0	0	0	1	0	0	0
methoxy-phenyl-Oxime	0	0	1	0	1	1	1	0
Methyl 2,2-dimethyl-3-hydroxypropionate	0	0	0	0	1	1	0	0
Phenylethyne	0	0	0	0	1	1	0	1
Unknown 1	0	0	0	0	1	1	0	0
Unknown 2	0	0	0	0	1	0	0	0
Unknown 3	0	0	0	0	1	0	0	0
Unknown 4	0	0	0	0	1	1	0	0
Unknown 5	0	0	0	0	1	1	0	0
Unknown 6	0	0	0	0	1	0	0	0
2,4-dimethyl-2-nitro-pentane	0	0	0	0	0	0	1	0
2-methyl-Decane	0	0	0	0	0	0	1	0
3,4-dimethyl-Heptane	0	0	0	0	0	0	1	1
3-ethylhexane	0	0	0	0	0	0	1	0
3-methyl-Heptane	0	0	0	1	0	0	0	0
1,1-dimethyl-Cyclopentane	0	0	0	0	0	1	0	0

1-ethyl-2-methyl-Cyclopropane	0	0	0	0	0	0	1	1
1-methyl-4-(1-methylethyl)-7-Oxabicyclo[2.2.1]heptane	0	0	0	1	0	0	0	0
1-Methylpentyl cyclopropane	0	0	1	0	0	0	0	0
1α,2β,3α,4β-Tetramethylcyclopentane	0	0	0	0	0	0	1	0
2,5-dimethyl-Furan	0	0	0	0	0	0	0	1
2-chloro-2-nitro-Propane	0	0	0	0	0	1	1	1
2-ethoxy-2-methyl-Propane	0	0	0	0	0	1	1	0
2-ethyl-Furan	0	0	0	1	0	0	0	0
2-methyl-Butane	0	0	1	0	0	0	0	0
2-nitro-Butane	0	1	0	0	0	0	0	0
3,3-dimethyl-Hexane	0	0	0	0	0	0	1	0
4-methyl-Decane	0	0	0	0	0	0	1	1
4-methyl-Octane	0	0	1	1	0	0	0	0
Decane	0	0	1	1	0	0	0	0
ethenylmethylene-Cyclopropane	0	0	1	1	0	0	0	0
methyl-Cyclooctane	0	0	0	0	0	1	0	0
tetrahydro-Furan	0	0	0	1	0	0	0	0
1,2-dimethoxy-Benzene	0	0	0	0	0	0	0	1
1-methyl-2-(1-methylethyl)-benzene	0	0	0	1	0	0	0	0
3-ethyl-2-Pentene	0	1	0	0	0	0	0	0
4-methyl-1-Decene	0	0	0	0	0	1	1	0
5-Tridecene	0	0	1	1	0	0	0	0
Methoxy-Benzene	0	1	0	0	0	0	0	0
(1-methylenepropyl)-Benzene	0	0	0	0	0	0	1	0
(1-methylethyl)-Benzene	0	0	0	0	0	0	1	0
1,3-bis(1,1-dimethylethyl)-Benzene	0	0	0	0	0	0	1	0
1,3-Pentadiene	0	0	0	0	0	0	1	1
1-ethyl-3,5-dimethyl-Benzene	0	0	0	1	0	0	0	0
1-ethyl-4-(1-methylethyl)-Benzene	0	0	0	0	0	0	0	1
1-Heptene	0	1	0	0	0	0	0	0
1-methyl-4-(1-methylethyl)- 1,4-Cyclohexadiene	0	0	0	1	0	0	0	0
2,4-dimethyl-1,3-Pentadiene	0	0	0	0	0	0	1	1
2-methyl-1,4-Pentadiene	0	0	0	0	0	0	1	1
2-methyl-1-Propene	0	0	0	0	0	0	0	1
2-methyl-2,4-Hexadiene	0	0	0	1	0	0	0	0
3-(ethenyloxy)-1-Propene	0	0	1	0	0	0	0	0
3,4-Dimethoxytoluene	0	0	0	0	0	0	0	1
3,5-dimethyl-Cyclohexanol	0	0	0	0	0	0	1	1
3-Hexene	0	1	0	0	0	0	0	0
6-methyl-1-Octene	0	0	1	0	0	0	0	0
7,7-dimethyl-1,3,5-Cycloheptatriene	0	0	1	1	0	0	0	0
7-Tetradecene	0	0	0	0	0	0	1	0
alpha-Cubebene	0	0	0	1	0	0	0	0
Alpha-Methylstyrene	0	0	0	0	0	0	1	1
Benzene, 1,4-dichloro	0	0	0	0	0	1	0	0
Tetrachloroethylene	0	0	0	0	0	1	1	1
Trichloroethylene	0	0	0	0	0	1	0	0
2,2,4-trimethyl-3-Penten-1-ol	0	0	1	0	0	0	1	1
1-Hepten-4-ol	0	0	1	0	0	0	0	0
1-Hexyn-3-ol	0	1	0	0	0	0	0	0
1-Pentanol	0	0	0	0	0	0	0	1
2,5-Dimethyl-5-hexen-3-ol	0	0	0	0	0	0	0	1
2-Buten-1-ol	0	1	0	0	0	0	0	0
2-ethyl-1-Hexanol	0	0	1	0	0	0	0	0
2-Hexyl-1-octanol	0	0	0	0	0	0	0	1
2-methoxy-Ethanol	0	0	1	1	0	0	0	0
2-methyl-2-nitro-1-Propanol	0	0	0	0	0	0	1	1
2-Methylene cyclopentanol	0	0	0	0	0	0	1	1

3,4-Dimethylcyclohexanol	0	0	1	0	0	0	0	0
3-Methylpenta-1,4-diene-3-ol	0	0	1	0	0	0	0	0
3-Nonen-1-ol	0	0	0	0	0	1	1	1
alpha,alpha-dimethyl-Benzenemethanol	0	0	0	0	0	1	0	1
Cyclodecanol	0	0	0	0	0	0	1	1
Cyclopentanol, 2-methyl-, acetate	0	0	1	0	0	0	0	0
Isoborneol	0	0	0	1	0	0	0	0
α,α,4-trimethyl-3-Cyclohexene-1-methanol	0	0	0	1	0	0	0	0
1-phenyl-1-Propanone	0	0	0	0	0	0	1	1
1,3,3-trimethyl-Bicyclo[2.2.1]heptan-2-one	0	0	0	1	0	0	0	0
1-Hepten-3-one	0	0	0	0	0	1	0	0
2,4-dimethyl-3-Pentanone	0	0	0	0	0	0	1	0
2-Pentanone	0	1	1	1	0	0	1	1
3,4-dimethyl-2-Hexanone	0	0	0	0	0	0	1	1
3,5-Dimethyl-4-octanone	0	0	0	0	0	0	1	0
7-Decen-2-one	0	0	0	0	0	0	1	0
Undecanal	0	0	0	0	0	0	1	1
2,2-dimethyl-Propanoic acid	0	0	0	0	0	0	1	0
Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	0	0	1	0	0	0	0	0
Propanoic acid, 2-methyl-, 2-ethylhexyl ester	0	0	0	0	0	1	0	0
Benzeneacetic acid, 2-tridecyl ester	0	0	0	0	0	0	1	1
Dichloroacetic acid, 2-pentadecyl ester	0	0	1	1	0	0	0	0
Oxalic acid, allyl nonyl ester	0	0	0	0	0	0	1	0
2-Buten-1-ol, 3-methyl-, acetate	0	0	0	0	0	0	1	0
2-methyl-1,3-Dioxolane	0	0	0	0	0	1	1	1
2-methyl-2-Undecanethiol	0	0	1	1	0	0	0	0
3-Hexyne	0	0	0	0	0	0	1	1
Allyl methallyl ether	0	0	0	0	0	0	1	1
Artemiseole	0	0	0	1	0	0	0	0
Carbon disulfide	0	0	0	0	0	0	1	0
Linalyl isobutyrate	0	0	0	0	0	0	0	1
Methylglyoxal	0	0	0	0	0	0	1	1
N-methyl-3-Pyridinecarboxamide	0	0	0	0	0	0	1	0
Pyrrolidine	0	0	0	0	0	0	1	1
Vinylcyclohexyl ether	0	0	0	0	0	1	0	0
Unknown 10	0	0	0	0	0	0	1	1
Unknown 7	0	0	0	0	0	1	0	0
Unknown 9	0	0	1	1	0	0	0	0
Unknown 8	0	0	0	0	0	1	1	1

Among the six potential markers identified on fiberglass and highlighted in yellow in Table 26, none is emitted on all three other tested materials. Nevertheless all of them were produced on WP.

Among these 6 molecules, limonene, being also produced by the mutant strain on FWP and Fir, cannot be relevant for signing the production of MPA.

Four components, including 2,4-dimethyl-hexane, undecane, 1-butoxy-2-propanol and 3-methyl-2-pentanone, revealed toxinogenesis on WP and FG. Only the 2-methyl-1-Pentene could detect MPA on WP, FG and fir. None of them was produced on FWP.

At this stage, the construction of a VOC biochemical fingerprint would require the creation of an index that takes into account these 5 tracers completed with 44 VOCs, issued exclusively by parent strain and taking into account their dependence on the support.

2.3.2 Study of chemical emissions from Pb16 strain (04891) on various materials

To test the relevance of our approach we compared the chemical emissions of two *Penicillium brevicompactum* strains (parent and mutant) with those of another TOX+ strain of this species (Pb16). Table 27 lists the emissions from these three strains on all studied materials. Application of the Fungal Contamination Index on chromatograms of this third strain confirmed fungal activity.

Table 27: MVOCs emitted during development of three *P. brevicompactum* strains according to the growth material (not identified components are labelled « unknown ») (presence of one component is noted with « 1 » and its absence with « 0 »)

	Pb25 Parent (TOX+)				**Pb19 Mutant (TOX-)**				**Pb16 (04891) (TOX+)**			
	FG	**WP**	**FWP**	**Fir**	**FG**	**WP**	**FWP**	**Fir**	**FG**	**WP**	**FWP**	**Fir**
9-hexyl-Heptadecane	0	0	0	0	0	0	0	0	1	0	0	0
2,3-dihydro-4-(1-methylethyl)-Furan	0	0	0	0	0	0	0	0	1	0	0	0
2-Bromononane	0	0	0	0	0	0	0	0	1	0	0	0
3-methyl-Pentane	0	0	0	0	0	0	0	0	0	0	1	0
Tetrahydro-2,2,4,4-tetramethyl-Furan	0	0	0	0	0	0	0	0	0	0	1	0
3-methyl-1-Hexene	0	0	0	0	0	0	0	0	1	0	0	0
4-Acetyl-1-methylcyclohexene	0	0	0	0	0	0	0	0	0	0	0	1
5,5-Dimethyl-1,3-hexadiene	0	0	0	0	0	0	0	0	0	0	1	0
2-Butanol	0	0	0	0	0	0	0	0	1	0	0	0
2-Pentanol	0	0	0	0	0	0	0	0	0	1	0	0
4-Hexen-1-ol	0	0	0	0	0	0	0	0	0	0	1	0
9-Tetradecen-1-ol	0	0	0	0	0	0	0	0	0	1	0	0
1-phenyl-1-Butanone	0	0	0	0	0	0	0	0	0	0	1	1
2-methyl-3-Hexanone	0	0	0	0	0	0	0	0	0	0	1	1
6-Methyl-3,5-heptadiene-2-one	0	0	0	0	0	0	0	0	0	1	0	0
3-methyl-2-Butenal	0	0	0	0	0	0	0	0	0	0	1	0
Acetic acid, pentyl ester	0	0	0	0	0	0	0	0	0	0	0	1
Butanedioic acid, 2,3-diethyl-, dimethyl ester	0	0	0	0	0	0	0	0	1	0	0	0
Butanoic acid, 2-ethyl-, methyl ester	0	0	0	0	0	0	0	0	1	0	0	0
Butanoic acid, octyl ester	0	0	0	0	0	0	0	0	0	0	1	0
m-Toluic acid, 4-nitrophenyl ester	0	0	0	0	0	0	0	0	0	0	0	1
Undecanoic acid, 2-ethyl-, methyl ester	0	0	0	0	0	0	0	0	1	0	0	0
2-Ethoxytetrahydrofuran	0	0	0	0	0	0	0	0	1	0	0	0
3-Furaldehyde	0	0	0	0	0	0	0	0	0	1	0	0
Pyrrole	0	0	0	0	0	0	0	0	0	0	1	0
2-methyl-Decane	0	0	0	0	0	0	1	0	0	0	1	0
3-ethylhexane	0	0	0	0	0	0	1	0	0	0	1	0
3-methyl-Decane	0	0	0	0	1	1	1	1	1	1	1	1
pentyl-cyclopropane	0	0	0	0	1	1	1	1	1	1	1	1
1-ethyl-2-methyl-Cyclopropane	0	0	0	0	0	0	1	1	0	0	1	1
1α,2β,3α,4β-Tetramethylcyclopentane	0	0	0	0	0	0	1	0	0	0	1	0
2,5-dimethyl-Furan	0	0	0	0	0	0	0	1	0	0	0	1

3,5-dimethyl-Octane	0	0	0	0	0	0	0	0	0	0	0	0
butyl-Cyclopropane	0	0	0	0	1	1	1	1	1	1	1	1
methyl-Cyclooctane	0	0	0	0	0	1	0	0	0	1	0	0
nitroso-Methane	0	0	0	0	1	1	1	1	1	1	1	1
Phthalan	0	0	0	0	1	1	1	0	1	1	1	0
1,2-dimethoxy-Benzene	0	0	0	0	0	0	0	1	0	0	0	1
1,3-dimethyl-Benzene	0	0	0	0	1	1	1	0	1	1	1	0
(1-methylenepropyl)-Benzene	0	0	0	0	0	0	1	0	0	0	1	0
(1-methylethyl)-Benzene	0	0	0	0	0	0	1	0	0	0	1	0
1,2,3-trimethyl-Benzene	0	0	0	0	1	1	1	0	1	1	1	0
1,3,5,7-Cyclooctatetraene	0	0	0	0	1	1	1	1	1	1	1	1
1,3-bis(1,1-dimethylethyl)-Benzene	0	0	0	0	0	0	1	0	0	0	1	0
1,3-Pentadiene	0	0	0	0	0	0	1	1	0	0	1	1
2-methyl-1,4-Pentadiene	0	0	0	0	0	0	1	1	0	0	1	1
2-methyl-1-Propene	0	0	0	0	0	0	0	1	0	0	0	1
3,4-Dimethoxytoluene	0	0	0	0	0	0	0	1	0	0	0	1
3,5-dimethyl-Cyclohexanol	0	0	0	0	0	0	1	1	0	0	1	1
4-methyl-1,4-Hexadiene	0	0	0	0	0	0	0	0	0	0	0	0
4-methyl-1-Undecene	0	0	0	0	1	1	1	1	1	1	1	1
Alpha-Methylstyrene	0	0	0	0	0	0	1	1	0	0	1	1
Benzene, 1,4-dichloro	0	0	0	0	0	1	0	0	0	1	0	0
1,9-Nonanediol	0	0	0	0	1	1	1	1	1	1	1	1
1-Pentanol	0	0	0	0	0	0	0	1	0	0	0	1
2,2'-oxybis-, diacétate Ethanol	0	0	0	0	1	1	0	0	1	1	0	0
2,5-Dimethyl-5-hexen-3-ol	0	0	0	0	0	0	0	1	0	0	0	1
2,6-dimethyl-7-Octen-2-ol	0	0	0	0	1	1	0	0	1	1	0	0
2-Hexyl-1-octanol	0	0	0	0	0	0	0	1	0	0	0	1
2-Hexyn-1-ol	0	0	0	0	1	1	1	1	1	1	1	1
2-methyl-2-nitro-1-Propanol	0	0	0	0	0	0	1	1	0	0	1	1
2-Methylene cyclopentanol	0	0	0	0	0	0	1	1	0	0	1	1
2-Octyn-1-ol	0	0	0	0	1	1	0	0	1	1	0	0
2-propyl-1-Heptanol	0	0	0	0	1	1	1	0	1	1	1	0
3-Hexanol	0	0	0	0	1	1	1	1	1	1	1	1
3-Octen-1-ol	0	0	0	0	1	1	1	1	1	1	1	1
alpha,alpha-dimethyl-Benzenemethanol	0	0	0	0	0	1	0	1	0	1	0	1
alpha-Bisabolol	0	0	0	0	0	0	0	0	0	0	0	0
Cyclodecanol	0	0	0	0	0	0	1	1	0	0	1	1
Isotridecanol	0	0	0	0	1	1	0	0	1	1	0	0
Phenol	0	0	0	0	1	1	1	1	1	1	1	1
(1α,2β,5α)-2,6,6-trimethyl-Bicyclo[3.1.1]heptan-3-one	0	0	0	0	1	1	0	1	1	1	0	1
1-phenyl-1-Propanone	0	0	0	0	0	0	1	1	0	0	1	1
1-Hepten-3-one	0	0	0	0	0	1	0	0	0	1	0	0
2,2-dihydroxy-1-phenyl-Ethanone	0	0	0	0	1	1	1	1	1	1	1	1
2,4-dimethyl-3-Pentanone	0	0	0	0	0	0	1	0	0	0	1	0
2-methyl-Cyclopentanone	0	0	0	0	1	1	1	1	1	1	1	1
3,4-dimethyl-2-Hexanone	0	0	0	0	0	0	1	1	0	0	1	1
3,5-Dimethyl-4-octanone	0	0	0	0	0	0	1	0	0	0	1	0
6-methyl-5-Hepten-2-one	0	0	0	0	1	1	0	1	1	1	0	1
7-Decen-2-one	0	0	0	0	0	0	1	0	0	0	1	0
2-Heptenal	0	0	0	0	1	1	1	1	1	1	1	1
Hexanal	0	0	0	0	1	1	1	1	1	1	1	1
Undecanal	0	0	0	0	0	0	1	1	0	0	1	1
2,2-dimethyl-Propanoic acid	0	0	0	0	0	0	1	0	0	0	1	0
2-Propylnonanoic acid	0	0	0	0	1	1	0	0	1	1	0	0
Butanoic acid, 2-ethyl-2,3,3-trimethyl-, methyl ester	0	0	0	0	1	0	0	0	1	0	0	0
Butanoic acid, 3,3-dimethyl-2-(1-methylethyl)-, methyl ester	0	0	0	0	1	1	0	0	1	1	0	0
Propanoic acid, 2-hydroxy-2-methyl-,	0	0	0	0	1	1	1	1	1	1	1	1
Benzeneacetic acid, 2-tridecyl ester	0	0	0	0	0	0	1	1	0	0	1	1

Heptanoic acid, 2,2-dimethyl-6-oxo-, methyl ester	0	0	0	0	1	0	0	0	1	0	0	0
Methyl 2-methylhexanoate	0	0	0	0	1	1	0	1	1	1	0	1
Oxalic acid, allyl nonyl ester	0	0	0	0	0	0	1	0	0	0	1	0
O-decyl-Hydroxylamine	0	0	0	0	1	1	1	1	1	1	1	1
3,4-dihydro-2H-Pyran	0	0	0	0	1	1	0	1	1	1	0	1
1-Pentadecyne	0	0	0	0	1	1	0	0	1	1	0	0
2-Buten-1-ol, 3-methyl-, acetate	0	0	0	0	0	0	1	0	0	0	1	0
2-propyl-Tetrahydrofuran	0	0	0	0	1	1	1	1	1	1	1	1
Acetic anhydride	0	0	0	0	1	1	1	1	1	1	1	1
Benzonitrile	0	0	0	0	1	1	1	1	1	1	1	1
N-methyl-2-amino-Propanamide	0	0	0	0	1	1	1	1	1	1	1	1
Phenylethyne	0	0	0	0	1	1	0	1	1	1	0	1
Pyrrolidine	0	0	0	0	0	0	1	1	0	0	1	1
Unknown 1	0	0	0	0	1	1	0	0	1	1	0	0
Unknown 3	0	0	0	0	1	0	0	0	1	0	0	0
Unknown 4	0	0	0	0	1	1	0	0	1	1	0	0
Unknown 5	0	0	0	0	1	1	0	0	1	1	0	0
Unknown 6	0	0	0	0	1	0	0	0	1	0	0	0
Unknown 8	0	0	0	0	0	1	1	1	0	1	1	1
2,4-dimethyl-2-nitro-pentane,	0	0	0	0	0	0	1	0	0	0	0	0
3,4-dimethyl-Heptane	0	0	0	0	0	0	1	1	0	0	0	1
3-methyl-Heptane	0	0	0	1	0	0	0	0	0	0	0	0
1,1-dimethyl-Cyclopentane	0	0	0	0	0	1	0	0	0	0	1	0
1-methyl-4-(1-methylethyl)-7-Oxabicyclo[2.2.1]heptane	0	0	0	1	0	0	0	0	0	0	0	0
1-Methylpentyl cyclopropane	0	0	1	0	0	0	0	0	0	0	0	0
2,4-dimethyl-Furan	0	0	0	0	1	1	1	1	0	1	0	1
2,4-dimethyl-Hexane	1	1	0	0	0	0	0	0	0	0	0	0
2,6,7-trimethyl-Decane	0	0	0	0	1	1	0	0	0	1	0	0
2-chloro-2-nitro-Propane	0	0	0	0	0	1	1	1	1	1	0	1
2-ethoxy-2-methyl-Propane	0	0	0	0	0	1	1	0	1	1	0	0
2-ethyl-Furan	0	0	0	1	0	0	0	0	0	0	0	0
2-methyl-Butane	0	0	1	0	0	0	0	0	0	0	0	0
2-methyl-Furan	1	1	1	1	1	1	1	1	1	1	1	1
2-nitro-Butane	0	1	0	0	0	0	0	0	0	0	0	0
2-pentyl-Furan	0	0	0	1	1	1	1	1	1	1	1	1
3,3-dimethyl-Hexane	0	0	0	0	0	0	1	0	1	1	1	0
3-ethyl-hexane	1	1	1	1	1	1	1	1	1	1	1	1
3-methyl-Furan	1	1	1	1	1	1	1	1	1	1	1	1
3-methyl-Hexane	1	1	0	1	1	1	1	1	1	1	1	1
4-methyl-Decane	0	0	0	0	0	0	1	1	0	1	1	0
4-methyl-Octane	0	0	1	1	0	0	0	0	0	0	0	0
Decane	0	0	1	1	0	0	0	0	0	0	0	0
ethenylmethylene-Cyclopropane	0	0	1	1	0	0	0	0	0	0	0	0
ethyl-cyclobutane	0	0	0	0	1	1	0	0	1	0	0	0
tetrahydro-Furan	0	0	0	1	0	0	0	0	0	0	0	0
Undecane	1	1	0	0	0	0	0	0	0	0	0	0
1-methyl-2-(1-methylethyl)-benzene	0	0	0	1	0	0	0	0	0	0	0	0
3-ethyl-2-Pentene	0	1	0	0	0	0	0	0	0	0	0	0
4-methyl-1-Decene	0	0	0	0	0	1	1	0	0	1	0	0
5-Tridecene	0	0	1	1	0	0	0	0	0	0	0	0
Methoxy-Benzene	0	1	0	0	0	0	0	0	0	0	0	0
1,2,3-Trifluorobenzene	0	0	0	0	1	1	1	0	1	1	0	0
1,3-Octadiene	1	1	1	1	1	1	1	1	1	1	1	1
1-ethyl-3,5-dimethyl-Benzene	0	0	0	1	0	0	0	0	0	0	0	0
1-ethyl-4-(1-methylethyl)-Benzene	0	0	0	0	0	0	0	1	0	0	0	0
1-Heptene	0	1	0	0	0	0	0	0	0	0	0	0
1-methyl-4-(1-methylethyl)- 1,4-Cyclohexadiene	0	0	0	1	0	0	0	0	0	0	0	0
2,4-dimethyl-1,3-Pentadiene	0	0	0	0	0	0	1	1	0	0	1	0
2-methyl-1-Pentene	1	1	0	1	0	0	0	0	0	0	0	0

2-methyl-2,4-Hexadiene	0	0	0	1	0	0	0	0	0	0	0	0
3-(ethenyloxy)-1-Propene	0	0	1	0	0	0	0	0	0	0	0	0
3-Hexene	0	1	0	0	0	0	0	0	0	0	0	0
6-methyl-1-Octene	0	0	1	0	0	0	0	0	0	0	0	0
7,7-dimethyl-1,3,5-Cycloheptatriene	0	0	1	1	0	0	0	0	0	0	0	0
7-Tetradecene	0	0	0	0	0	0	1	0	0	0	0	0
alpha-Cubebene	0	0	0	1	0	0	0	0	0	0	0	0
Alpha-Pinene	1	1	1	1	1	1	1	1	1	1	1	1
Cyclohexene, 3-methyl-6-(1-methylethyl)	0	0	0	0	1	1	0	1	1	1	1	1
Ethylbenzene	0	1	0	0	1	1	1	1	1	1	1	1
Limonene	1	1	0	1	0	0	1	1	0	0	1	1
Tetrachloroethylene	0	0	0	0	0	1	1	1	1	1	1	1
Trichloroethylene	0	0	0	0	0	1	0	0	1	1	0	1
Xylene	0	1	0	0	1	1	1	1	1	1	1	1
1-butoxy-2-Propanol	1	1	0	0	0	0	0	0	0	0	0	0
2,2,4-trimethyl-3-Penten-1-ol	0	0	1	0	0	0	1	1	0	0	1	1
3,4-dimethyl-1-Pentanol	0	0	0	0	1	0	0	0	0	0	0	0
1-Hepten-4-ol	0	0	1	0	0	0	0	0	0	0	0	0
1-Hexyn-3-ol	0	1	0	0	0	0	0	0	0	0	0	0
1-Octen-3-ol	1	1	1	1	1	1	1	1	1	1	1	1
2-Buten-1-ol	0	1	0	0	0	0	0	0	0	0	0	0
2-ethyl-1-Hexanol	0	0	1	0	0	0	0	0	0	0	1	1
2-methoxy-Ethanol	0	0	1	1	0	0	0	0	0	0	0	0
2-methyl-1-Butanol	1	1	1	1	1	1	1	1	1	1	1	1
2-methyl-2-Propanol	0	0	1	1	1	1	1	1	1	1	1	1
3,4-Dimethylcyclohexanol	0	0	1	0	0	0	0	0	0	0	0	0
3-methyl-1-Butanol	1	1	1	1	1	1	1	1	1	1	1	1
3-Methylpenta-1,4-diene-3-ol	0	0	1	0	0	0	0	0	0	0	0	0
3-Nonen-1-ol	0	0	0	0	0	1	1	1	0	0	1	1
Cyclopentanol, 2-methyl-, acetate	0	0	1	0	0	0	0	0	0	0	0	0
Isoborneol	0	0	0	1	0	0	0	0	0	0	0	0
α,α,4-trimethyl-3-Cyclohexene-1-methanol	0	0	0	1	0	0	0	0	0	0	0	0
1,3,3-trimethyl-Bicyclo[2.2.1]heptan-2-one	0	0	0	1	0	0	0	0	0	0	0	0
2-Butanone	0	0	1	1	1	1	1	1	1	1	1	1
2-Pentanone	0	1	1	1	0	0	1	1	0	0	1	1
3-methyl-2-Pentanone	1	1	0	0	0	0	0	0	0	0	0	0
4-Amino-1,5-pentandioic acid	0	0	0	0	1	0	0	0	1	1	0	0
Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	0	0	1	0	0	0	0	0	0	0	0	0
Propanoic acid, 2-methyl-, 2-ethylhexyl ester	0	0	0	0	0	1	0	0	0	1	1	0
Benzeneacetic acid, octyl ester	0	0	0	0	1	1	0	0	0	1	0	0
Dichloroacetic acid, 2-pentadecyl ester	0	0	1	1	0	0	0	0	0	0	0	0
Hexanoic acid, 2-ethyl-, methyl ester	0	0	1	0	1	0	0	0	1	0	0	0
2-methyl-1,3-Dioxolane	0	0	0	0	0	1	1	1	1	1	1	1
2-methyl-2-Undecanethiol	0	0	1	1	0	0	0	0	0	0	0	0
2-n-Butyl furan	0	0	0	0	1	1	1	1	1	1	1	0
2-Octen-1-ol, 3,7-dimethyl-, isobutyrate	0	0	0	0	1	0	0	0	1	1	0	0
3-Hexyne	0	0	0	0	0	0	1	1	0	0	0	0
Allyl methallyl ether	0	0	0	0	0	0	1	1	0	0	1	0
Artemiseole	0	0	0	1	0	0	0	0	0	0	0	0
Carbon disulfide	0	0	0	0	0	0	1	0	0	0	0	0
Dimethyldisulfide	1	1	1	1	1	1	1	1	1	1	1	1
Linalyl isobutyrate	0	0	0	0	0	0	0	1	0	0	0	0
methoxy-phenyl-Oxime	0	0	1	0	1	1	1	0	1	1	1	1
Methyl 2,2-dimethyl-3-hydroxypropionate	0	0	0	0	1	1	0	0	1	1	1	0
Methylglyoxal	0	0	0	0	0	0	1	1	0	0	0	1
N-methyl-3-Pyridinecarboxamide	0	0	0	0	0	0	1	0	0	0	0	0
Vinylcyclohexyl ether	0	0	0	0	0	1	0	0	0	0	0	0

Unknown 10	0	0	0	0	0	0	1	1	0	0	0	0
Unknown 2	0	0	0	0	1	0	0	0	0	0	0	0
Unknown 7	0	0	0	0	0	1	0	0	1	1	0	0
Unknown 9	0	0	1	1	0	0	0	0	0	0	0	0

Whatever pair strain/concerned support, the identified compounds belong to many chemical families (carboxylic acids, esters, alkenes, alkanes, aldehydes, alcohol, sulfur molecules, halogenated molecules, amino and cyclic molecules).

The analysis of emissions showed that:

- 153 VOCs were emitted from Pb16 strain, from which twenty five exclusively by it, all materials confronted (highlighted blue in the table)

- TOX+ strains Pb25 and Pb16 have qualitatively different chemical emissions, which is compromising the construction of an index. There is just one MVOC, 2-ethyl-1-Hexanol, that is not emitted by TOX- strain but by two TOX+ strains, on FWP.

Furthermore, the mutant strain (Pb19) and the wild type strain (Pb16) have, interestingly, many common emissions (84 VOCs are emitted by these two strains on the same materials, highlighted in pink), indicating more metabolic similarities between these two strains than between the two toxinogenic strains (Pb25 et Pb16).

2.3.3 Determination of global footprint for production of mycophenolic acid

Since the analysis of emitted MVOCs seem unsufficient to identify specific emission during mycotoxin synthesis, more in depth analysis of data was performed, using fragmentation spectra of emitted compounds at the CSTB (Dr S. Moularat).

From the mass spectrometry results of each of the tests achieved with the TOX+ strains Pb25 and Pb16 and TOX- strain Pb19, the strength of signal associated with each fragment ion was summed over the entire analysis period, fixed at 80 min. A matrix fragment ions/test was then constructed. These results were then analysed using a Principal Component Analysis (PCA). Results are summarized in Annex 2.

This discrimination using PCA allowed the identification the 90 ions, which can present good chemical footprint for production of MPA. These ions are specifically associated with the growth of TOX+ strains. They are essentially ions whose ratio between mass and charge is greater than 200, indicating molecules with high molecular weight, for which the used analytical method is not

optimal. This finding may explain the fact that no discriminating MVOCs was identified during the first approach.

2.3.4 Identification of MVOCs issued from the fungal growth of *A. versicolor* and *S. chartarum*

The experimental and analytical approach adopted for *P. brevicompactum* was applied on the two other species, *Aspergillus versicolor* and *Stachybotrys chartarum*, with, for each of them, two strains whose toxinogenic potential differs by a factor higher than 100. Indeed, for that two species, it was not possible to identify strains that did not produce any toxin. Therefore, for each species we have tested weakly toxinogenic strain and highly toxinogenic strain.

2.3.4.1 Study of VOCs emission for Aspergillus versicolor strains on referent material

Emissions from two strains (highly and weakly toxinogenic) of *Aspergillus versicolor* during development on fiberglass were analysed. Table 28 presents the MVOCs that are exclusively emitted either by TOX+ or TOX- strain. Common MVOCs, as well as VOCs issued from the contamination-free media were excluded from the table.

Table 28: MVOCs emitted during development of *A. versicolor* strains on FG (presence of one component is noted with « 1 » and its absence with « 0 »)

	Weakly toxinogenic strain (Av13)	**Highly toxinogenic strain (Av9)**
1-bromo-Pentane	0	1
3-Trifluoroacetoxypentadecane	0	1
1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-Naphthalene	0	1
1,2,4-Trimethoxybenzene	0	1
1,3-dimethoxy-Benzene	0	1
1-cyclopropyl-2-nitro-Benzene	0	1
2-Hexyl-1-octanol	0	1
2-Hexyn-1-ol	0	1
Heptanal	0	1
9-Octadecenoic acid-, hexyl ester	0	1
2-Heptanol	1	0
2-Octen-1-ol	1	0
4-(3,4,5-trimethoxybenzylidenamino)-1,2,4-Triazole	1	0
2-methyl-, 2-(acetyloxy)-1,1a,2,3,4,6,7,10,11,11a-decahydro-7,1-2-Butenoic acid	1	0
1,2-Benzenedicarboxylic acid, diisooctyl ester	1	0

The identified compounds belong to several chemical groups (carboxylic acids, esters, alkenes, alkanes, aldehydes, alcohol, sulfur-containing molecules, halogenated, amino and cyclic molecules).

Among the 15 MVOCs emitted by *Aspergillus versicolor*:

- 10 are specific for the highly toxinogenic strain (highlighted in blue)
- 5 are specific for weakly toxinogenic strain (highlighted in pink).

2.3.4.2 Study of VOC emission for Stachybotrys chartarum strains on referent support

The same test was performed with two strains (highly and weakly toxinogenic) of *Stachybotrys chartarum* during development on fiberglass. Table 29 presents the MVOCs exclusivelly emitted by each fungal strain, common MVOCs and those issued from the contamination-free media were excluded from the table.

Table 29: MVOCs emitted during development of *S. chartarum* strains on FG (presence of one component is noted with « 1 » and its absence with « 0 »)

	Weakly toxinogenic strain (ST81)	**Highly toxinogenic strain (ST82)**
2-Hexyl-1-octanol	0	1
2-Hexyn-1-ol	0	1
2-Heptanol	0	1
1-bromo-Pentane	1	0
3-Trifluoroacetoxypentadecane	1	0
trimethylphenoxy-Silane	1	0
1-methyl-4-(1-methylethylidene)-Cyclohexene	1	0
alpha-pinene	1	0
Carene	1	0
Toluene	1	0
1-octen-3-ol	1	0
2-Octen-1-ol	1	0
Santalol	1	0
trans-2-Ethyl-2-hexen-1-ol	1	0
3,4-dihydro-8-hydroxy-3-methyl-1H-2-Benzopyran-1-one	1	0
2-Heptanone	1	0
Cyclobutylamine	1	0
2-methyl-1-Hexen-3-yne	1	0

The identified compounds belong to different chemical groups (carboxylic acids, esters, alkenes, alkanes, aldehydes, alcohol, sulfur-containing molecules, halogenated molecules, amino and cyclic molecules).

In comparison to *A. versicolor*, in the case of *Stachybotrys chartarum,* more MVOCs were differentially produced according to toxinogenic potential of strains, among which:

- 3 are specifically issued by highly toxinogenic strain (highlighted in blue)
- 15 are specifically issued by weakly toxinogenic strain (highlighted in pink).

According to Tables 28 and 29, both toxinogenic strains of *Aspergillus versicolor* and *Stachybotrys chartarum* produced 2 common MVOCs:

- 2-hexyl-1-octanol
- 2-hexyn-1-ol.

Remaining MVOC, that could be specific to mycotoxin production, depended on considered species.

In addition to the identification of common chemical molecules, analysis of fragment ions, adopted for *Penicillium*, should distinguish other correlations. A statistical analysis of results for these 4 strains on fir, FWP, WP and FG was performed by PCA.

2.3.5 Determination of global footprint for production of mycotoxins

To search this global footprint, the method described before for MPA was used for MVOCs produced by *A. versicolor* and *S. chartarum*. Detailed results are presented in Annex 2.

41 specific ions were retained as fragment ions that may constitute a chemical fingerprint of the mycotoxinogenesis, “signing” highly toxinogenic strains. Only two ions were common to the two fingerprints (obtained for *A. versicolor* and *S. chartarum*).

2.4 Discussion and conclusion

The analysis of the emission of specific VOC by fungi during their development has been the subject of many works aiming to use these compounds as marker of fungal development (Lavine *et al.*, 2012; Polizzi *et al.*, 2012b; Ryan and Beaucham, 2013; Vishwanath *et al.*, 2011). In particular, works by Moularat and Joblin (Joblin *et al.*, 2010; Moularat *et al.*, 2008b, 2008c, 2008d) allowed determination of a Fungal Contamination Index that is used to identify a fungal contamination of indoor environments before macroscopic development or in areas where visual inspection is not possible.

Other studies were performed in order to determine MVOCs specific to certain fungal species (Claeson *et al.*, 2002; Mason *et al.*, 2010; Van Lancker *et al.*, 2008). This could be very useful to highlight the presence of species known for their allergic or toxinogenic potential and subsequent consequences on habitants' health. For instance, Mason et al. (2010) highlighted ethyl-furan as MVOC indicating development of *A.versicolor*. However, in our study, this molecule was also produced by TOX+ strain of *P. brevicompactum* on fir. In the same study dimethyldisulfide was proposed as indicator of *S. chartarum* and *A. versicolor* growth. In our study, this compound was found to be produced by all tested *P. brevicompactum* strains, on all materials. Therefore, these MVOCs don't seem specific to any species.

In another study (Gao *et al.*, 2002) 3-methyl-1-butanol; 2-methyl-1-propanol and 2-heptanone were proposed as markers of common *Aspergillus* species grown indoors. Our results revealed that first two compounds are produced by strains of *Penicillium brevicompactum* and latest one, 2-heptanone, was produced by *Stachybotrys chartarum*. Once again, these MVOCs are probably not specific to species.

Our results are also in agreement with several studies that demonstrated the influence of growing support on MVOCs emissions. For instance, *A. versicolor* emissions of MVOCs varied if grown on MEA or on wallpaper (Van Lancker *et al.*, 2008); as confirmed by Mason (2010) for same species growing on gypsum board, ceiling tile, kraft paper and strand board. Same author reported that *S. chartarum* showed important metabolite differences when grown on those 4 materials.

Since MVOCs production is highly substrate-dependent, it makes it more difficult to identify molecules that could serve as markers in all situations. As an example, Matysik et al. (2008) studied VOC profiles for *Penicillium expansum, P. chrysogenum, Aspergillus versicolor, A. fumigatus, A. niger* and *Cladosporium cladosporoides* on WP and DG18 and demonstrated that MVOCs production on agar are very different than on building material. They proposed 1,3-dimethoxybenzene for *A. versicolor* presence on wallpaper, without any relation to toxinogenic potential. In our study this MVOC was emitted exclusively by toxinogenic *A. versicolor*. Closely, 1,2-dimethoxybenzene was produced by one of TOX+ strains of *P. brevicompactum* on fir.

The aim of this study was to identify MVOCs that could be specific of toxinogenic strains, independently of the species. If differential expression of MVOCs was observed when comparing TOX+ and TOX- of each species of interest, a very few number of compounds was found to be produced by all toxinogenic strains. Moreover, if combined none MVOC was emitted by all TOX+ strains on all tested materials. This result is in agreement with the few available data.

For instance, Jelen and Grabarkiewicz-Szczęsna (2005) studied the profiles of volatile compounds produced on wheat by a toxinogenic strains of *A. ochraceus* (producing ochratoxin A) and non-

toxinogenic *Aspergillus* strains. The pattern of MVOCs produced by toxinogenic strains did not allow their differentiation from non-toxinogenic strains. In the same way, Polizzi and al. (2009) studied produced mycotoxins on building materials and emitted MVOCs in water-damaged environments. In this study no MVOC was directly related to mycotoxin production.

By contrast, Betancourt and al. (2013) highlighted a correlation between certain MVOCs (3,4-dihydro-8-hydroxy-3-methyl- (R)-1H-2-Benzopyran-1-one and 1-octen-3-ol) and toxinogenic strains of *S. chartarum.* In our study, first component was found to be emitted by weakly toxinogenic strain and not by toxinogenic. Moreover, second compound, 1-octen-3-ol, was produced by all *P. brevicompactum* strains on all materials. Therefore maybe these components can't be specific indicator for presence of toxinogenic strain of *S. chartarum*, as also sugested by Gao and Martin (2002).

In the study of Wilkins and al. (2003) trichodiene was showed to be produced in significantly larger amounts by *Stachybotrys chartarum* isolates, which produced satratoxins. However, it was difficult to use this metabolite to detect the presence of toxic isolates in buildings due to the relatively small amounts excreted. Moreover, during various experiments about VOCs emissions in the laboratory of CSTB, this compound is usually found to be emitted by several sources and therefore probably doesn't exclusively predict presence on *Stachybotrys*.

As a conclusion, the low number of MVOCs that can be specifically related to mycotoxin production by all TOX+ strains and on all materials, makes impossible the building of a toxinogenic index in a comparable way to the existing fungal contamination index.

That is why a more deepened analysis of data was done by S. Moularat at the CSTB using PCA and fragment ions analysis. Results of this approach were more encouraging. Indeed, the "fragment ions" approach has allowed the discrimination of the strains according to their toxinogenic potential for each species, but also the identification of 7 fragment ions (56, 58, 59, 71, 143, 152 and 199) that appeared correlated to a high level of mycotoxin production for the three tested fungal species. These results shall now be confirmed by testing the emission using other toxinogenic strains.

PCA results also suggest that, the identification of MVOCs that could serve as indicators of mycotoxins' synthesis in indoor environments, could require an optimization of the analytical methodology in order to allow access to molecules with higher molecular weight.

CHAPTER THREE.

Aerosolization of mycotoxins from contaminated building material

3.1 Introduction

In the first part of our work, we demonstrated that the development of some toxinogenic fungi on building materials can go with the active synthesis of quite high amounts of mycotoxins. Even if the detection of fungal presence in indoors and even more the presence of their ability to produce toxic compounds is very important in terms of human health, it does not, however, prejudge the occupants' exposure by inhalation. Indeed, the propensity for aerosolization of toxinogenic fungi and contaminated particles from supports could strongly vary as a function of the species (Afanou *et al.*, 2015; Madsen *et al.*, 2016; Pasanen *et al.*, 1991).

Within this context, the aim of this work was to characterize the possible transfer of mycotoxins from a contaminated support to the air. For that, a specific device was set up at the CSTB to be able to characterize the nature of aerosols generated under controlled airflows.

Since final objective is a practical application to living spaces, it was therefore essential to work under realistic conditions. We have chosen to test the impact of possible aeraulic solicitations on the emission of fungal particles from a contaminated material and on the associated toxic load according to the size of emitted particles.

For that, three previously chosen fungal species were used (toxinogenic strains) and since wallpaper was found to be a good support for both development and toxinogenesis of these three species, it was chosen as the reference material.

In practice, in a closed chamber, the contaminated medium was placed, on which was applied, thanks to a blowing device, aeraulic solicitations - variable in terms of intensity and duration. Those solicitations could represent the opening of a window, airflows due to heating systems or ventilations, human activities etc.

After adaptation, this assembly was then used to determine the propensity of toxinogenic strains to aerosolize, as well as the possible transfer of mycotoxins to the air.

3.2 Development of the experimental system

Before performing experiments of aerosolization of fungal particles and eventual toxins, experimental system had to be developed and calibrated. In the next paragraphs will be presented the different devices that were included in the system and the preliminary adaptation of the system itself.

3.2.1 Description of the experimental system

The developed device is able to reproduce, in the laboratory, the mechanical action of airflows between 0 and 10 m/s over contaminated medium. The general principle is shown on Figure 27. The contaminated material was placed in a closed chamber and subjected to a controlled (intensity and time) aeraulic solicitation by the blowing device. Next to this aerosolization assembly, necessary metrology was installed to provide characterization of aerosols, in size and in number. To ensure the safety of the operator, the entire assembly is disposed within a microbiological safety cabinet (Figure 27).

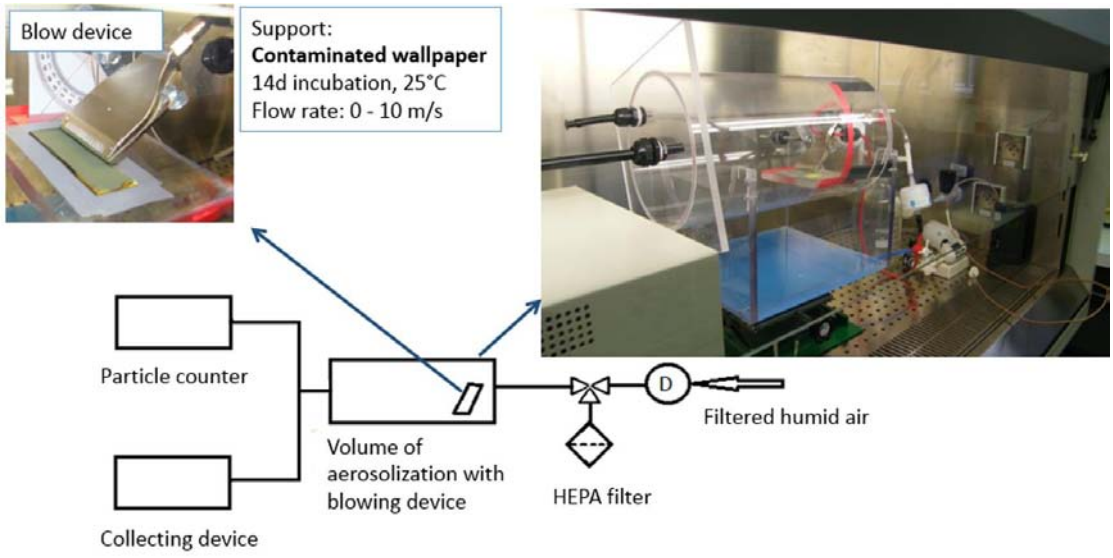


Figure 27: Schema and photography of the whole experimental assembly used for aerosolization of mycotoxins from wallpaper

Blowing device

The developed assembly presents cylindrical volume of 10.5 dm^3 equipped with a blowing device provided with filtered humidified air (50 %RH at 22 °C) to ensure the aerodynamic solicitations on contaminated medium. Experimental measurements were carried out to assure/define the

precise angle of air stream respected to the contaminated surface as well as distance between surface and blowing device.

The blowing device contains 16 semi-circular orifices of 1 mm diameter, as showed on figure below (Figure 28). It is positioned so the air stream forms an angle of 45 ° with respect to the fungal culture.

Moreover, the assembly is positioned that the distance between the bottom of the blowing nozzles and the fungal cultures was 1 cm that appeared to be the best experimental conditions as revealed by preliminary assays conducted at the CSTB (see annex 3).

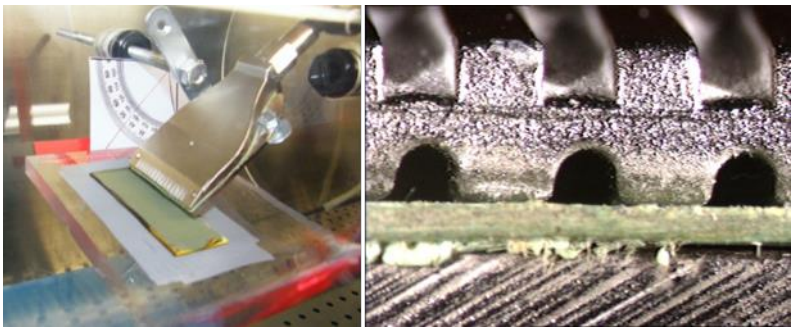


Figure 28: Blowing device and its placement

The blowing device displayed on the medium aeraulic solicitations of 5 seconds, repeated until the measured concentration of aerosolized particles decrease to 1 particle/dm^3.

The physical characterization of the produced aerosols was carried out using particle counters, as well as a multi-stage impactor, for capturing particles according to their size and aerodynamic characteristics on fiberglass media.

Aerodynamic Particle Counter (APS 3320, TSI)

The principle of this counter is to measure the time of flight of the particles between two points after acceleration of the flow carrier through an orifice. Because of their inertia, aerosols will adjust faster or slower their speed to that of air. This delay in adjustment is proportional to the relaxation time and therefore to the aerodynamic diameter of the particle. This diameter may be determined by measuring the speed of each particle just after the orifice. This speed is measured using a split laser beam forming two points when traversing the particles.

The characteristics of this device are: Aspiration flow: 5 L/min; sampled volume: 1 L/min; 52 measurement channels can detect particle sizes between 0.3 and 20 microns; maximal sampled concentration: 1000 particles/cm^3.

Laser spectrometer (Model 3340, TSI)

This counter works on the principle that the light broadcasted by a particle in an active laser cavity is in direct function of its size. The particles produce light pulses in their transit through the laser beam. These pulses are detected by two detectors and analysed by four stages of amplification in cascade coupled to the analog converters - converting digital to the size.

This optical counter allows to distinguish particle diameter between 90 nm and 7.5 µm. The sampling rate is fixed at 0.1 L/min. The time step of data acquisition can be changed by the operator, it was set to 5 seconds.

Six-Stage Viable ANDERSEN Cascade Impactor

This collecting device uses the inertia of the particles which, when pass through holes that make up the sieve, either follow flow or leave the air flow which has been accelerated. So the finest particles follow the air stream up to the next stage while the largest will be captured on the collection material (fiberglass filters) which is situated under the sieve. Therefore the collected particles are finer and finer from stage 1 to stage 6 as shown in Figure 29 (Andersen, 1958). The amount of aerosolized toxin was measured by UPLC-MS/MS from the fiberglass filters of each stage giving the toxic load of emitted particles in relation with their size.

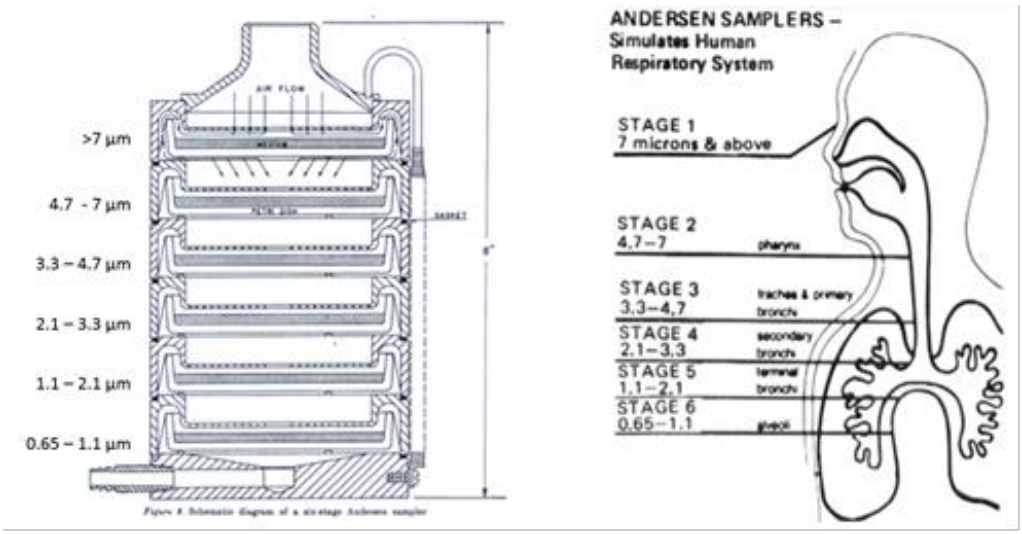


Figure 29: Illustration of the cascade impactor and domain of particle's size investigated in different stages of Andersen collector and its correlation with human respiratory system

On figure 29 is presented correlation between stages of Andersen collector and human respiratory system. It is important to note that particles found on third and fourth stage of Andersen can penetrate up to primary and secondary bronchi, furthermore particles captured on fifth and sixth stage are able, thanks to their small size, to penetrate up to terminal bronchi – bronchioles as well as to alveoli.

3.2.2 Characterization of aerosolization conditions

Firstly, we studied the propensity of fungal species to emit particles by applying increasing speeds on highly contaminated culture media. The results of these tests are detailed for *Penicillium brevicompactum*, and applied for *Aspergillus versicolor* and *Stachybotrys chartarum* as well, with any corrections in orientation if needed.

Penicillium brevicompactum

Penicillium brevicompactum cultures on PDA were subjected to increasing air speeds. These tests showed that below 0.3 m/s, the particulate emission was sporadic with a maximum of 10 particles measured in the experimental volume (10.5 dm^3). Figure 30 indicates the number of particles sampled following a blowing of 5 seconds, with solicitation speeds of 0.3, 1 and 2 m/s.

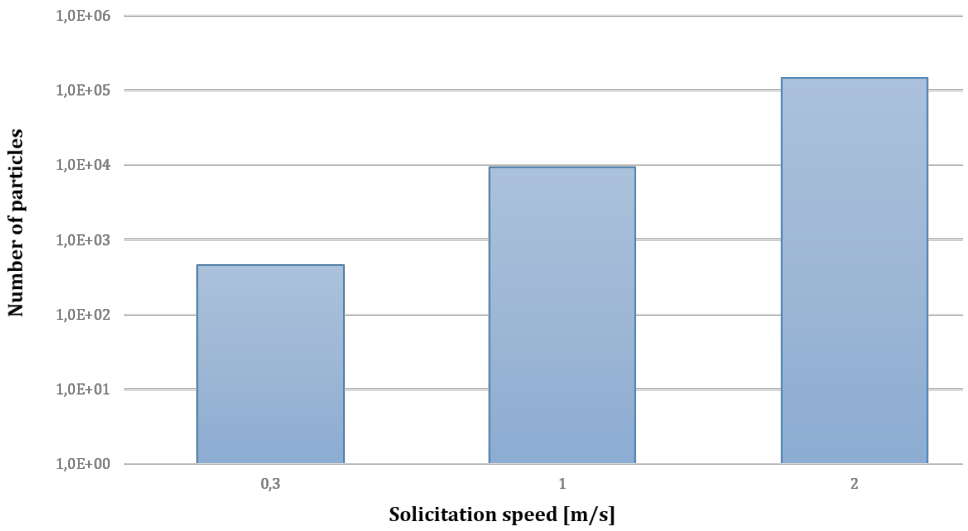


Figure 30: Number of particles aerosolized from *P. brevicompactum* culture, after applying different solicitations

These tests demonstrate, in our test conditions, that:

- An air velocity of 0.3 m/s is sufficient to significantly aerosolize particles from a culture of *Penicillium*
- As expected, the emission of particles increases as the blowing speed increases, with a number of sampled particles greather than 10^5 particles when solicitation speed is 2 m/s.

The study of the granulometric profiles of the emitted particles, for each speed applied on cultures of *Penicillium,* was also performed (Figure 31).

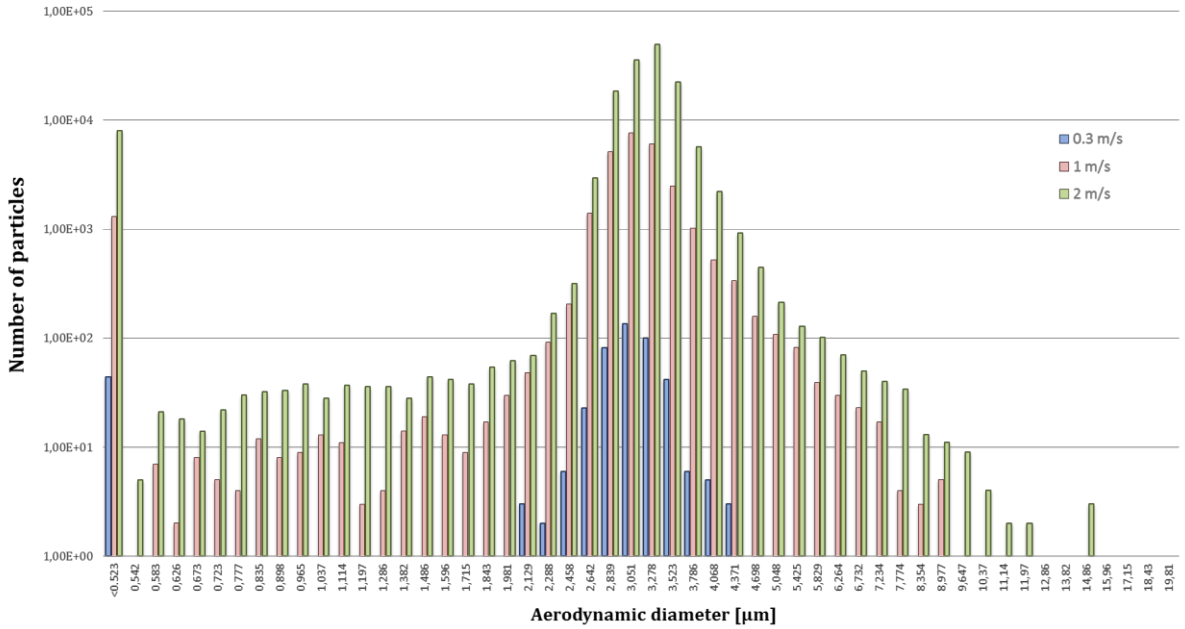


Figure 31: Granulometric profiles of *Penicillium brevicompactum* aerosolized from PDA culture, for different air solicitations

For the lowest speed, the particles are divided into two areas of size:

- The sub-micron particles of aerodynamic diameter less than 523 nm

- The super-micronic particles of aerodynamic diameter of between 2 and 4.4 µm, presumably corresponding to the spores of *Penicillium brevicompactum*.

For blowing speeds of 1 and 2 m/s, the particle size profiles are polydisperse. Thus the particles are counted in almost all of the channels up to 9 µm. Solicitation of 2 m/s allows the aerosolization of few larger particles.

Regarding the fine particles (aerodynamic diameter less than 523 nm), issued regardless of the applied air velocity, they represent 9.6, 4.8 and 5.4% of the total number of particles sampled when air speed of 0.3, 1 and 2 m/s were applied, respectively.

Three independent solicitations were performed on same culture. Figure 32 illustrates the evolution of the overall particle concentration generated during the three successive aeraulic solicitations of 1 m/s, on culture of *Penicillium brevicompactum*.

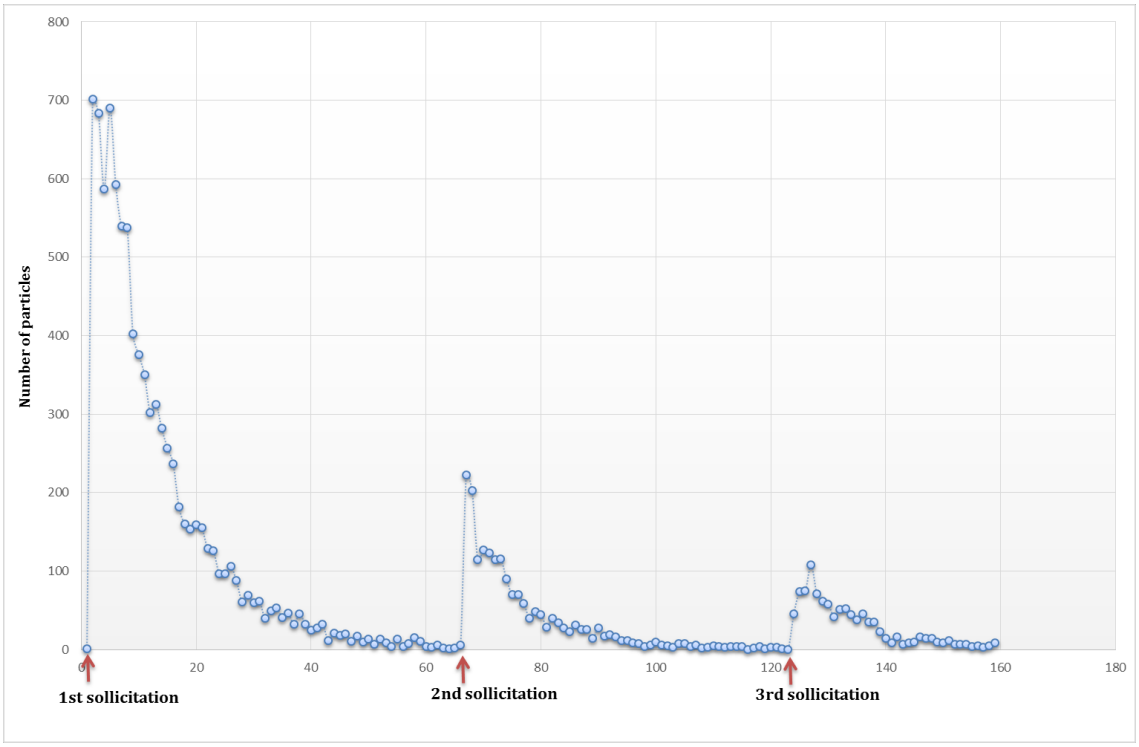


Figure 32: Evolution of the overall particle concentration generated during the three successive aeraulic solicitations of 1 m/s, on culture of *Penicillium brevicompactum*

If, as expected, the first solicitation allows the aerosolization of a large amount of particles, this procedure ensures the progressive exhaustion of culture. Thus, the particulate load emitted at the tenth solicitation of culture represents only 2% of the first, which provides itself 70% of the total particulate load.

Regarding the size profile of aerosolized particles, first applied stress produces particles which are distributed in almost all counter channels up to 9 µm with a peak at 3 microns. The following two solicitations produce particles which are divided into a number of smaller channels (20 against 37) with a constant for finer particles and those whose aerodynamic diameter is between 2.3 and 5.5 µm (Figure 33).

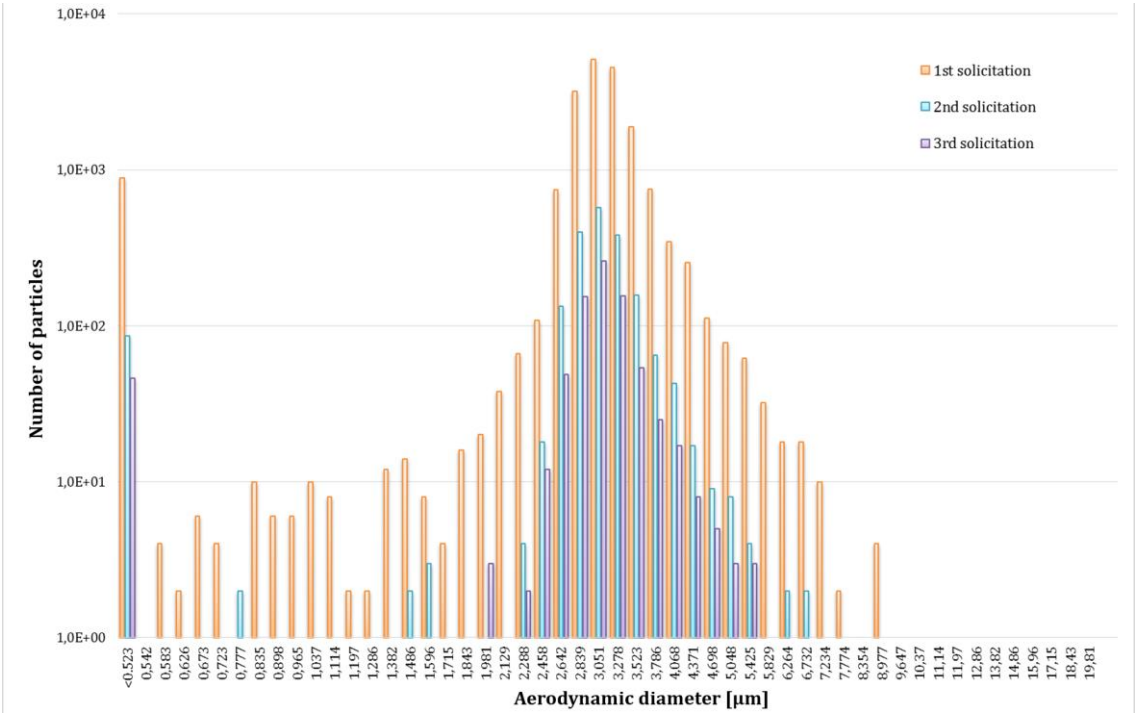


Figure 33: Granulometric profiles of aerosols generated from *Penicillum brevicompactum* during solicitations of 1 m/s

This test shows that the first aerosolized structures have different sizes, with probably aerosolization of spores and propagules of which stage of development is different.

Aspergillus versicolor

Solicitations applied on fungal culture at speeds of 0.3 and 1 m/s, carried out in accordance with the procedure described previously, did not allow the particles' emission. Therefore speed had been increased. Figure 34 reflects the evolution of the generated particle concentration by the applied solicitations.

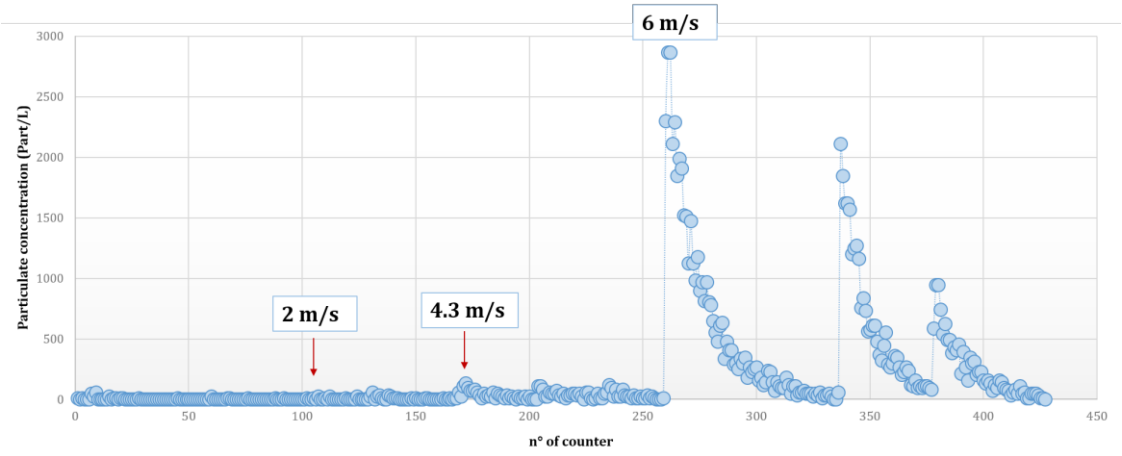


Figure 34: Evolution of the particulate concentration generated by the different air velocity applied to a culture of *Aspergillus versicolor* on PDA

The first particles were observed from speed of 2 m/s, with particulate concentrations of about twenty particles per liter. In order to set the air flow conditions that would lead to significant particle concentrations which can induce a strong exposure to toxic particles, we increased the air speed. Thus, the application of stresses generated by an air speed of 4.3 m/s enabled aerosolization of more particles (132 particles/L). Increasing the speed to 6 m/s, with a concentration of around $3*10^3$ particles/L, resulted in a massive emission of potentially toxic particles whose particle size profile is shown in Figure below. The renewal of this solicitation induced, as expected, the exhaustion of the support, and the maximum particle concentration was reduced by about 1 log between 2 solicitations. The size profile of the particles generated in these conditions is shown in Figure 35.

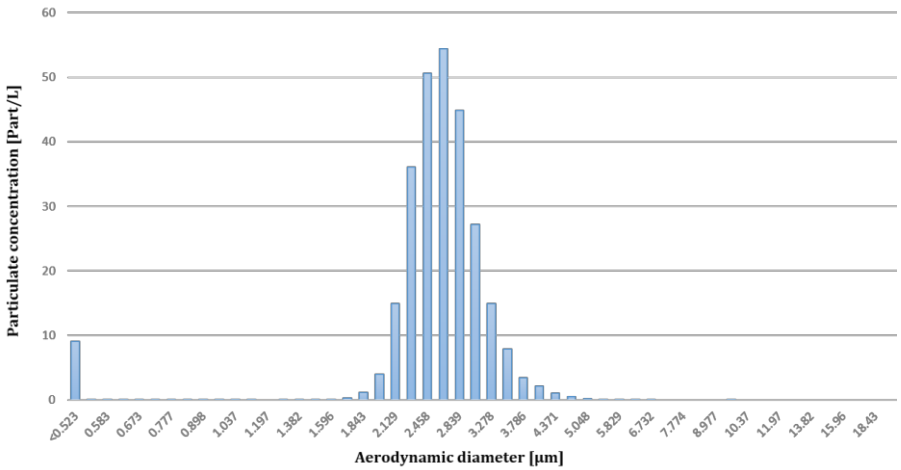


Figure 35: Granulometric profile of the fungal aerosols emitted from a culture of *Aspergillus versicolor* on PDA generated after aerodynamic solicitations of 6 m/s

If the majority of generated particles had aerodynamic diameters between 1.8 and 5 µm, with a peak at 2.6 µm, finer particles were emitted as well. Thus, 3.3% of the produced aerosol had an aerodynamic diameter less than 523 nm.

Stachybotrys chartarum

As for *Aspergillus versicolor* and *Penicillium brevicompactum*, aeraulic stresses were applied to cultures of *Stachybotrys chartarum*, incubated for 14 days on MEA.

No aeraulic solicitation allowed the particle aerosolization from the fungal culture until air speed reached 6 m/s. At this speed, with a maximum particle concentration of 120 particles/L, the propensity to aerosolization of *Stachybotrys* is weaker than for *Aspergillus versicolor* (average concentration of $3*10^3$ particles/L).

In the logic of obtaining large quantities of particles, we increased the air speed to 8.4 m/s. The evolution of the particle concentration in the test, during successive solicitations of 5 seconds, is shown in Figure 36.

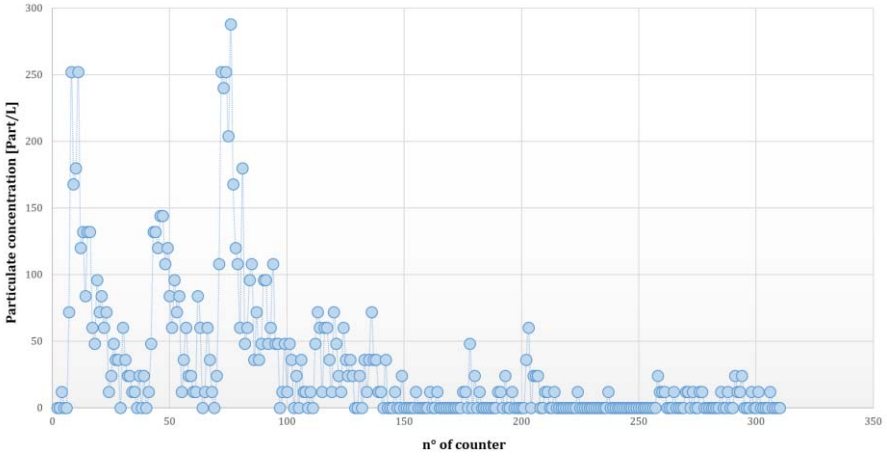


Figure 36: Evolution of the particulate concentration in successive solicitations of 8.4 m/s on a culture of *Stachybotrys chartarum*

With a maximum particle concentration of less than 300 particles/L, this test confirmed the difficulty to aerosolize a culture of *Stachybotrys chartarum*. It also highlighted the rapid exhaustion of the culture, since average particle concentration was 70 particles/L at the first solicitation and decreased to 10 at the fifth. As regards granulometric profiles, Figure 37 reflects the dispersion of aerosolized particles as different air velocities were applied to the culture.

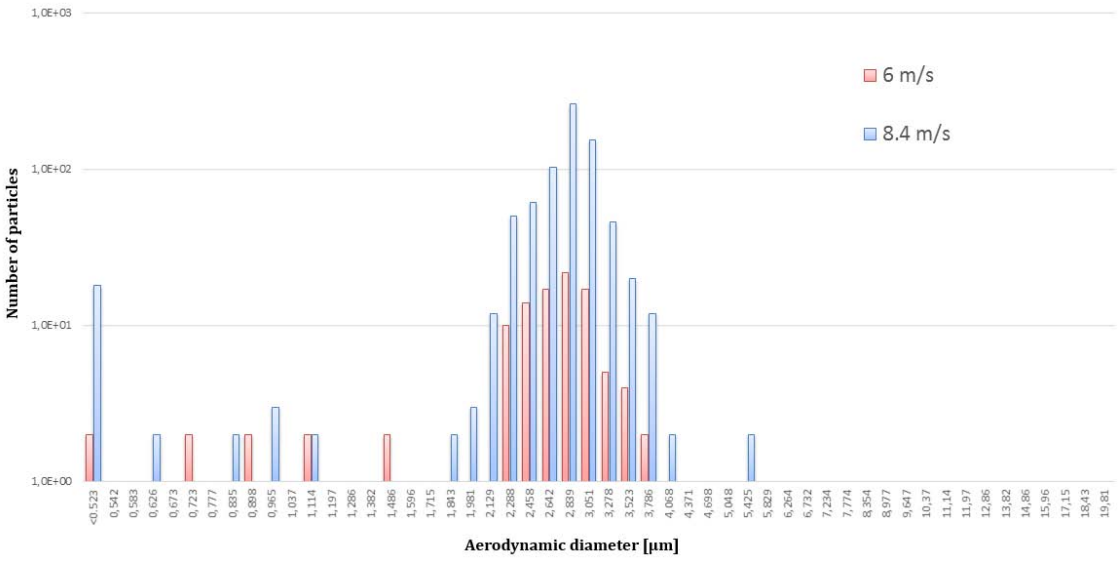


Figure 37: Profile size of the aerosol produced from a culture of *S. chartarum* on MEA according to the applied air speeds

The majority of aerosolized particles had an aerodynamic diameter between 2 and 4 µm, even if it appears that finer particles were also emitted, and on several channels (while they were mainly found in the channel <523 nm for *Aspergillus versicolor* and *Penicillium brevicompactum*).

These tests revealed that the propension to aerosolization of the three species from agar media are different according to the species:

- *Penicillium brevicompactum* appeared to be the most conducive to the particulate emission, an air speed of just 1 m/s is sufficient to achieve a concentration of $8*10^3$ particles/L
- Speed of 2 m/s and 6 m/s are necessary to allow the particle aerosolization from *Aspergillus* and *Stachybotrys* cultures, respectively; with maximum particle concentrations of $3*10^3$ and 120 particles/L, respectively.

3.3 Aerosolization of mycotoxins after development of toxinogenic fungi on wallpaper. Brankica Aleksic, Marjorie Draghi, Sebastien Ritoux, Sylviane Bailly, Marlène Lacroix, Isabelle P. Oswald, Jean-Denis Bailly, Enric Robine. *Submitted to Indoor Air*

Aerosolization of mycotoxins after development of toxinogenic fungi on wallpaper

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Abstract

Fungi are frequent indoor contaminants and some are known to be potent mycotoxin producers. The frequent isolation of toxinogenic species in indoor environments raises the question of the possible exposure of inhabitants to these toxic compounds by inhalation after aerosolization.

The study investigated the toxinogenesis of *Penicillium brevicompactum*, *Aspergillus versicolor* and *Stachybotrys chartarum* during their development on wallpaper and the possible subsequent aerosolization of produced mycotoxins from contaminated supports.

We demonstrated that mycophenolic acid, sterigmatocystin and macrocyclic trichothecenes (sum of 4 major compounds) could be produced at levels of 1.8, 112.1 and 27.8 mg/m^2, respectively on wallpaper. Part of the produced toxins could be aerosolized from support. The propensity to aerosolization differed according to the fungal species. Thus, *P. brevicompactum* was aerosolized from 0.3 m/s, when *S. chartarum* required airflow of 5.9 m/s. *A versicolor* was intermediate with aerosolization under 2 m/s airflow.

Quantification of the toxic content revealed that, for considered toxins, toxic load was mostly associated with micronic and super-micronic particles' fractions, whose sizes may correspond to spores. However, some macrocyclic trichothecenes (especially satratoxin H and verrucarin J) can be found in smaller and easily inhalable particles.

KEYWORDS

Indoor air, mycotoxins, exposure, aerosolization, wallpaper, fungi

PRACTICAL IMPLICATIONS

- Development of toxinogenic fungi in indoors may lead to mycotoxin production
- Mycotoxins can be aerosolized from contaminated supports
- Proportion of aerosolized toxin vary according to fungal species
- Mycotoxin load is mostly found on micronic particles
- Some macrocyclic trichothecenes (especially satratoxin H and verrucarin J) were found on particles smaller than spores and easily inhalable

1. INTRODUCTION

In industrialized countries, people spend 80-95 % of their time inside buildings [1]. Many agents of physical, chemical or microbiological nature can have detrimental effects for occupants, such as allergies, infections or poisoning [2-5]. Among the microbiological pollutants of indoor environments, micromycetes are ubiquitous microorganisms capable of growing on most construction and decoration materials if appropriate environmental conditions are present [6-9]. It is estimated that, in Northern Europe and North America, 20 to 40 % of buildings display macroscopically visible fungal development [10]. As an example, in France, a national campaign by the Observatory of the Quality of Indoor Air revealed that more than 610 000 homes had mouldy surfaces of more than 1 m [11].

Among the fungal contaminants commonly observed in habitats, some species are known to produce toxic secondary metabolites called mycotoxins [6, 12, 13]. For instance, *Aspergillus versicolor*, a potent producer of sterigmatocystin (STG), is one of the most frequent contaminant of indoor environments [6, 14]. *Stachybotrys chartarum* is often isolated from homes that have suffered from water damages [15-17]. This species is known to be able to produce different toxic compounds belonging to the family of macrocyclic trichothecenes (MCT) (namely satratoxins G (SG) and H (SH), roridin L2 (RL2), verrucarin J (VerJ)) [18, 19]. On the same way, *Penicillium brevicompactum* was also frequently identified as indoor contaminant [20].

These findings raise the question of the possible occupants' exposure to these toxic compounds by contact or inhalation following their aerosolization. Indeed, it has been shown that mycotoxins can be found in fungal spores [12] and could therefore subsequently be inhaled [21, 22].

To evaluate presence of these contaminants in indoor environments, some studies have measured mycotoxins on contaminated materials [23-26] or settled dust [27-29]. Thus, STG could be found in more than 20 % of analyzed samples as well as MCT on material samples from water-damaged homes [16].

However the toxin quantification from material or settled dust does not prejudge the airborne toxic load nor toxin quantities potentially inhaled by the occupants. Indeed, the relationship between contaminated surfaces, mycotoxin production and transfer to the air of these toxic substances is poorly documented. Most researches focus primarily on aerosolization of conidia or fungal fragments [30-33] without associating them with mycotoxins. Only one previous work demonstrated the possible aerosolization of MCT [34].

Within this context, the aim of this study was to quantify mycotoxin production by three frequent fungal contaminants of indoor environments that are *P. brevicompactum*, *A. versicolor*, and *S. chartarum* during their development on wallpaper, and to evaluate possible aerosolization of produced toxins according to airflow rate and size of emitted particles.

2. MATERIAL AND METHODS

2.1. Mycotoxin standards

Standards of satratoxin G (SG), satratoxin H (SH), roridin L2 (RL2) and verrucarin J (VerJ) were gracious gift from Professor J.J. Pestka (Department of Microbiology and Molecular Genetics, Michigan State University, USA). Sterigmatocystin (STG), mycophenolic acid (MPA), verrucarin A (VerA), o-methylsterigmatocystin (o-mSTG) and mycophenolic acid-d_3 (MPA-d_3) were purchased from Sigma (Saint-Quentin Fallavier, France). Standards were dissolved in methanol (MeOH) to obtain stock solutions that were stored at -20 °C.

2.2. Solvents and reagents

All reagents and solvents were purchased from ICS (Lapeyrouse-Fossat, France) and were analytical grade. Acetonitrile (AcN) used for mobile phase was LC/MS grade and purchased from Thermo Fischer Scientific (Illkirch, France) and water was obtained from an ultrapure water (18.2 MΩ) system (Elga Labwater Veolia, Anthony, France).

Wallpaper (WP) (Papier Peint BLAN BLA 0 INSP, Leroy Merlin) was purchased in a specialized store. It was cut into 2×5 cm pieces and then sterilized (121 °C, 20 min) before use as described by Górny et al. [32] and Peitzsch et al. [35].

2.3. Fungal strains

P. brevicompactum IBT 23078 strain was a gracious gift from Professor J.B. Nielsen (Technical University of Denmark, Lyngby, Denmark), *A. versicolor* NCPT 54 was a gift from Dr O. Puel (INRA, Toulouse, France), and *S. chartarum* 82 (ST82) strain was isolated from wheat straw. These three strains were selected for their ability to produce mycophenolic acid, sterigmatocystin and macrocyclic trichothecenes, respectively. All strains were maintained in the laboratory on malt extract agar (MEA, Biokar, France) at 4 °C and were regularly checked for viability by culturing on MEA.

2.4. Growth and toxinogenesis of fungi on wallpaper

The fungal strains were grown on potato dextrose agar (PDA, Biokar, France) for 14 days at 25 °C to obtain highly sporulating cultures. Spore suspensions were prepared from these fungal cultures by adding 10 mL of Tween 80 (0.05 %) to the Petri dish. The number of spores was quantified by direct counting on a Malassez cell. Spore suspensions were then diluted to obtain the required concentration. Contamination was achieved by applying 100 µL of those suspensions (10^7 spores/mL) on sterile wallpaper.

Contaminated wallpaper pieces (2 x 5 cm) were then incubated for 10 days at 25 °C in darkness. After incubation, fungal development was assessed by examining samples under stereomicroscope (magnification from 12 to 120) (Olympus SZX9) and under Scanning Electron Microscopy (SEM) (Jeol JSM 5600LV) (magnification from 40 to 30 000). Both hyphae development (density and colonized surface) and density of sporulated conidial heads on the whole sample surface (10 cm^2) have been evaluated.

Some samples were used for mycotoxin determination whereas others, incubated in the same conditions, were used for aerosolization as described below.

In order to measure mycotoxin base line at the beginning of experiment due to inoculum on materials (= T0 value), control material samples were frozen immediately after spores' deposit, without incubation to avoid fungal development, and analysed as other samples.

All analysis were done in triplicate and three independent experiments were carried out.

2.5. Aerosolization of mycotoxins from wallpaper

To evaluate aerosolization of particles and toxins from wallpaper, a specific experimental device capable of reproducing controlled aerodynamic solicitations from 0 to 10 m/s over contaminated surfaces was developed. The principle of this device is shown on Figure 1. To ensure the safety of the operator, the entire assembly is disposed within a microbiological safety cabinet.

The developed assembly presents cylindrical volume of 10.5 dm^3 equipped with a blowing device provided with filtered humidified air (50 % RH at 22 °C) to ensure the aerodynamic solicitations on contaminated material. The blowing device placed in the closed space consisted of 16 semicircular holes of 1 mm diameter. It is placed so that the air stream forms an angle of 45 ° with respect to the contaminated material. Moreover, the assembly is leveled so the distance between the bottom of the blowing nozzles and the fungal cultures was 1 cm (Figure 1).

Air solicitations (or air jets) of 5 seconds each were performed and repeated until the measured concentration of particles decrease to 1 particle/dm^3.

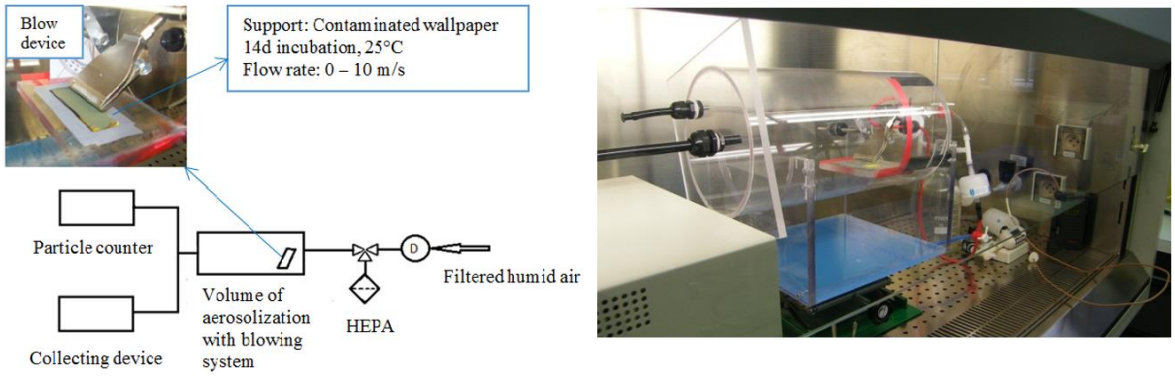


Figure 1. Experimental assembly used for aerosolization of mycotoxins from wallpaper

The physical characterization of the produced aerosols was carried out using an optical counter (Model 3340, TSI). An Andersen-like multi-stage impactor (ACFM, Graseby), was used for capturing particles according to 6 ranges of size and aerodynamic characteristics (Table 1). Each stage of the impactor was equipped with fiberglass disk to collect particles and allow mycotoxin determination as described below.

Table 1. Domain of particle's size collected on the 6 stages of Andersen collector

Stage	Size range [µm]	Corresponding penetration in respiratory tract
1	>7	Nose
2	4.7 - 7	Pharynx
3	3.3 - 4.7	Trachea and primary bronchi
4	2.1 - 3.3	Secondary bronchi
5	1.1 - 2.1	Terminal bronchi
6	0.65 - 1.1	Alveoli

2.6. Mycotoxin determination

Extraction procedure

MPA, STG and four MCT (SG, SH, VerJ and RL2) were extracted from samples (wallpaper and fiberglass disks) by gentle mechanical agitation on an agitation table (Reciprocating Shaker, IKA HS501 Digital, Grosseron, France) in chloroform:methanol (2:1). For each toxin, an internal standard was added before starting extraction procedure at known concentration in order to take into account possible matrix effect and losses during extraction procedure. These internal standards were mycophenolic acid-d_3 for MPA, o-methyl

sterigmatocystin for STG and verrucarin A for MCT [36]. These compounds were chosen for their structural similarity with toxins of interest, their availability and the fact that they are not produced by used toxigenic strains.

After 4 hours, extracts were centrifuged for 5 min at 3500 rpm and filtered through a phase separator filter (Whatman 1 PS). Filtered extracts were evaporated to dryness and suspended in 1mL of methanol.

UPLC - MS/MS analysis of mycotoxin

Quantification of mycotoxins of interest was performed as previously described by Aleksic et al. [36], using an Acquity ultra performance liquid chromatography (UPLC) system coupled to a Xevo triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The desolvation temperature and nitrogen flow rate were set at 650°C and 800 L/h, respectively. Argon was used as the collision gas at a flow rate of 0.12 mL/min.

In brief, mycotoxins (5 µL of samples) were eluted on an Acquity BEH C18 column (2.1 x 100 mm; 1.7 µm; Waters) with an AcN/H_2O gradient (*t*(0-0.5 min): 10 % AcN; *t*(0.5-4 min): 90 % AcN) at a flow rate of 0.35 mL/min. These chromatographic conditions allowed the separation of the mycotoxins within 5 minutes with a relative standard deviation on the retention times of less than 1 % for each analyte. Quantification was carried out by Multiple Reaction Monitoring (MRM) mode in positive electrospray ionization (ESI+). MRM transitions, cone voltage and collision energies used for the different toxins are listed in Table 2. Chromatographic data were monitored by Masslynx 4.1 software (Waters, Milford, MA, USA).

Table 2. MRM transitions, cone voltages and collision energies used for mycotoxins detection

Toxin	Molecular weight	Parent ions	MRM fragments	Cone voltage (V)	Collision energy (eV)
MPA	320	321	159	16	36
		321	207	16	22
STG	324	325	115	40	64
		325	310	40	24
RL2	530	553	249	42	16
		553	305	42	26
SG	544	545	81	20	34
		545	231	20	16
SH	528	529	249	24	16
		551	303	48	28
VerJ	484	523	151	46	32
		523	293	46	34

The lowest limit of detection (LOD) was defined as the lowest concentration that could be reliably differentiated from background noise (signal to noise > 3). LOD was determined from 3 injections of the different mycotoxins standards at the lowest concentration that could be detected with a signal to noise ≥ 3. The limit of quantification (LOQ) was determined and validated for the lowest concentration of the calibration curve chosen for its relevance to mycotoxin investigation on wallpaper.

The lowest concentrations of calibration standard that could be detected and differentiated from the baseline with S/N>3 were 1 ng/mL for MPA and STG, 0.2 ng/mL for RL2, 5 ng/mL for VerJ, 10 ng/mL for SH and SG; these values were considered as the LODs of each mycotoxin. The LOQs were set at 10 ng/mL for MPA, STG, RL2 and VerJ, and 100 ng/mL for SG and SH.

2.7. Statistical analysis

Data were analysed with GraphPad Prism statistical software version v4.0. Student's t-test was used to analyse the differences between initial concentration of toxins on materials (T_0) and toxins' concentrations after incubation period. The differences were considered to be statistically significant when p-value was lower than 0.05.

3. RESULTS

3.1. Development and toxinogenesis of fungal strains on wallpaper

After 10 days of incubation at 25 °C, the three tested fungal species grew and sporulated on wallpaper. Nevertheless some differences could be observed between species (Figure 3).

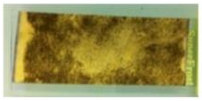
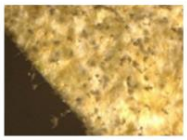
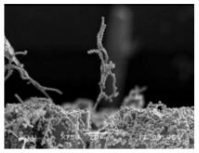
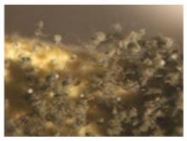
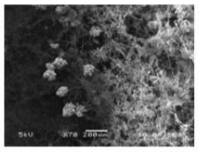
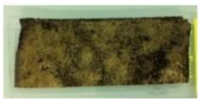
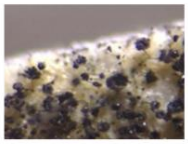
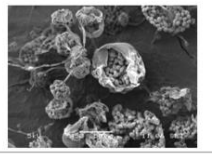
Species	Mycelial development	Sporulation	Macroscopic aspect of contaminated wallpaper		Microscopic aspect of contaminated wallpaper
			Observation with naked eye	Observation under stereomicroscope	Observation by SEM
Penicillium brevicompactum	+++	+++			
Aspergillus versicolor	+++	++++			
Stachybotrys chartarum	++++	++++			

Figure 3. Evaluation of mycelial development and sporulation by macroscopic, under stereomicroscope and SEM observations of wallpaper contaminated with different species
Number of + indicates the relative importance of mycelial development and sporulation

P. brevicompactum colonized almost all the piece of wallpaper, with a loosened mycelium. Numerous large and compact *penicilli* were observed under stereomicroscope and, at microscopic level, long terverticillate conidiophores with adjoined branches sometimes bent away from the axis. Inflated *metulae* bore divergent phialides' clusters and very long, dry and disordered chains of spores.

As for *P.brevicompactum, A. versicolor* development covered almost all the sample but with a heterogeneous density. Stereomicroscope examination revealed a dense field of aerial and closely interwoven hyphae bearing conidiophores. Classical microscopic features were observed: radiate and biseriate conidial heads, closely packed *metulae* and phialides bearing short chains of spores.

S. chartarum displayed an intense and regular development with abundant hyphae colonizing the whole sample's surface with many sporulated heads. Conidiophores were simple or branched. Phialides, organized in clusters, bore black ellipsoidal conidia covered with dry slime.

Mycotoxin measurement revealed that all three species produced mycotoxin(s) during their development on wallpaper (Table 3). STG was produced in larger quantities with more than 110 mg/m^2. The four analysed MCT were also found. SH was the most abundant one, followed by SG and RL2. Only mild amounts of VerJ were measured after development of *S. chartarum* ST82 strain on wallpaper.

Table 3. Toxin(s) production on wallpaper contaminated by three different toxigenic fungal strains

Species	Toxin	Concentration after 10 days [mg/m]	Initial concentration (T0) [mg/m^2]	P value
P. brevicompactum	MPA	1.8 ± 0.86	0.21 ± 0.09	<0.0001
A.versicolor	STG	112.1 ± 30.08	0.12 ± 0.004	0.0008
S.chartarum	Total MCT	27.8	1.7	
	RL2	5.9 ± 1.04	0.3 ± 0.01	<0.0001
	VerJ	0.6 ± 0.18	0.08 ± 0.02	<0.0001
	SG	7.1 ± 3.92	ND	0.0143
	SH	14.2 ± 6.97	1.3 ± 0.33	0.0018

ND: not detected

3.2. Characterization of bioaerosols

To define the conditions leading to particle aerosolization from support as a function of the fungal species, contaminated wallpaper samples were submitted to different aeraulic solicitations ranging from 0.1 to 10 m/s, which can correspond to movement of people in a room, speed of air in ceiling diffusers to slamming doors or air drafts when opening windows. It appeared that for *P. brevicompactum,* airflow of 0.3 m/s was sufficient to aerosolize some particles from support. However, a solicitation of 2 m/s strongly increased the number of particles emitted from wallpaper (Figure 4). In that case, the overall particles production was around 5.6x10^4 counted particles that were distributed mainly in:

- Fine aerosols with optical diameter about 100 nm (maximal concentration of 10^3 particles/L)
- Particles with optical diameter between 2 and 8 µm (maximal concentration of 2.3x10^3 particles/L).

For *A. versicolor*, a solicitation of 2 m/s also allowed the aerosolization of 1.5x10^4 counted particles that were mostly made of fine aerosols with optical diameter about 100 nm (maximal

concentration of $1.2x10^3$ particles/L) and few particles with optical diameter between 2 and 8 µm (maximal concentration of 700 particles/L).

By contrast, for *S. chartarum*, air speed of almost 6 m/s was needed for sufficient aerosolization of particles (Figure 4). In that case, the overall production of $7x10^3$ counted particles presented a poly-dispersed distribution of particle size. The production of sub-micronic particles represented 77.5 % of the total particle emissions.

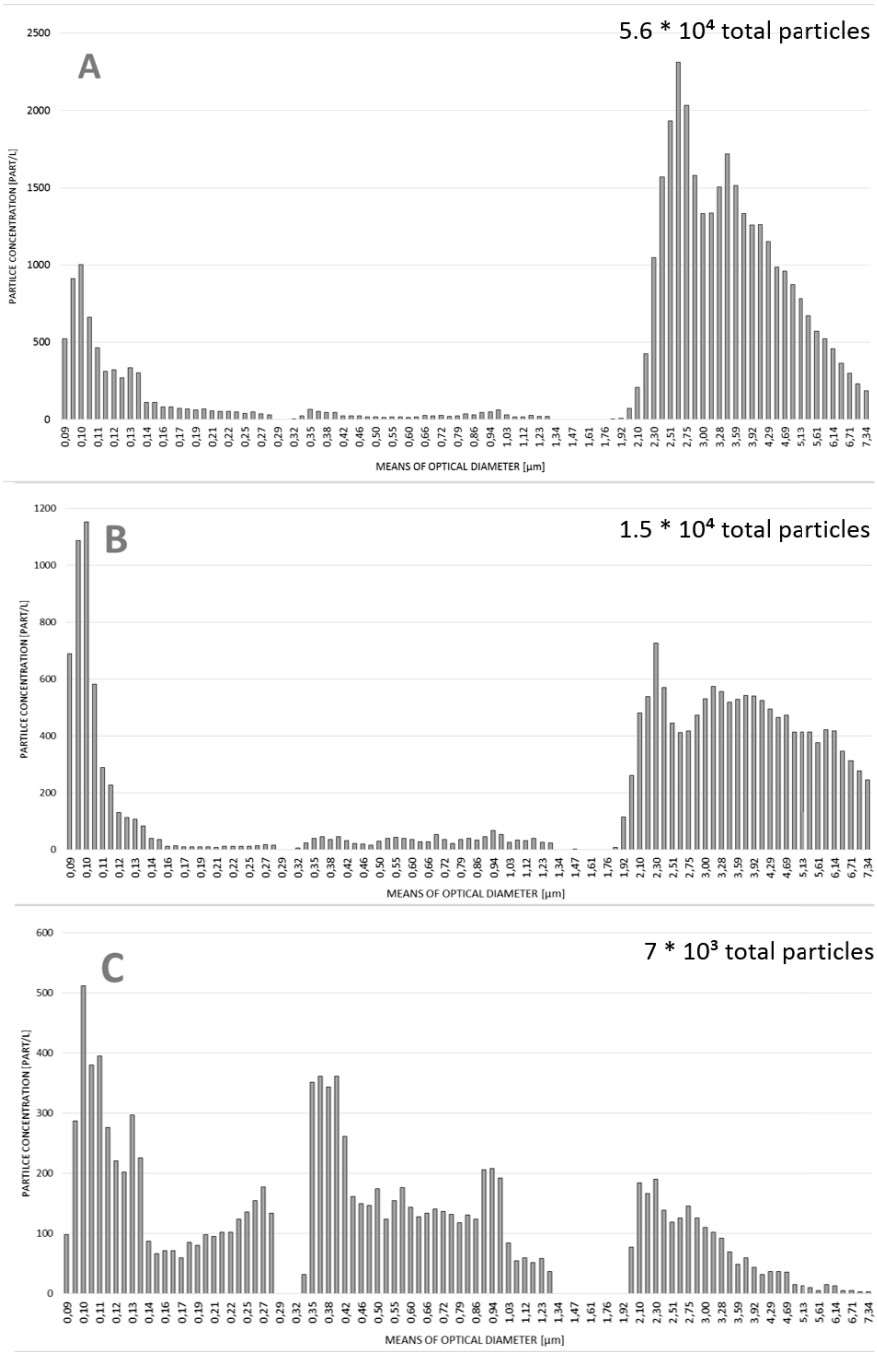


Figure 4. Granulometric profiles of aerosols from *P. brevicompactum* (A), *A. versicolor* (B) and *S. chartarum* (C) following aeraulic solicitations on contaminated wallpaper with airflows of 2, 2 and 6 m/s, respectively

3.3 Aerosolization of mycotoxins

To evaluate the aerosolization of mycotoxin, airflows of 2, 2 and 6 m/s were used for *P. brevicompactum, A. versicolor* and *S. chartarum*, respectively. The global mycotoxin loads of aerosols from the three fungal species are reported in Table 4.

Table 4. Global mycotoxin content of aerosols generated from wallpaper

Toxin(s)	Air flow [m/s]	Aerosolized toxin [ng]	% of aerosolized toxin
MPA	2	271	15
STG	2	179	0.2
Total MCT	6	1260	4.5
RL2		64	1.1
VerJ		80	13.3
SG		102	1.4
SH		1014	7.1

All analysed toxins were aerosolized from material but it appeared that the percentage of aerosolized toxin strongly differed. MPA was the most aerosolized compound with 15 % of the initial contamination transferred to air after solicitation. By contrast, the percentage of aerosolized STG was only 0.2 %. However, since it was also the most produced toxin, total quantity of aerosolized toxin reached almost 180 ng.

The proportion of total aerosolized MCT was 4.5 %. It has to be noted that *S. chartarum* required a higher air speed than the two other species to be aerosolized, and the total quantity of aerosolized toxins was the most important for that species.

Among the 4 analysed trichothecenes, VerJ was the most aerosolized with 13.3 % of the initial toxic load, followed by SH, SG and RL2. However, when considering the quantities that were transferred from support to air, SH appeared predominant, representing almost 80 % of the overall toxic load.

In order to analyse the repartition of mycotoxins as a function of particle sizes and subsequent risk of inhalation, the mycotoxin loads of each domain size of emitted aerosols were quantified and results are presented in Table 5.

Table 5. Quantification of mycotoxins in the different stages of the Andersen collector

Stage	Size range [µm]	Quantity of aerosolized toxin [ng]						
		MPA	**STG**	**MCT (Total)**	RL2	VerJ	SG	SH
1	>7	20.5	74.7	380.5	15.5	ND	55.3	309.7
2	4.7 – 7	79.6	49.8	58.7	27.6	ND	31.1	ND
3	3.3 – 4.7	138.7	45.2	522.1	8.4	10.8	15.7	487.2
4	2.1 – 3.3	26.5	9.2	4.4	4.4	ND	ND	ND
5	1.1 – 2.1	5.8	ND	226.3	7.2	59.8	ND	159.3
6	0.65 – 1.1	ND	ND	68.7	1.4	9.4	ND	57.9

ND: not detected

MPA was quantifiable on 5 of the 6 considered granulometric ranges, the maximum (about 140 ng) being associated with the particles collected on the third stage of the impactor, with a granulometric domain of 3.3 to 4.7 µm. For STG, similar toxic profile of aerosol was observed, since no toxin was found on stages corresponding to the particles with size below 2.1 µm and total mycotoxin load was associated with bigger particles.

Macrocyclic trichothecenes produced by *S. chartarum* and aerosolized from wallpaper were detected in all stages of Andersen collector, even on the stages 5 and 6 that correspond to sub-micronic particles. Nevertheless, 90 % of the total toxic load (1129 ng) was found on stages 1, 3 and 5.

The four analysed macrocyclic trichothecenes were differently distributed within Andersen collector's stages. RL2 was found on all stages. SG was exclusively found on stages 1 to 3, SH on stages 1, 3, 5 and 6. VerJ was found on stages 3, 5 and 6 with 86 % of the total toxic load being associated with these two later stages whereas no toxin was measured on stages 1 and 2. Stage 3 that corresponds to particles ranging from 3.3 to 4.7 µm, was the most contaminated with 41 % of the total MCT load. It was also the only one containing the four tested macrocyclic trichothecenes, and moreover it contained MPA and STG.

4. DISCUSSION

The presence of fungi as indoor contaminants may have several detrimental effects for inhabitants. In particular, a risk of respiratory symptoms such as rhinitis, pneumonitis, and

exacerbation of asthma has been demonstrated [18, 37]. These evidences led to the setup of air quality guidelines on dampness and mould in indoor environments [38].

The possible implication of mycotoxins in mouldy homes inhabitants' troubles is also suspected. These compounds are low molecular weight, toxic secondary metabolites, produced by some fungi during their development. The possible exposure to those toxic metabolites by inhalation emerged in the late 90's, when macrocyclic trichothecenes produced by *S. chartarum* were implicated in the appearance of pulmonary haemorrhages in infants in the USA [39]. More recently, these mycotoxins were also suspected to play a role in the sick building syndrome [40, 41].

Data on the direct relationship between mycotoxin production on materials and their transfer to air are missing and therefore do not allow precise risk assessment.

The present study evaluated the ability of different mycotoxins produced by frequent indoor fungal contaminants, namely *A. versicolor, P. brevicompactum* and *S. chartarum*, to be aerosolized from mouldy wallpaper.

Firstly, we investigated the ability of these three toxinogenic species to grow and produce toxic compounds on wallpaper. This frequently used material for indoor decoration allowed both mycelium development and sporulation. This is in agreement with surveys reporting a frequent contamination of such materials by moulds, particularly in case of moistening following water damage [17, 24, 42, 43]. Of note, for *P. brevicompactum* some morphological features were peculiar when this species grown on wallpaper compared to agar culture media. Indeed, usual aspect of *P. brevicompactum* colony on agar medium is dominated by a dense felt of large and compact conidiophores and a velutinous aspect of the thallus with only few trailing [44]. On wallpaper, colony displayed more abundant aerial mycelium with conidiophores borne by aerial hyphae. Such a structure may have an important role in facilitating aerosolization of fungal structures.

Wallpaper also allowed mycotoxin production by the tested species. Concentrations as high as 112 mg/m^2, 14 mg/m^2 and 7 mg/m^2 were found for STG, SH and SG respectively. These findings are in agreement with previous studies about production on wallpaper of SG and SH by Gottschalk et al. [17] and STG by Polizzi et al. [24].

The investigation of the aerosolization of mycotoxins produced on wallpaper firstly showed that particle emission strongly differed from one species to another, possibly related to mycelium organization and conidial structures.

Both *A. versicolor* and *P. brevicompactum* are fungal species characterized by the presence of small and light spores organized in chains at the extremity of phialides [45]. For

these two species, airs flows of 2 m/s, which matches the minimal air speed pulsed by ceiling diffusers in tertiary buildings, allowed the aerosolization of numerous particles from wallpaper. These particles were distributed in two main categories: one made with very small particles lower than 0.15 µm and the second with particles ranging from 2 to 6 µm. This second group may correspond to spores, groups of spores or mycelium debris [44, 45], in agreement with previous data on aerosolization of these fungal species [46-48]. One can note that for *P. brevicompactum*, total number of particles emitted from support was higher than for *A. versicolor*. This is in relation with the disposition of spores on mycelium structures. In *P. brevicompactum*, long chains of spores are borne by aerial conidiophores and may easily be aerosolized. For *A. versicolor*, spores' chains are shorter and located on tight and compact phialides, making them mildly more difficult to hook from material.

For *S. chartarum*, a poly-dispersed particle cloud was observed. There was an important cluster made of particles ranging from 0.4 to 1 µm that is smaller than spores. It could correspond to micro-fungal particles, debris of wallpaper released from support due to cellulolytic activity of *S. chartarum* or exudate droplets from culture [49]. Such finding is important since these small particles could easily penetrate deeply in human respiratory tract in case of inhalation.

All tested mycotoxins were found in aerosols generated from mouldy wallpaper and the proportion transferred to air varied with fungal species. MPA was the most aerosolized with 15 % of the produced toxin. This is relevant with the higher facility of *P. brevicompactum* to release its particles from support compared to other species. By contrast, the proportion of STG aerosolized was low (0.2 %). Since *A. versicolor* emitted numerous particles from support, this suggests that STG could be located in fungal parts that are strongly adherent to the support and probably mainly in mycelium [8, 10]. However, since STG was also the major toxin produced, the total quantity aerosolized was comparable to MPA.

For MCT, even if the required solicitation for aerosolization was higher, it has to be highlighted that the four analyzed toxins were aerosolized and total aerosolized toxic load was 5 times higher than that of other toxins.

The analysis of the toxin distribution according to the aerosol profile and size of emitted particles also brought some important information. For MPA and STG, maximal toxic load was found on Andersen collector's stages that can correspond to the size of spores, groups of spores or mycelium debris (stages 1 to 3). Low proportion of MPA was also found in stage collecting particles lower than spores (stage 5). It could also be related to the excretion of part of the toxin

in exudate droplets as previously demonstrated for other *Penicillia* [50]. The excreted toxin could be then adsorbed on small particles of dust.

The distribution of MCT was different. Toxins were found in all stages of Andersen collector, even those collecting particles smaller than spores. This result is in agreement with a study by Brasel et al. [34]. As for *P. brevicompactum*, it could be the result of the excretion of MCT by fungus in droplets outside the mycelium [49] and their adsorption on dust particles or wallpaper debris generated by cellulolytic activity of *S. chartarum*.

Of note, the analyzed MCT differed regarding their repartition in the various particles' sizes. On stages 1 and 2, corresponding to spores and mycelium pieces, RL2, SG and SH were detected, but not VerJ. The four compounds were present in stage 3, which may correspond to small (or young) spores. In stages 5 and 6, collecting particles smaller than spores, no SG was found whereas 86 % of VerJ was detected. Taken altogether, these results suggest that the different MCT analyzed in this study are differently distributed/excreted within fungal structures.

Further studies are required to characterize the distribution of macrocyclic trichothecenes in *S. chartarum* mycelium. It would help better understanding the biosynthetic pathway and processing of these compounds in fungal cells.

All these results on mycotoxin aerosolization according to particle size bring important insight for risk assessment and possible subsequent toxicity after inhalation.

Although no clear dose-effect relationship has been established for these mycotoxins in case of inhalation, it has been demonstrated that intranasal exposure could be highly toxic. For instance, Carey et al. [51] showed that exposure to 5 µg SG for 4 days led to widespread apoptosis of olfactory sensory neurons and to epithelial and olfactory nerve atrophy as well as acute neutrophilic rhinitis in Rhesus monkey.

5. CONCLUSION

This study demonstrated that, during their development on wallpaper, *P. brevicompactum*, *A. versicolor* and *S. chartarum* that are frequent indoor contaminants produce mycotoxins. These toxins can subsequently be aerosolized, at least partly, from mouldy material. This transfer to air requires aeraulic solicitations that can be encountered in building since

correspond to movement of people in a room (0.2 m/s), air speed in ceiling diffusers (2 m/s), slamming door or air drafts from opening the window (6 m/s).

Most part of the aerosolized toxic load is found in particles whose size corresponds to spores or mycelium fragments. However, for MPA and mainly MCT, toxins were also found on particles smaller than spores, that could be easily inhaled by inhabitants and deeply penetrate into respiratory tract. It seems important to take these data in consideration for risk assessment related to fungal contamination of indoor environment and precise the possible toxicity associated to inhalation of these toxins.

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3.4 Conclusion

In this section, we studied aerosolization of three species of interest, *Penicillium brevicompactum, Aspergillus versicolor* and *Stachybotrys chartarum* after development on wallpaper. Results showed that, as expected, their different mycelium and conidial structures influenced behaviors.

We determined profiles of aerosolized particles from contaminated WP in terms of particle size, number and toxic load. *P. brevicompactum* revealed to be the easiest one to aerosolize, where air speed of just 0.3 m/s was sufficient to aerosolize important quantity of particles. Concerning related toxic load, more than 15% of produced MPA was found in the air, highlighting not just easy aerosolization of *P. brevicompactum* particles but its toxin as well.

A. versicolor needed air speed of 2 m/s for significant aerosolization of fungal fragments, and interestingly just one small part of STG (0.2 % of total STG produced) was transferred to the air under tested conditions.

By contrast, *S. chartarum* was much more difficult to aerosolize. For this species, airflow of 6 m/s was required to release particle from contaminated wallpaper. However, this air speed is easily reachable in indoor environments and furthermore, aerosolized toxic load revealed to be more than 4% of total MCT produced on wallpaper.

Analysis of aerodynamic profiles of generated aerosols was very important, since it indicated that when aerosolized, these three fungal species released numerous sub-micronic fragments, particles of very small size able to penetrate deeply into respiratory tract, more precisely up to terminal bronchi – bronchioles and even to alveoli.
In terms of risk evaluation of fungal contamination in indoors may have on health of occupants, these information are crucial. Determination of the smallest particles as well as their associated toxic load gives first information about eventual penetration of different toxins deep into respiratory tract.

Further examinations are needed in order to determine precise impact that those compounds may have. First approach will be to evaluate cytotoxicity of those components on human lung cells, to roughly simulate exposure via inhalation.

CHAPTER FOUR.

Toxicity of sterigmatocystin, mycophenolic acid and macrocyclic trichothecenes on human pulmonary cells

4.1 Introduction

We showed that the development of toxinogenic fungal strains on building material could lead to the accumulation of several mycotoxins, at quite high levels. Moreover, we also showed that mycotoxins could also be aerosolized from contaminated materials. All mycotoxins of interest were able to be partly transferred to indoor air in case of aeraulic solicitation consistent with commonly found airflows in indoor environments (opening the window, heating, human activities, etc.) and a toxic load could be observed in particles whose size is compatible with deep penetration in aerial tract.

The question of the possible impact of exposure to those toxic metabolites by other routes than ingestion, especially by inhalation, raised in the late 90's, when macrocyclic trichothecenes produced by *Stachybotrys chartarum* were suspected to be responsible for pulmonary hemorrhages in infants in the USA (Dearborn *et al.*, 1999). Moreover, it was shown that toxicity of mycotoxins after inhalation can be more important than after ingestion (Creasia *et al.*, 1987; Van Vleet *et al.*, 2002).

In order to perform an accurate risk assessment for these biological (moulds) and chemical (mycotoxins) contaminants in indoor environments and to evaluate the significance of aerosolized toxin quantities, the aim of this work was to evaluate the cytotoxicity of studied mycotoxins (sterigmatocystin, mycophenolic acid, verrucarin A and mixture of macrocyclic trichothecenes) on human lung cells A549 as a classical model of human pulmonary epithelium.

Data about toxicity of these mycotoxins on pulmonary cells are very limited and only one study by Bünger et al. (2004) was available on toxicity of STG and MPA on A549 cell line. It demonstrated that sterigmatocystin was 80-folds more toxic on A549 lung cell line compared to Hep-G2 liver cells, indicating a specific susceptibility of pulmonary cells to this mycotoxin. Moreover, sterigmatocystin appeared more cytotoxic than aflatoxin B1 (Palanee *et al.*, 2000) on A549 cell lines.

There are no data about cytotoxicity of MCT produced by *Stachybotrys chartarum* on lung cells. Only one work reported the toxicity of VerA on A 549 cell line (Yan *et al.*, 2014). However, Carey et al. (2012) demonstrated that intranasal exposure of Rhesus monkeys to 5 µg SG for 4 days led to widespread apoptosis of olfactory sensory neurons and to epithelial and olfactory nerve atrophy as well as acute neutrophilic rhinitis. This dose corresponds to the quantity of SG that we measured on only 10 cm^2 of contaminated wallpaper in the first part of our work.

Within this context, we tested the effects of studied mycotoxins on A549 cells. In order to compare the risks that could be associated to inhalation vs. ingestion, we simultaneously characterized their toxicity on Caco-2 digestive cells.

4.2 Preliminary results: methodological developments

4.2.1 Choice of method

Before determination of the effects of mycotoxins on chosen cell lines, we firstly evaluated the CellTiter-Glo luminescent cell viability assay kit (Promega, Madison, WI), which is based on measuring the amount of ATP to assess the metabolic activity of living cells and thus to determine impact of one compound on cell viability by comparing ATP produced by control cells to that produced by treated cells.

A549 cells were seeded at concentration of $3 * 10^3$ cells/well in 100µL of adequate solution (see section 4.3) in 96-well flat-bottom cell culture plates. After 24 h of culture, various concentrations of solvent up to 5% in culture medium were added to the cells. After 48 h of incubation, the CellTiter-Glo assay was used as supplier instructed. Briefly, treated cell suspension was mixed with 100 µl assay reagent (freshly prepared), and the luminescence was recorded with a microtiter-plate reader Thermo Scan Elisa TECAN. The results were obtained by comparing the cell viability of each condition and the control condition and expressed as % of control.

Unfortunately, we failed to achieve satisfactory results since no dose-effect relationship was observed using this test to determine the best solvent for toxin solubilisation. Indeed, exposure to very low concentrations of solvents was found to be more toxic than higher ones before the appearance of a dose-effect curve (Figure 38). This bi-phasic response couldn't be explained; we decided to turn to the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega Corporation, Madison, USA) - MTS test, based on the measurement of the activity of mitochondrial enzymes.

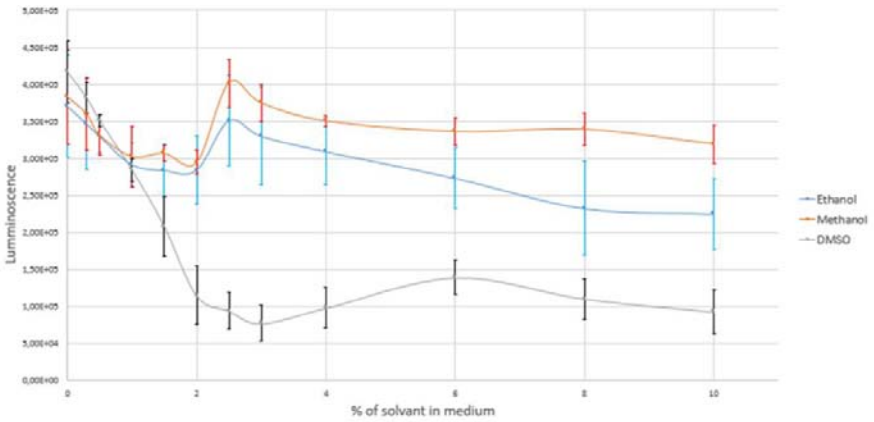


Figure 38: Effects of different solvents on cell viability measured by Luminescent Cell Viability Assay

4.2.2 Choice of solvent

We tested different solvents (dimethyl sulfoxide, ethanol and methanol) at concentrations between 0% and 5% in the culture medium, to measure their own toxicity on the cells and to choose the most suitable solvent for toxin solubilisation and dilution.

A549 cells were seeded at concentrations of $3 * 10^3$ cells/well in 100µL of adequate medium (see section 4.3) in 96-well flat-bottom cell culture plates and exposed to different concentrations of solvent.

Results were obtained by comparing the cell viability of each condition and the control condition. Results are presented on Figure 39, expressed as % of control.

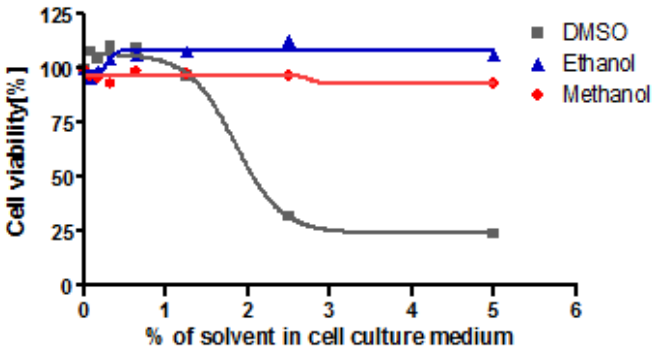


Figure 39: Effects of different solvents on proliferation of A549 lung cells

From this assay, it appeared that:

- Dimethyl sulfoxide (DMSO) showed an effect on the growth of cells starting from the concentration of 1%
- Ethanol and methanol showed no negative effect on cells up to a concentration of 5%.

Therefore, for further experiments, we have chosen the use of methanol, which proved to be non-toxic to our cells, is easy to handle and in which our toxins can easily be dissolved.

4.3 Materials and methods

4.3.1 Mycotoxins

Stock solutions of STG, MPA and VerA (Sigma, St Quentin Fallavier, France) were dissolved in methanol (MeOH) at 0.39 mg/mL (1.2025µM), 25 mg/mL (78.13 mM) and 0.1 mg/mL (0.1988 mM), respectively. The mixture of 4 toxins from macrocyclic trichothecenes family (SG, SH, RL2 and VerJ) was obtained from hay sample highly contaminated by highly toxinogenic *S.chartarum* ST82 strain. The mixture was prepared in methanol at 0.04 mg MCT Mix/mL (59.22% of SH, 31.81% of SG, 6.94% of RL2 and 2.03% of VerJ). Stock solutions were stored at -20 °C, and working dilutions were prepared in culture medium. A final concentration of 0.5% MeOH in the culture medium, corresponding to the highest concentration of MeOH in working dilutions, was tested and showed no significant differences from controls.

4.3.2 Cell lines and culture conditions

The A549 cell line is a human cell line derived from lung cancer with properties of alveolar type II pneumocytes (ATCC CCL-185, Rockville, MD, USA). The Caco-2 cell line has been derived from a human colon adenocarcinoma (ATCC HTB-37).

A549 and Caco-2 cells were cultured in 75 cm^2 culture flasks in DMEM Glutamax culture medium (Sigma) (supplemented with 2 mM L-glutamine) with 10% heat-inactivated fetal calf serum (Perbio Sciences, Bezons, France) at 37 °C in a humidified atmosphere with 5% CO_2. Medium was supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin (Eurobio, Courtaboeuf, France) or 0.5% of gentamycin and 1% non-essential amino acids for A549 and Caco-2, respectively.

4.3.3 Cytotoxicity assay (MTS test)

The effects of chosen mycotoxins on the proliferation and viability of A549 and Caco-2 cells were studied using the MTS test. The MTS tetrazolium (3- (4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium) is bio-reduced by living cells into a colored product, the formazan, soluble in the cell culture medium. NADPH or NADH molecules produced by dehydrogenase enzymes in metabolically active cells are responsible for this

conversion. Formazan is quantified by measuring the optical density at 492 nm and comparison between the untreated control cells and cells exposed to treatment allow the determination of the impact on cell viability (Figure 40).

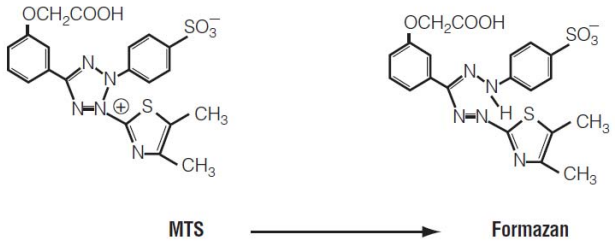


Figure 40: Structure of MTS tetrazolium and formazan product

In practice, A549 and Caco-2 were seeded at concentrations of 3 * 10^3 and 5 * 10^3 cells/well in 100 µL of adequate medium in 96-well flat-bottom cell culture plates. After 24 h of culture, various concentrations of mycotoxins ranging from 0.001 to 3200 µg/mL were added to the cells. After 48 h of incubation, the MTS test was used according to the supplier's instructions. Briefly, 20 µL of a freshly prepared MTS/PMS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/ phenazine methosulfate] solution was added to each well (20 µl of mixture to 100 µL of medium), and the cells were further incubated at 37°C for 3 h. Absorbance was measured at 492 nm with a spectrophotometer Thermo Scan Elisa TECAN. The results were obtained by comparing the cell viability of each condition to the control condition (results expressed as % of control).

4.3.4 Statistical analysis

Reported values present the mean ± standard deviation (SD) of three independent experiments. Each treatment was performed in triplicate. Statistical analysis was performed using GraphPad Prism software version 4.0. The differences between all mycotoxins' treatments were analysed statistically by analysis of variance (one way ANOVA) followed by a multiple comparison test Bonferroni's. P value <0.05 was considered statistically significant. For the IC50 determination, a non-linear regression analysis was applied.

4.4 Results and discussion

The cytotoxicity of sterigmatocystin, mycophenolic acid, verrucarin A and a natural mixture of macrocyclic trichothecenes containing satratoxins G and H, roridin L2 and verrucarin J was determined on A549 human lung cell lines and compared to results obtained on Caco-2 human intestinal cell lines. Results are presented toxin by toxin.

4.4.1 Sterigmatocystin

On Figure 41 is summarized the viability of A549 cells according to the presence of different STG concentrations in the culture medium. A concentration of 0.19 µg/mL (0.58 µM) led to a statistically significant decrease ($P < 0.05$) of cell proliferation. The metabolic activity of cells dropped very significantly ($P < 0.001$) after exposure to STG concentration from 0.313 µg/mL (0.965 µM).

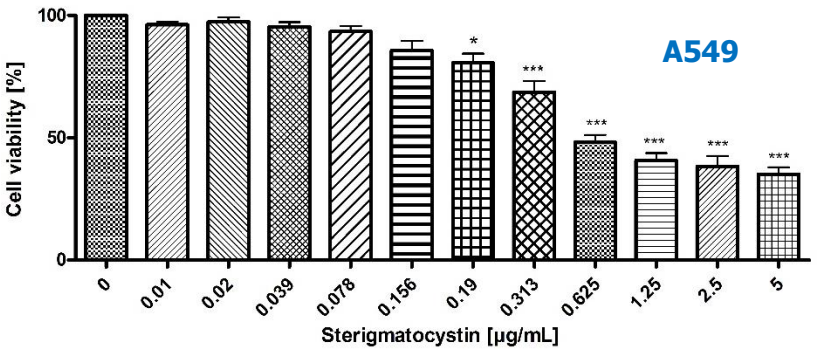


Figure 41: Cytotoxicity of STG on human lung cells A549
Results are presented as mean ± standard deviation (SD) of three individual experiments, each experimental condition being carried out in triplicate. The stars represent statistically significant differences between the treated samples and control (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

On human intestinal cells (Figure 42), concentrations of STG from 0.125 µg/mL had very significant impact ($P < 0.001$) on cell proliferation.

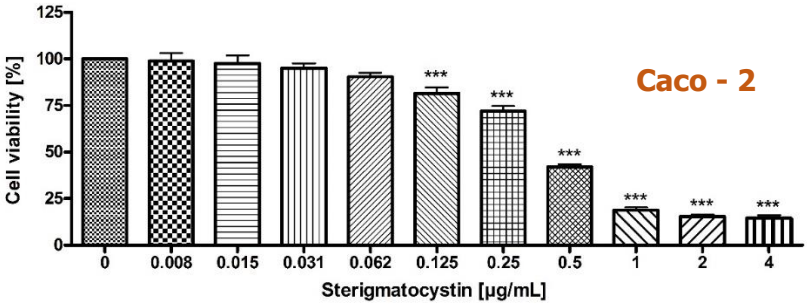


Figure 42: Cytotoxicity of STG on human intestinal cells Caco-2
Results are presented as mean ± standard deviation (SD) of three individual experiments, each experimental condition being carried out in triplicate. The stars represent statistically significant differences between the treated samples and control (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

4.4.2 Mycophenolic acid

On Figure 43 are reported the cell viability values for A549 as a function of the concentration of MPA in culture medium. The metabolic activity of cells dropped very significantly (P <0.001) after exposure of A549 cells to MPA concentration of 3.125 µg/mL.

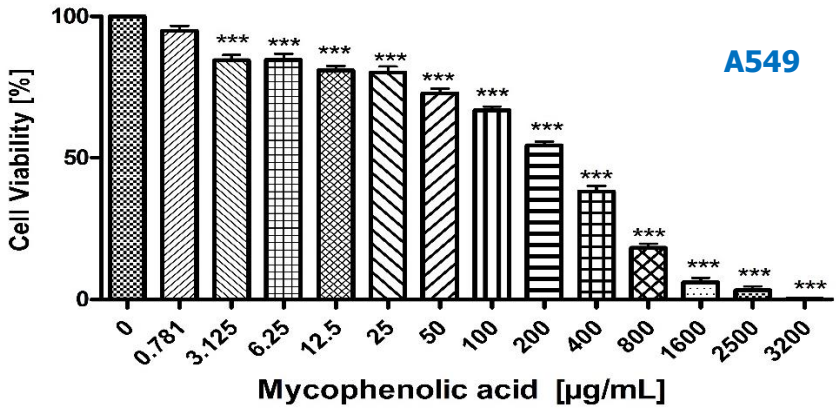


Figure 43: Cytotoxicity of MPA on human lung cells A549
Results are presented as mean ± standard deviation (SD) of three individual experiments, each experimental condition being carried out in triplicate. The stars represent statistically significant differences between the treated samples and control (* P <0.05; ** P <0.01; *** P <0.001)

Results on Caco-2 cells (Figure 44) showed different sensibility to MPA. In fact, cell proliferation dropped very significantly (P <0.001) after exposure of Caco-2 cells to MPA concentration of 0.195 µg/mL, showing higher sensibility to this molecule than A549 cells. Interestingly, starting from concentration of 12.5 µg/mL cells seemed to resist to toxicity with a viability remaining around 50 % independently of any increase in MPA concentration.

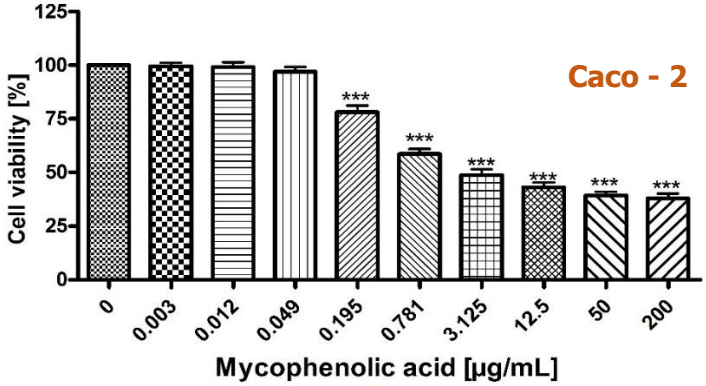


Figure 44 : Cytotoxicity of MPA on human intestinal cells Caco-2
Results are presented as mean ± standard deviation (SD) of three individual experiments, each experimental condition being carried out in triplicate. The stars represent statistically significant differences between the treated samples and control (* P <0.05; ** P <0.01; *** P <0.001)

4.4.3 Macrocyclic trichothecenes

In this study, cytotoxicity of VerA alone and a natural mixture of four macrocyclic trichothecenes (SG, SH, RL2 and VerJ) were estimated. For now, determination of cytotoxic effect of those compounds taken individually is not possible, since no pure toxins are commercially available. VerA is not produced by *S. chartarum* but some other species (*Myrothecium* for instance) and can sporadically be found in indoor environments. It should be noted that tested values for those components are expressed in ng/mL, demonstrating a strongly higher toxicity of MCT than previously tested mycotoxins (tested dilutions in µg/mL).

Figure 45 summarizes values of cell viability for A549 cell line according to different VerA concentration in the culture medium. From a concentration of 0.781 ng/mL we observed a statistically significant decrease (P <0.01) of cell proliferation and very significantly (P <0.001) after exposure to VerA concentration of 1 ng/mL.

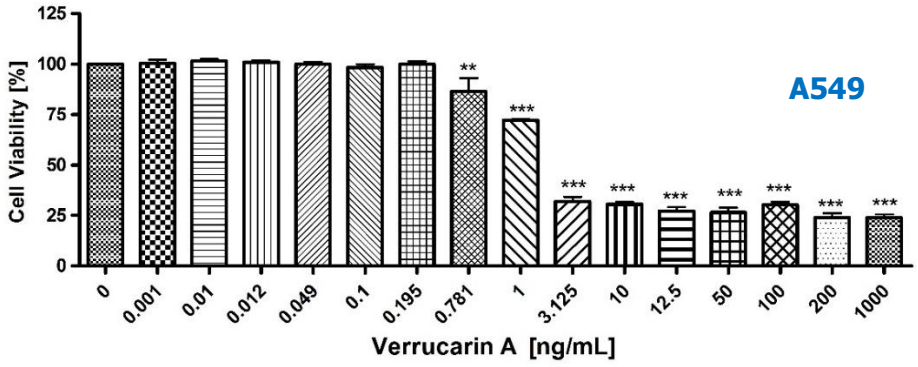


Figure 45: Cytotoxicity of VerA on human lung cells A549
Results are presented as mean ± standard deviation (SD) of three individual experiments, each experimental condition being carried out in triplicate. The stars represent statistically significant differences between the treated samples and control (* P <0.05; ** P <0.01; *** P <0.001)

Results on intestinal cells, showed very significant decrease of cell proliferation at concentrations of 3.125 ng/mL and higher (Figure 46).

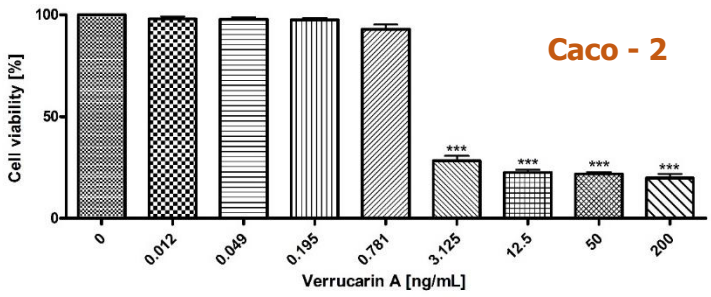


Figure 46: Cytotoxicity of VerA on human intestinal cells Caco-2
Results are presented as mean ± standard deviation (SD) of three individual experiments, each experimental condition being carried out in triplicate. The stars represent statistically significant differences between the treated samples and control (* P <0.05; ** P <0.01; *** P <0.001)

Mixture of MCT influenced cell proliferation of A549 cell line starting from 3.125 ng/mL. Results are shown on figure below (Figure 47). Interestingly, the same concentration showed to be very significant on Caco-2 cell line as well (Figure 48).

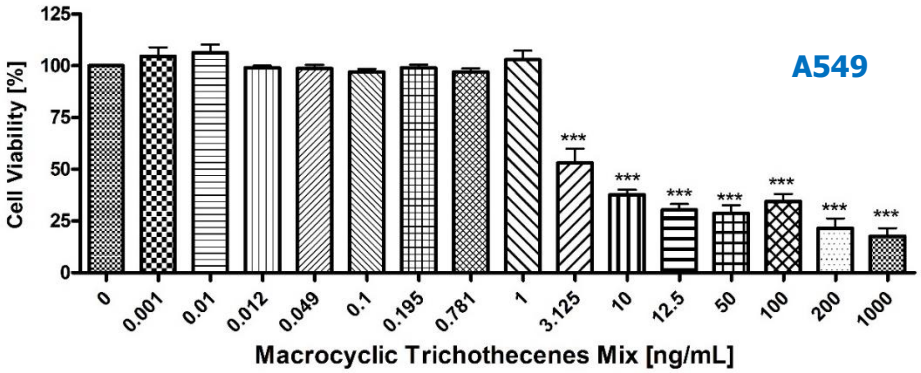


Figure 47: Cytotoxicity of MCT mixture on human lung cells A549
Results are presented as mean ± standard deviation (SD) of three individual experiments, each experimental condition being carried out in triplicate. The stars represent statistically significant differences between the treated samples and control (* P <0.05; ** P <0.01; *** P <0.001)

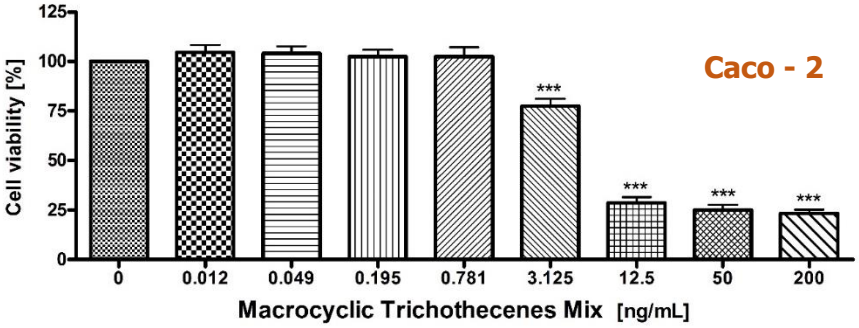


Figure 48: Cytotoxicity of MCT mixture on human intestinal cells Caco-2
Results are presented as mean ± standard deviation (SD) of three individual experiments, each experimental condition being carried out in triplicate. The stars represent statistically significant differences between the treated samples and control (* P <0.05; ** P <0.01; *** P <0.001)

4.4.4 Determination of IC50 values

To highlight eventual different sensitivity of cells to toxins, the values of IC50 were defined. Use of Software GraphPad Prism 4 allowed, from experimental data, to calculate the concentration corresponding to the IC50. The cytotoxic concentrations of mycotoxins that inhibit 50% of cell viability (IC50) after 48 hours of exposure are listed in Table 30.

Table 30: IC50 values for various mycotoxins on two tested cell lines

Mycotoxin	IC 50	
	A549	Caco-2
STG	0.32 µg/mL	0.36 µg/mL
MPA	406.9 µg/mL	0.37 µg/mL
VerA	1.20 ng/mL	1.64 ng/mL
MCT Mix	2.61 ng/mL	4.15 ng/mL

For MPA on lung cells, it has to be noted that, probably because the dose-effect curve was very flat, determination of IC50 was difficult and this value may be overestimated. Same problem was observed by Rasmussen et al (2011), suggesting that unusual dose-effect curve didn't allow precise determination of IC50. Nevertheless, there is one study determining IC50 for MPA on A549 cells at 114 µg/mL, but using DMSO as solvent and evaluation after 24h of treatment by NRU assay (Bünger *et al.*, 2004).

Our results showed that pulmonary cells are less sensitive to toxicity compared to digestive cells. This finding is of interest since MPA can be used for its immune-suppressive properties in patients that will undergo organ transplantation (Elbarbry and Shoker, 2007; Nguyen Thi *et al.*, 2015; Tönshoff *et al.*, 2011). In such case, the usual route of administration is by oral route. Taking into account the high sensitivity to epithelial digestive cells to that compound, it is likely that other route of administration could have less detrimental effects and allow a better therapeutic use of MPA.

From our data, it clearly appears that the toxicity of STG and VerA on pulmonary cells is comparable to that on digestive cells. The very few published data about cytotoxicity of those molecules on A549 are comparable to our results, revealing IC50 of STG and VerA at 1.2 µg/mL (Bünger *et al.*, 2004) and 3.18 ng/mL (Yan *et al.*, 2014), respectively. To the best of our knowledge there are no publication about toxicity of those toxins on Caco-2 cells.

For MCT mixture, the IC50 is 2 fold lower for pulmonary cells. This indicates that inhalation can be as toxic as ingestion for these compounds. To the best of our knowledge, this is the first time that cytotoxicity of mixture of MCT toxins, being produced simultaneously by *Stachybotrys chartarum*, was studied on A549 human hung cells.

Nevertheless, it was shown by Nielsen et al (2009) that IC50 on Caco-2 cells for satratoxin G and H were 6.4 ng/mL and 5.5 ng/mL, respectively. Patent proposed by Liu and co-workers (2009) evaluated cytotoxicity of verrucarin J on A549 cells and determined IC50 as 2.34 ng/mL.

4.5 Conclusion

In this study, we aimed to analyse and compare the cytotoxicity of mycotoxins that can occur in indoor environments on pulmonary cells in order to estimate the possible consequences on health of an exposure to that compounds following inhalation. Moreover we compared those results to values obtained for cytotoxicity on digestive cells.

Altogether, the results demonstrated that toxicity on pulmonary cells was quite similar (or even mildly higher for MTC) to that observed on digestive cells. It means that the presence of these contaminants in indoor environments and subsequent possible inhalation after aerosolization could represent health problem, especially in populations that are more sensitive to contaminants (children, old people, and immune-compromised persons).

It is very difficult to extrapolate the quantities of mycotoxin that were measured during aerosolization assays to define concentrations that could be inhaled and therefore to make correspondence with toxic concentrations reported here. Nevertheless, these results highlight the need for further experiments on pulmonary toxicity of mycotoxins that could be found in indoor environments.

Moreover, MCT showed very strong cytotoxic effect on pulmonary cells with IC50 values of few nanograms. This is important since cytotoxicity of those components had been poorly studied and several studies highlighted presence of satratoxins in indoor environments (Bloom *et al.*, 2009b; Täubel *et al.*, 2011) and moreover in the air of water-damaged dwellings (Brasel *et al.*, 2005b; Charpin-Kadouch *et al.*, 2006; Gottschalk *et al.*, 2008). It is also of great concern for people involved in remediation of contaminated buildings as well as for farmers that manipulate forages. Indeed, *S. chartarum* is also a frequent contaminant of hay or straw in case of moistening during storage (Bailly *et al.*, 2016) and can be responsible of acute animal poisoning due to the strong production of MCT on such cellulose rich substrates. Manipulation of hay or straw usually goes with the aerosolization of many particles that could be inhaled by manipulator and therefore lead to an aerial exposure to toxic particles. Such occupational exposure should also probably be investigated in the future.

Finally, all previous experiments done during this thesis also highlight the need to define protocols able to decontaminate materials in case of moulds/mycotoxin contamination (Chapter 5).

CHAPTER FIVE.

Evaluation of most used procedure of decontamination in homes on persistence of fungal contamination

5.1 Introduction

In previous sections, we demonstrated that the development of toxinogenic moulds on building materials can lead to the accumulation of quite large quantities of mycotoxins. The presence of these toxic secondary metabolites may represent a health hazard related to possible aerosolization and subsequent inhalation of contaminated particles. It may also be hazardous for people in charge of remediation of mouldy homes, some toxins having a demonstrated dermal toxicity (EFSA, 2013; Johanning *et al.*, 2014).

In case of fungal contamination of indoor environment, it is therefore important to assess the efficacy of possible remediation procedures on the persistence of both microorganisms and toxins.

There are several commercially available products and methods for fungal decontamination of indoor environments. Some of them are listed below:

- *Chemical methods*: chlorine and sodium hypochlorite, chlorine dioxide, ethanol (Bundgaard-Nielsen and Nielsen, 1996; Castegnaro *et al.*, 1980) boron-, chloride- and ammonium chloride - based chemicals.
- *Thermal methods*: Hot air, dryings, steaming
- *Gamma and UV radiation* (Peitzsch *et al.*, 2012a)
- *Oxidation* - Ozone (Menetrez *et al.*, 2009) etc.

Bleach that contains sodium hypochlorite with antimicrobial activity is recommended by the US Environmental Protection Agency (EPA) (Bundgaard-Nielsen and Nielsen, 1996) as well as CSTB (2015).

However, several studies showed that none of those treatments has absolute efficacy. For example, efficacy of UV light was directly function of fungal species. Indeed, it was shown that 90% of *Aspergillus versicolor* died after UV exposure (4.38×10^4 µWs/cm^2 during 5min), while the same conditions did not have effect on *Stachybotrys chartarum* (Menetrez *et al.*, 2010).

Similarly, Chakravarty and Kovar (2012) investigated the effect of five antifungal agents used in fungal remediation practices on the growth and spore germination of six commonly occurring indoor fungal species. They concluded that all tested agents showed fungistatic effect. This inhibition is due to inhibitory effect of antifungal compounds when applied on the fungal contaminated surfaces. The inhibitory effect is reversible once the inhibitory substances are removed or become diluted; spores can again germinate and mycelia can resume growth. Most of

the fungicides are effective only on hard non-porous surfaces. Viable spores hiding in porous surfaces may be unaffected and can go dormant when fungicides are applied.

The data reporting the effect of decontamination procedures on the mycotoxins are quite rare. To our knowledge, only one study investigated the effect of different treatments on the persistence of both moulds and produced toxins. Indeed, Peitzsch et al. (2012a) tested 10 different treatments on *Stachybotrys chartarum* and *Aspergillus versicolor* growing on gypsum board and pinewood, respectively. He concluded that none of applied treatments was able to completely eliminate viable moulds, nor toxins produced on materials. Elimination of sterigmatocystin ranged from 0 to 60% compared to positive control. Elimination level of three tested macrocyclic trichothecenes (trichodermol, satratoxins G and H) was variable depending on treatment, and could reach 80%. Moreover, they observed that after re-moisturising of materials fungal growth increased rapidly.

On the other side, roridine A, verrucarine A and T-2 persistence, after three decontamination procedures (bleach/detergent washing, gamma irradiation or steam cleaning) was evaluated in work of Wilson et al. (2004). However, in this study, toxins were not naturally produced on materials by fungal species but spiked as pure compounds. This study was therefore far from realistic conditions.

In order to further investigate the persistence of moulds on contaminated material, we aimed to test the efficiency of bleach, since it is one of the most widely used products to eliminate fungal contaminations and is recommended in various remediation guides proposed to the general public.

To perform these tests, we have chosen to firstly work on *Aspergillus versicolor* and three building materials identified previously as favorable for fungal development and mycotoxin production: wallpaper, fir and painted fiberglass wallpaper.

The remediation process was performed according to good practices recommendations (CSTB, 2015) and its effectiveness was evaluated on both the elimination of an initial contamination with *Aspergillus versicolor* and the ability to prevent a secondary development with time due to remaining viable spores.

5.2 Materials and methods

Experimental design was set to:

- Confirm initial potential of chosen strain to develop on different materials of interest during 21 days at 25°C and evaluate efficiency of decontamination procedure (washing materials with diluted bleach)
- Evaluate potential of strain to regrow after application of treatment and re-incubation for 21 days at 25°C without introducing new sources of nutrients or fungal contamination.

At each step of the protocol, fungal development was evaluated by:

- Examination of samples under stereo-microscope: evaluation of both hyphae development (density and colonized surface) and density of sporulated heads on the whole sample (10 cm^2)
- Examination of samples under SEM
- Determination of total CFU (cultivation on MEA)
- Determination of total spore numbers (spore counting using Malassez cell).

Overall experimental protocol is schematically presented on Figure 49.

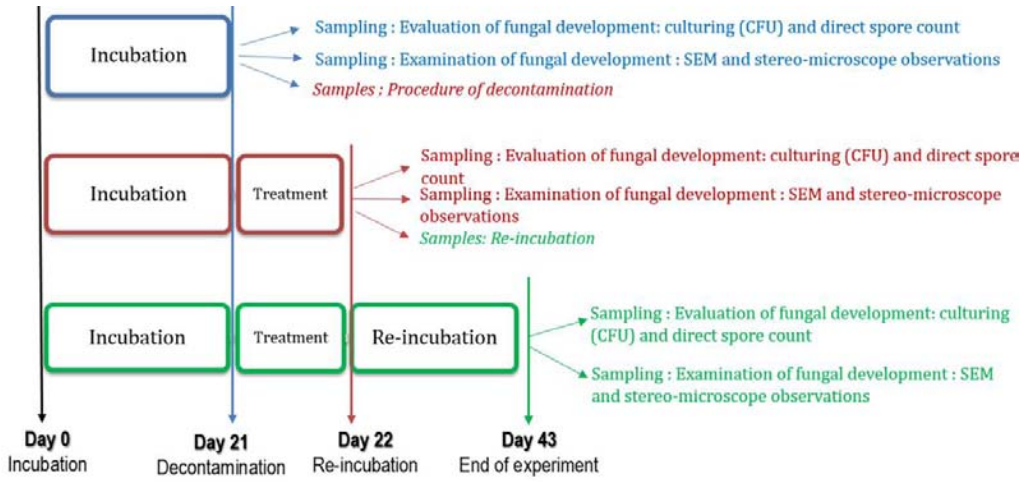


Figure 49: Schema of different sampling steps and obtainment of samples

5.2.1 Fungal strain

Experiments were performed with toxinogenic *Aspergillus versicolor* NCPT54 strain. It was stored at 4 °C and its viability was checked regularly by culturing on MEA.

5.2.2 Building materials

Three commercially available materials, frequently encountered in indoor environments were tested. These materials were purchased in a specialized store and were: painted fiberglass wallpaper (FWP) (Toile de verre, maille chevron, BATCH N_S2009061492 þ Paint MS SAT LUXENS, Leroy Merlin, France), wallpaper (WP) (Papier Peint BLAN BLA 0 INSP, Leroy Merlin, France) and fir (Leroy Merlin, France).

They were cut into 2 x 5 cm pieces and then sterilized (121 °C, 20 min) before use as described previously (Experimental work: 1.3.1.3).

5.2.3 Contamination procedure

The NCPT54 strain of *Aspergillus versicolor* was grown on potato dextrose agar (PDA, Biokar, France) for 14 days at 25 °C to obtain highly sporulated culture. Spore suspension was prepared from this fungal culture by adding 10 mL of Tween 80 (0.05%) to the Petri dish. The number of spores was quantified by direct counting on a Malassez cell. Spore suspension was then diluted to obtain the required concentration. Materials were placed in flasks, on a layer of 2 cm of glass beads with sterile water in order to maintain moisture level at saturation throughout the test. Contamination was achieved by applying 100 µL of this suspension (10^8 spores/mL) to each previously prepared material. Samples were then cultured at 25 °C in total darkness. Two material pieces were placed in each flask (Figure 50).

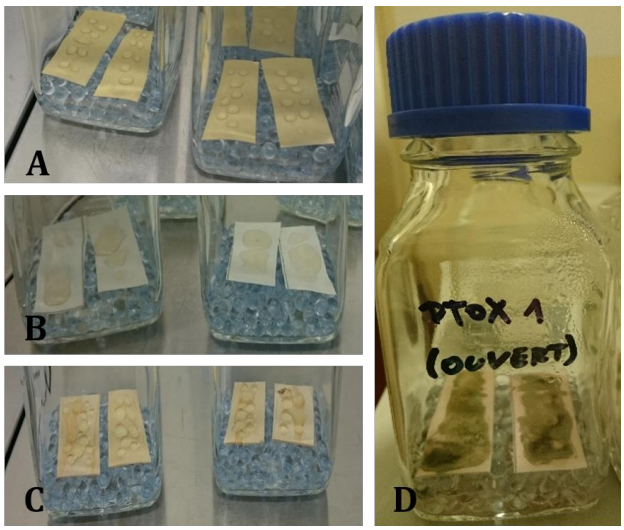


Figure 50: Flask system: wallpaper (A), painted fiberglass wallpaper (B) and fir (C) before and wallpaper (D) after fungal development

After 21 days of incubation, first batch of samples was obtained and samples were examined for fungal development:

- Examination of samples under SEM was done in microbiological laboratory of the CSTB

- Examination of samples under stereo-microscope (magnification from 12 to 120) (Olympus SZX9) to evaluate both hyphae development (density and colonized surface) and density of sporulated heads on the whole sample (10 cm^2) as well as general visual appearance (pigmentation).

To quantify fungal development, samples were transferred into plastic tubes with 20 mL of Tween 0.05 %, shaken 2 minutes on Vortex, and submitted to gentle mechanical agitation on a horizontal shaker (Reciprocating Shaker, IKA HS501 Digital, Grosseron, France) for 2 hours. Dilutions were made in order to obtain countable suspensions on Malassez cell. One hundred µL were also seeded on Petri dish containing MEA and incubated at 25 °C. After 3 days of incubation, CFU were counted by direct visual observation.

5.2.4 Decontamination procedure

Second batch of samples was incubated 21 days and was then submitted to decontamination procedure. Decontamination procedure was adapted from the good practices guide edited by CSTB (2015).

Two buckets with diluted bleach were prepared as recommended by producer: 60 mL of bleach (2.6 % active chlorine (AC) or 9 French chlorometric degree (°Cl)) in 2 L of water. Material samples were blocked with pins (1 to 2) and contaminated surfaces were cleaned using a sponge soaked in diluted bleach. This was repeated as many times as required to clear material from visible contamination, a new piece of sponge being used every time (Figure 51). After the decontamination procedure, materials were transferred in sterile Petri dishes and left under MSC at room temperature during 24 hours to dry. After that, same examinations were done as for batch one to evaluate the possible persistence of fungal contamination following treatment.

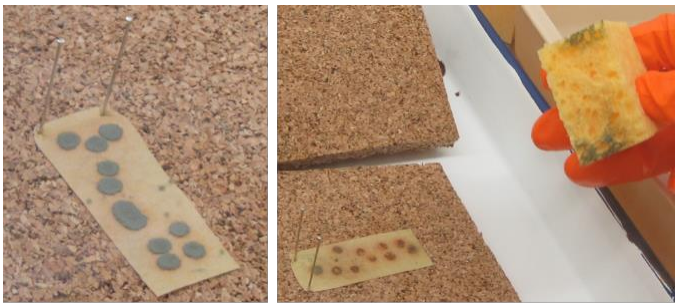


Figure 51: Decontamination of wallpaper with diluted bleach until disappearance of visible contamination

5.2.5 Re-incubation procedure

Third batch of samples received same treatment as second one and was re-incubated for 21 more days at 25°C after decontamination procedure. After this second incubation, examinations were performed as previously described to evaluate fungal re-development.

5.3 Results

To evaluate the efficacy of diluted bleach, two visual methods and two mycological methods were performed at each step of the experiment, giving us information about fungal growth after incubation, fungal resistance to treatment and fungal regrowth after a second incubation period.

5.3.1 Examination of fungal development under stereo-microscope and SEM

Visual examinations were performed to obtain information about fungal growth, sporulation etc., but also to examine changes on materials linked to fungal development as well as decontamination procedures.

5.3.1.1 Examinations after incubation period – day 21

Figure 52 shows the aspect of three different type of materials observed by naked eyes, under stereo-microscope or using SEM, following *A. versicolor* development during 21 days at 25°C.

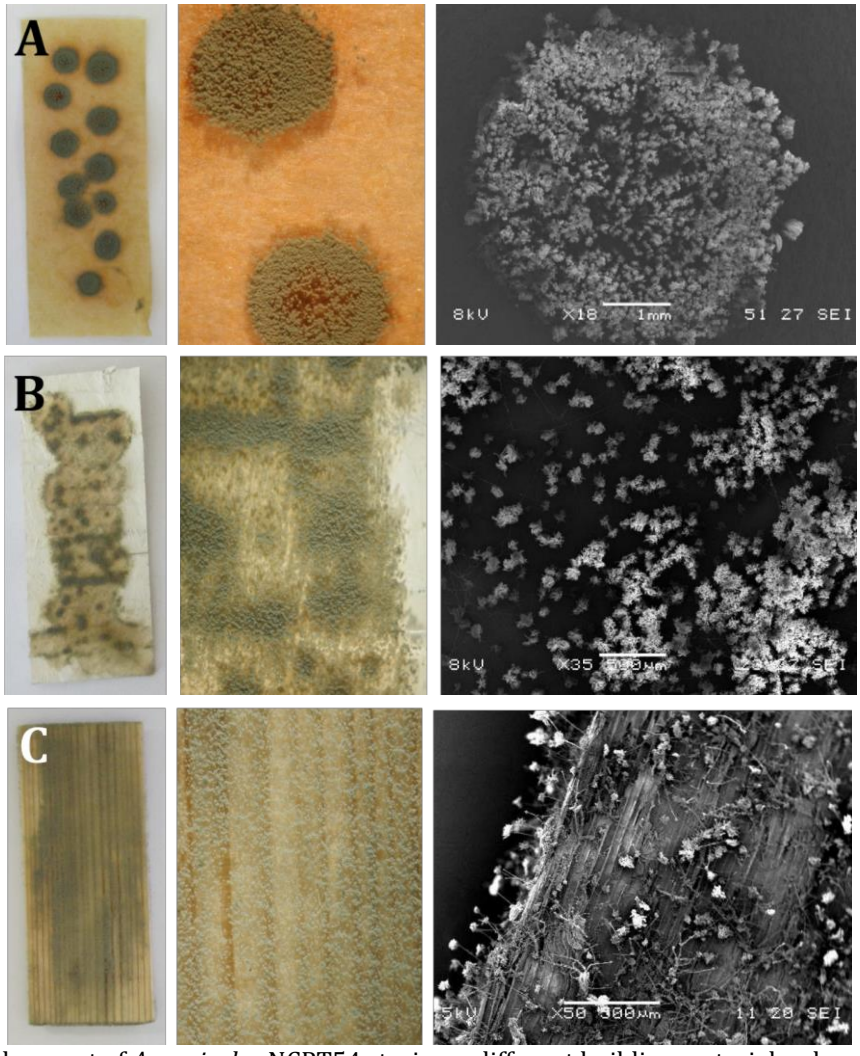


Figure 52: Development of *A.versicolor* NCPT54 strain on different building materials, observations made by naked eye (left), under stereo-microscope (middle) and by SEM (right). A: wallpaper. B: painted fiberglass wallpaper. C: fir

On wallpaper, observations of *Aspergillus versicolor's* growth after 21 days of incubation suggested that development occurred in limited areas corresponding to the initial inoculum deposit on the sample (around 10 spots per piece of material). Nevertheless, few isolated conidiophores are scattered outside spots. Edges of wallpaper were not colonized. Colonies were green, and synthesis of a pink-orange pigment that diffused on surface, even on reverse, was observed. Very strong sporulation was observed with relatively long chains of spores. However, colonized surface was moderate and represented about ¼ of the total surface. On reverse, we observed mild and localized contamination with few tufts of conidiophores bearing sporulated heads.

Aspect of painted fiberglass wallpaper was quite different. Development covered larger surface and appeared irregular. This is probably due to poor penetration of the spore suspension into the material (paint layer). Overall irregular density of fungal development was observed, due to the paint and to the composition of material (fibers). Edges are colonized on certain zones. Strong spore density was obtained on this type of material. Colonized surface was about ⅔ of the material. On reverse, without paint, level of contamination followed the relief of the fibers; on the "hills" very important development with strong sporulation was observed.

Finally, the development of *A.versicolor* NCPT54 strain on fir led to the colonization of almost all the surface and after incubation, fungal growth appeared globally uniform. An examination at higher magnification revealed that, in fact, development followed the wood fibers. Edges of samples showed to be highly colonized with development in "brushes" (Figure 53).

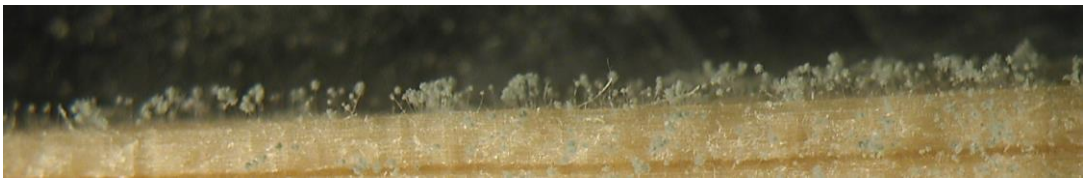


Figure 53: Edge of fir, highly colonized with development in "brushes"

Spore density seemed a little bit milder than that observed on previous materials. Colonized surface reached about 9/10 of samples. Contamination of reverse surface was important with a clear sporulation.

5.3.1.2 Examinations after treatment – day 22

After incubation, samples were decontaminated with bleach, and on 22nd day, after 24h of drying at room temperature, samples were analysed to evaluate the persistence of fungal contamination. Figure 54 shows the aspect of three different materials after treatment, observed by naked eyes, under stereomicroscope or by SEM.

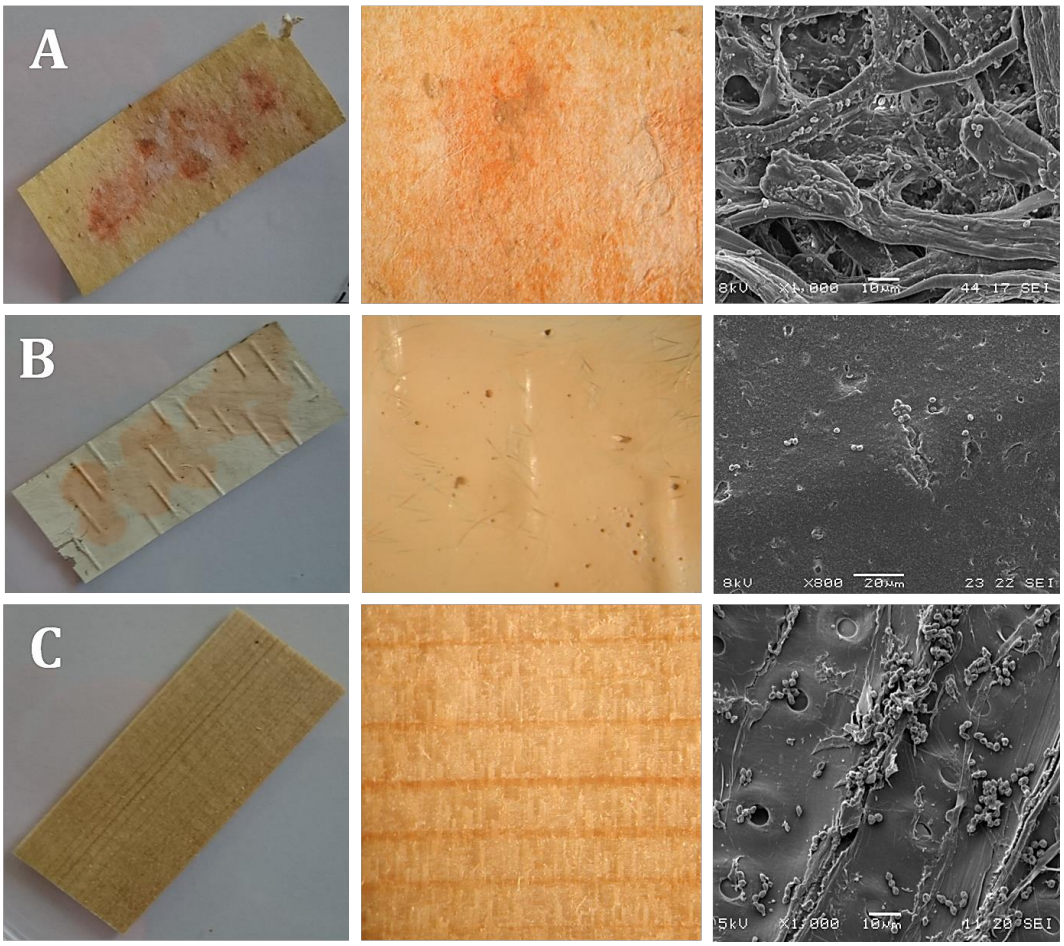


Figure 54: Observations of different building materials after treatment with bleach, made with naked eye (left), under stereo-microscope (middle) and by SEM (right). A: wallpaper. B: painted fiberglass wallpaper. C: fir

After treatment with bleach, observations by naked eye of wallpaper samples suggested that process to eliminate fungal contamination was in general successful (absence of visible fungal components). The orange pigmentation observed after development of *Aspergillus* persisted. It should be noted that cleaning process damaged wallpaper, the surface was crumbling and fibers were released. Damaged locations on wallpaper surface were as many "traps" for fungal fragments (spores, parts of mycelium etc.), which was confirmed by examination under stereomicroscope and SEM. These later showed certain persistence of fungal contamination but it was also observed that fungal structures were damaged (scattered spores, bonded conidiophores and absence of

intact mycelium). Reverse of material also revealed residual contamination, but the structures were also damaged by the cleaning process.

Concerning painted fiberglass wallpaper, the surface looked very well cleaned; without visible fungal residues when the paint is intact. However, residues persisted where paint layer was not continuous, making some "holes" that trapped fungal parts. Also, on the zones where paint was peeled by decontamination, the presence of some conidiophores was observed. It is also very important to note that edges resisted to cleaning process. The presence of many intact conidiophores (*Aspergillus* heads with spore chains) on the edges of material is showed on Figure 55. Reverse of painted fiberglass wallpaper revealed that contamination persists along the fibers, being slightly milder that after initial incubation.

Finally, observations on fir showed that surface looked very well cleaned without visible fungal residues. However, examination by SEM revealed the persistence of few fungal structures, mainly spores that were blocked by the unevenness of the surface. Reverse of this material, as for painted fiberglass wallpaper, still displayed a residual contamination, at lower level than initial one, with few conidiophores and presence of scattered piles of green spores.

As showed on figure below, contamination lying on the edges of painted fiberglass wallpaper and fir have circumvent the cleaning. This presents certain omission of our cleaning protocol but also correspond to what could be observed in practice, since, in homes edges of each band of wallpaper are not subjected to remediation process. Such observation could participate to understand the limits of decontamination procedures applied in mouldy homes.

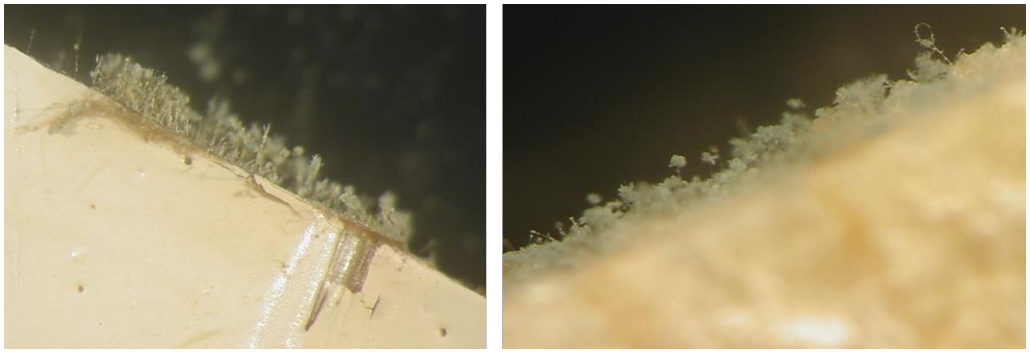


Figure 55: Residual *A.versicolor* contamination on edges of painted fiberglass wallpaper (left) and fir (right) after treatment with bleach

5.3.1.3 Examinations after re-incubation – day 43

After application of decontamination procedure, samples were re-incubated for 21 more days and in order to evaluate whether after this proposed decontamination process fungal growth is stopped (inhibited) or strain still had ability to re-colonize materials of interest. Figure 56 shows aspect of fugal re-contamination of different materials.

Observations on wallpaper indicated moderate recovery of fungal development, especially in round areas corresponding to the deposit of initial inoculum. Conidiophores were observed not only in these areas but also on little damaged parts of wallpaper, which served as traps for fungal fragments, but probably also as hideout. Reverse of wallpaper showed round areas of pigmentation, few conidiophores in the center and periphery of circle and mild sporulation.

On painted fiberglass wallpaper the presence of fungal structures on colored parts of sample (corresponding to initial development) was noted. Two types of structures could be distinguished: complete structures (conidiophores, vesicles and long chains of spores) but also sporulated microstructures in relatively high quantity. At microscopic level this structure showed to be only made of phialides or micro-vesicles with long chains of spores. Edges, as previously noted remained colonized (Figure 57), and reverse of material revealed few conidiophores.

After re-incubation period, surface of fir was completely re-colonized by scattered conidiophores, isolated or grouped by two or three. Some highly sporulated small fascicules were observed on whole surface. Chains of spores formed compact parallel thick columns (looking almost like *A. fumigatus*). Conidiophores seemed very long. On the edges, numerous sporulated conidiophores were present (Figure 57), as observed on painted fiberglass wallpaper as well. Reverse showed the presence of many long conidiophores with sporulated heads.

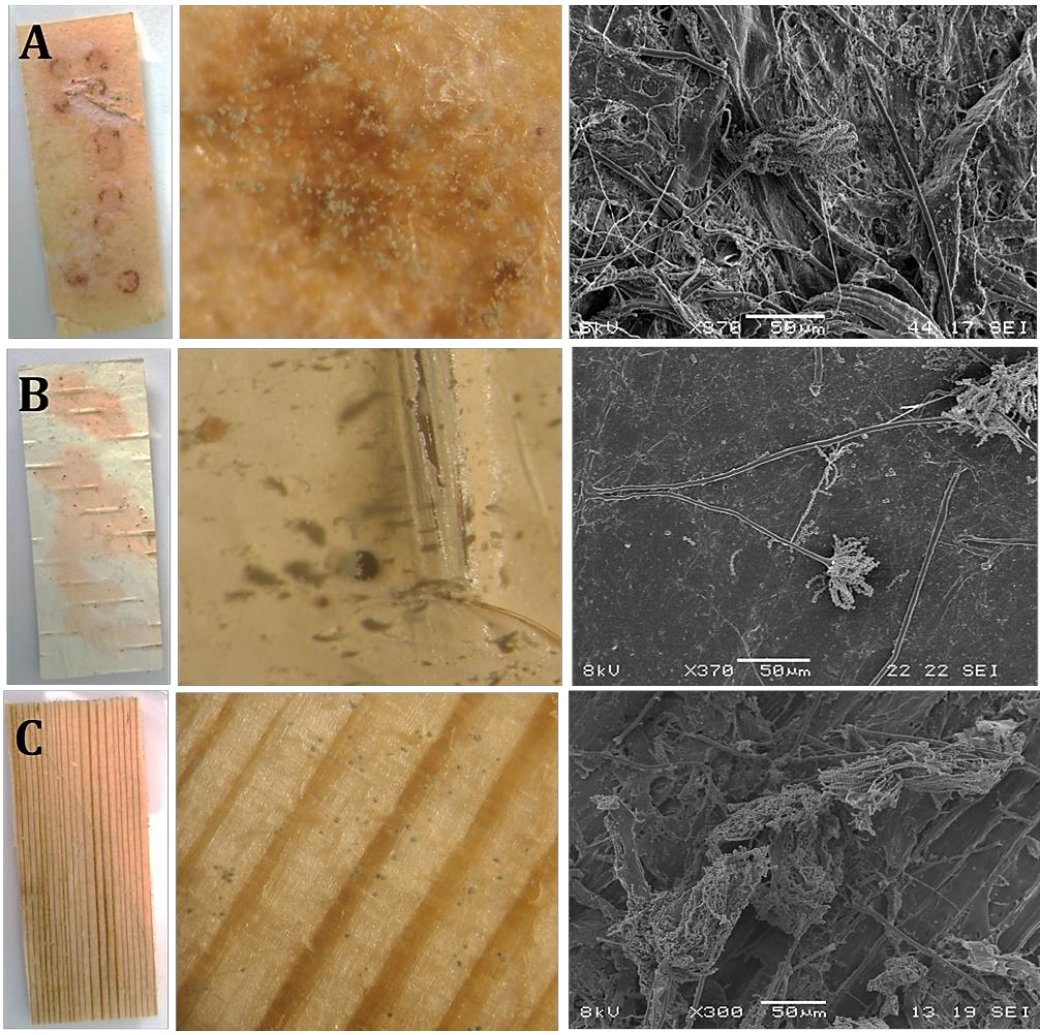


Figure 56: Observations of different building materials after re-incubation, made by naked eye (left), stereo-microscope (middle) and by SEM (right). A: growth on wallpaper. B: growth on painted fiberglass wallpaper. C: growth on fir

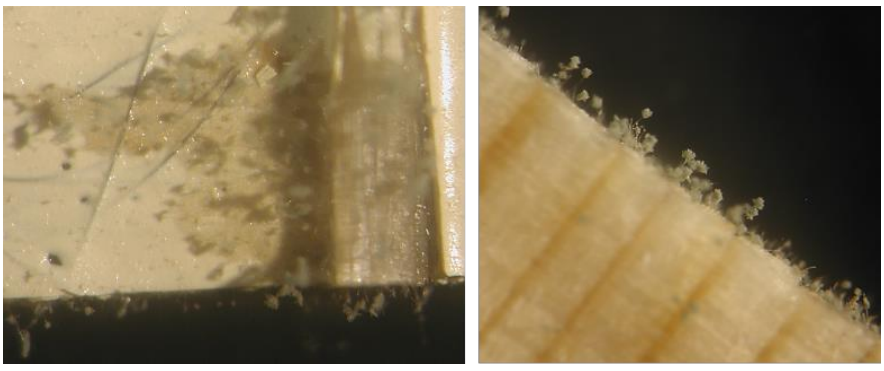


Figure 57: Development of *A.versicolor* during re-incubation on edges of painted fiberglass wallpaper (left) and fir (right)

5.3.2 Evaluation of fungal development by mycological methods

In order to quantify fungal development at each step of the experimental procedure, two mycological methods were performed: direct spore count and CFU determination.

Results for both are presented on figures 58 and 59 below.

It should be noted that weak differences between those two types of results were observed and could be related to two main reasons:

- Total spore count took into account all present spores, even if some of them were not viable (and therefore did not lead to a CFU after plating)
- While on microscope every spore can be distinguished and taken into account, by culturing on medium it is likely that aggregates of spores were not separated and subsequently have been counted as just one colony.

Nevertheless, results obtained by both methods are consistent.

It has to be highlighted that, after incubation period, the initial fungal contamination was very important (>10^8 CFU/material) and similar on all materials, despite different aspects, suggesting that in our case type of material did not strongly influenced global fungal development.

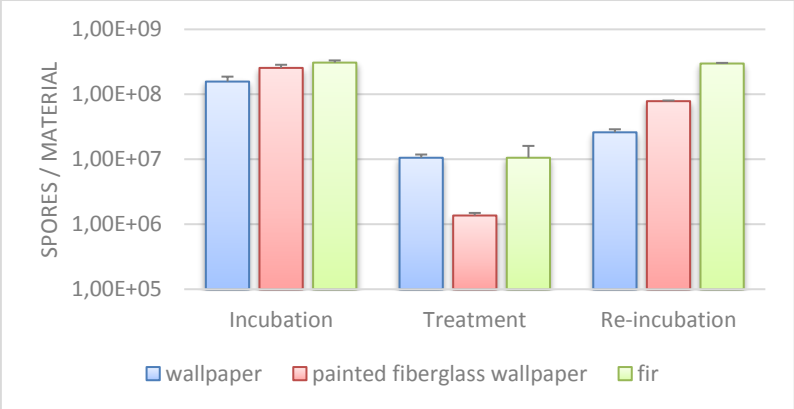


Figure 58: Total spore count for *A.versicolor* developed on three different materials at three different stages of experiment

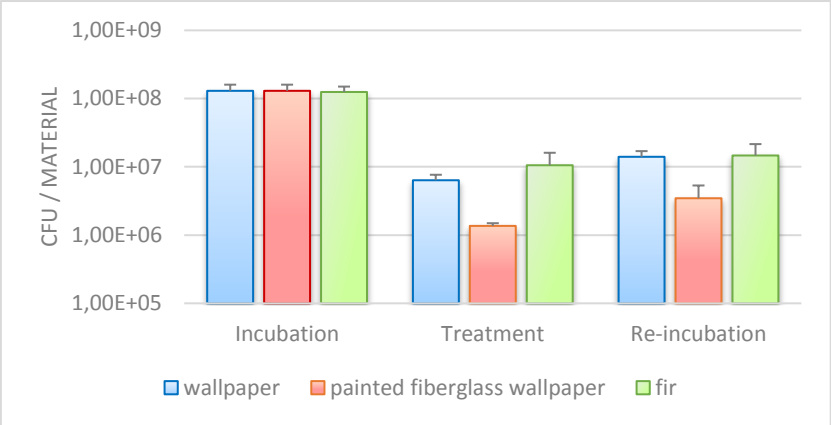


Figure 59: CFU count for *A.versicolor* developed on three different materials at three different stages of experiment

After treatment, cleaning process led to one log decrease in fungal load, going from 10^8 to 10^7 (or 10^6 for painted fiberglass wallpaper), which is in agreement with the observations made on microscopes, emphasizing that surface of FWP is well cleaned when intact. However, remaining fungal contamination was still important, as also observed on microscope, showing the partial efficiency of the decontamination method.

After three weeks of re-incubation period, partial recovery of fungal contamination was observed and *A. versicolor* showed ability to grow again on all three materials, as expected after samples' examination under stereomicroscope. It demonstrated that residual fungal structures observed after decontamination procedure were still viable and able to regrow when toxic agent activity decreased in the medium.

5.4 Discussion and conclusion

This chapter shows results for the study that was dedicated to evaluate efficiency of one of the most used methods for fungal decontamination in home conditions, efficiency of bleach.

Fungal ability to grow on different types of material was confirmed. Indeed, we previously demonstrated in chapter 1, in agreement with many available data, that WP, fir and FWP were good substrates for *A. versicolor* development (Andersen *et al.*, 2011; Nielsen, 2002; Polizzi *et al.*, 2009).

Moreover, despite different aspects of development, the quantification of CFU after 21 days revealed similar values. It would be interesting to evaluate such development on others materials with different composition especially with different cellulose content. However after treatment result were different according to the material.

For WP, the bleach treatment decreased by 20 folds the number of CFU. However, the soaking may alter WP and create lesions in which spores are trapped. This favors the re-development. Moreover, the pigmentation of material, formed as a result of pigment production of *A. versicolor* during its development, was not removed. It may suggest that this treatment did not allow elimination of fungal metabolites.

For FWP, treatment appeared more adequate in relation with the smooth paint layer that protects fibers and allows elimination of fungal structures. However any crack in the paint may hind spores.

Finally, for fir, the treatment was less effective despite a satisfactory visual aspect. In fact, spores may hook on the irregular surface of wood fibers.

Work by Betancourt et al. (2011) tested chlorine dioxide to inactivate viable *A.vesricolor* from building materials. A 4-log reduction in CFU was observed on gypsum wallboard, pine wood, glass, and carpet. Same treatment was applied by Burton et al. (2008) in real conditions of contaminated homes, and decrease of total fungi as well as culturable fungi was reported to be at least 85%. Even if this method shows very good efficiency, microscopic analyses of samples collected from surfaces after treatment showed that the fungal structures were still present on surfaces, which is in complete agreement with our observations.

Our results also demonstrated that the edges of materials may be heavily contaminated and not exposed to the treatment performed on the surface. In the same way, if material is unstuck from

support, reverse can be colonized and fungal structures protected. This may help to better understand failure of decontamination protocols in practice.

In all cases, cleaning did not allow a complete removal of fungal structures and few weeks after cleaning, fungal growth was again observed.

Our finding indicates that the commonly used method for remediation in the indoor environment, bleach, cannot completely kill all the fungal inoculum, in agreement with previous studies about its efficiency against fungal development (Chakravarty and Kovar, 2012; Peitzsch *et al.*, 2012a). Mostly, fungi will form dormant spores when exposed with fungicides (like Sanimaster, hydrogen peroxide, isopropyl alcohol, bleach, and Sporicidin). These dormant spores can germinate and resume growth when a favorable environment is available to them, as confirmed by previous authors (Chakravarty and Kovar, 2012). These results are observed even if no rinsing was applied, suggesting that bleach loses its activity with time. Nevertheless, work by Reynolds et al. (2012) suggested very good efficiency of bleach against *A. fumigatus* and some other species with 10 minutes contact of bleach with fungal growth (spore suspension was inoculated on ceramic carriers, treatment was performed by spreading solution with 2.4% NaOCl for direct contact). The results strongly suggest that complete removal of contaminated material is necessary for a successful remediation when dealing with indoor air quality problems. Moreover, the persistence of fungal structures even if they are dead or dormant may still have allergenic potential and represent a potential source of exposure to mycotoxins.

As main perspectives our team will continue studies in three directions:

- Evaluate efficiency not only on fungal growth but on elimination of produced toxins during initial fungal growth or during re-development following treatment.
- Evaluate the efficacy of bleach on other fungal species that are frequent indoor contaminant
- Evaluate others compounds or protocols.

GENERAL CONCLUSION AND PERSPECTIVES

General conclusion and perspectives

With people spending more than 80 % of their time inside the buildings, indoor air quality presents an important risk factor for human health. Fungi are frequent contaminants of dwellings and work facilities and can have various negative effects on human health. The role of mycotoxins may be an important parameter regarding occupant's health. However, a precise risk assessment still lacks some data on the nature and quantities of mycotoxins that can be produced in indoors, transferred to air and subsequently inhaled. We also lack some precise toxicological data following that route of exposure.

Overall objective of our work was to provide new insights about the contamination of indoor environments with toxinogenic fungi in order to better characterize the risk related to occupants' exposure to their secondary metabolites, mycotoxins.

This work tried to investigate all aspects of problematic related to mycotoxins in indoor environments, from production and aerosolization of these compounds to the evaluation of remediation procedures. Results and analytical developments that were conducted during this work may have several practical applications. They also highlight the need to further investigate some specific points.

Firstly, more fundamental perspectives related to the laboratory approach will be proposed, following precise and direct practical implication that obtained results could have.

To meet goals of this study, we have chosen to study different steps of this complex problem, using three toxinogenic species that are known as frequent indoor contaminants: *Aspergillus versicolor, Stachybotrys chartarum* and *Penicillium brevicompactum.*

Firstly, we characterized the ability of these species to colonize and produce their toxins on several types of building materials, usually used in indoor furnishing.

Our results revealed that produced amounts of mycotoxins varied depending on materials and fungal species but that no direct relationship with fungal development could be drawn.

Nevertheless, in all cases, wallpaper was very favorable for growth and sporulation of tested species. Moreover, same material showed to be the best support for toxinogenesis of *A. versicolor*

and *S. chartarum*, with the exception of *P. brevicompactum,* which preferred fiberglass. Fir and painted fiberglass wallpaper were also good supports for toxinogenesis in some cases.

Furthermore, quantities of produced toxins seem to be important, at least for some of them, like satratoxin H, reaching several dozen mg of toxins/m^2, and even up to more than 100 mg/m^2 in case of sterigmatocystin.

Contrary, vinyl wallpaper revealed to be resistant support for both fungal growth and mycotoxins' production. However, some development was noted on the edges, where vinyl layer was damaged.

These results raise several perspectives in order to better characterize this phenomenon. Firstly, further investigations should be performed in order to evaluate fungal development and toxinogenesis after a longer period (since this study limited experiments to 10 days and some species have highest toxin production after several weeks) and after damaging of materials, more precisely different protective layers. It is indeed likely that aging of material may facilitate fungal growth and toxinogenesis. Secondly, tests following a multi-contamination with different species growing together should be performed, in order to better evaluate conditions approaching to real conditions.

On a practical point of view, our results could also have important perspectives. First, they could be integrated in the methodology used to evaluate vulnerability of building materials. Indeed, in those type of evaluations mould development is used as a criteria to investigate vulnerability of materials. However, it was shown in this project that their ability to promote mycotoxin production shall also be included and used as an important criteria for safety evaluation. This should be especially the case for materials used in furnishing which are the closest to the room air and in fact in direct contact with occupants.

Following, our work aimed to further evaluate danger that shown toxinogenesis can present for occupants. For that, we evaluated toxins' abilities to aerosolize from contaminated material to the air, where they can be inhaled and pose real risk.

Since wallpaper revealed to be the most suitable support for toxinogenesis, it was used as a model material for aerosolization experiments. We showed these toxins could be partly aerosolized from mouldy wallpaper. Toxins' transfer to air required aeraulic solicitations that varied according to the species. These different behaviours, as expected, are probably due to differences in mycelium organisation. *Penicillium* and *Aspergillus* structures are easier to aerosolize than *S. chartarum*'s one, where air speeds of 0.3, 2 and 6 m/s, respectively, were required. One should have in mind

that these air velocities are easily achievable and present inhabitants' movements, airspeed in ventilation ducts, door slamming or opening of the window.

Studies concerning toxic load of aerosolized particles showed different results. Firstly, fifteen percent of produced mycophenolic acid was found in the air, highlighting not just an easy aerosolization of *P. brevicompactum* particles but its toxin as well. Aerosolized toxic load revealed to be more than 4% of total macrocyclic trichothecenes produced on wallpaper, and finally just 0.2 % of produced sterigmatocystin. This latter information should be considered along the fact that this toxin is produced in high quantity of more than 110 mg on one square meter of wallpaper, and even small percent can be transferred to air, it still presents an important quantity of toxin.

More importantly, analysis of aerodynamic profiles of generated aerosols revealed that highest part of the aerosolized toxic load is found in particles whose size corresponds to spores or mycelium fragments. However, for MPA and mainly MCT, toxins were also found even on particles smaller than spores. These contaminated particles, thanks to their small size, could be inhaled by inhabitants and deeply penetrate into respiratory tract, more precisely up to terminal bronchi – bronchioles and even to alveoli.

As main perspectives, higher air velocities should be tested on all species, since they are easily found in indoor environments. Probably they will lead to higher aerosolization of particles and here evaluation of associated toxic load will be crucial for risk assessment. In parallel, aged materials should be tested, since with time it is probable that materials will dry which can lead to better (higher) aerosolization of those toxic particles than observed with fresh samples that were used in this study.

Here, we showed that the most aerosolized toxins are not always the most produced ones, and moreover they are not equally distributed in different size ranges. It seems very important to take these data in consideration for risk assessment related to fungal contamination of indoor environment.

To analyze mycotoxin production and aerosolization, we have developed an analytical method able to quantify simultaneously several mycotoxins present in indoors. It seems now necessary to use such methodological advances to evaluate what can happen in real situations.
For that, field samples, made of materials from houses with mould damage shall be used to evaluate real situation in indoor environments. Especially it could allow evaluating to which toxins and at which levels people living in moldy homes are really exposed.

Moreover, such tools could also be useful to evaluate aerial exposure in some occupational situations. Indeed, *S. chartarum* is a frequent contaminant of forages. Its presence is well known to be responsible for acute poisoning in animals. So it is likely that manipulation of contaminated hay or straw by farmers may lead to aerosolization of contaminated particles that can be inhaled. Such possible exposure to mycotoxins shall be evaluated.

Also, our results suggest different toxins' distribution within fungal organizations. Indeed, we showed that several mycotoxins were found on particles smaller than spores. This could be in relation with the ability of some species to excrete toxins in exudate droplets. It would be interesting to more deeply analyze this phenomenon on building material, with special attention to toxin content in exudates and in spores to verify such hypothesis.

Moreover, analysis of repartition of the four tested MTC (satratoxins G and H, roridin L2 and verrucarin J) as a function of particles' size, have highlighted that toxins were differentially distributed. This could suggest that biosynthetic pathway of the different molecules could take place at different levels within fungal structures.
It would be therefore now very interesting to analyze more in depth the distribution of the different toxins within fungal structures to better understand the processing of these compounds and maybe their role in fungal biology.

After demonstrating that part of produced toxins on contaminated building materials can be relatively easily aerosolized to the air, we aimed to determine impact that they may have if inhaled. First approach was to evaluate cytotoxicity of those components on human lung cells, to roughly simulate exposure via inhalation.

In our study, we analysed and compared the cytotoxicity of different mycotoxins that can occur in indoor air on pulmonary cells in order to estimate the possible consequences on health of an exposure to that compounds following inhalation. Moreover we compared those results to values obtained for cytotoxicity on digestive cells in order to compare effect that could be related to inhalation *vs* ingestion.

Altogether, the results demonstrated that toxicity on pulmonary cells was quite similar (or even mildly higher for macrocyclic trichothecenes) to that observed on digestive cells. It suggests that the presence of these contaminants in indoor environments and subsequent possible inhalation after aerosolization could represent health problem, especially in populations that are more sensitive (children, old people *and immune-compromised* persons).

Moreover, macrocyclic trichothecenes showed very strong cytotoxicity on pulmonary cells with IC50 values of few nanograms. This is important since cytotoxicity of those components had been poorly studied even if several studies highlighted presence of satratoxins in indoor environments and moreover in the air of water-damaged dwellings. As main perspectives different composition of macrocyclic mixture should be tested.

Our results highlight the need for further experiments on pulmonary toxicity of mycotoxins that could be found in indoor environments. In the future other indicators of toxicity should be evaluated. All these data are needed in order to determine precise impact that those compounds may have.
All these data on pulmonary toxicity of mycotoxins are of course of direct importance to evaluate subsequent risk for habitants of mouldy environment. But they could also be of great interest to better assess professional risks for people involved in remediation of contaminated buildings along with farmers who manipulate forages, being the individuals directly exposed to those toxic components.

Finally, all previous experiments done during this thesis also highlight the need to define protocols able to decontaminate materials in case of moulds/mycotoxin contamination.

Our finding indicates that the commonly used method for remediation in the indoor environment, bleach, cannot completely kill fungi. In all cases, cleaning did not allow a complete removal of fungal structures and few weeks after cleaning, fungal growth was again observed.

As main perspectives in laboratory terms, our team will continue studies in three directions. We will evaluate efficiency not only on fungal growth but on elimination of produced toxins during initial fungal growth or during re-development following treatment. Efficacy of bleach on other fungal species that are frequent indoor contaminant will be also evaluated. Finally we will evaluate other compounds or protocols in order to identify the most effective one that shall be promoted.

Nevertheless, from our first findings, it seems that, for materials which are impossible to decontaminate by a cleaning procedure, complete removal may probably represent the safer solution, if, of course, it does not weaken the building structure.

In parallel, we also tried to identify markers that could be used to monitor an active toxinogenesis in indoor environment by studying the MVOCs that are emitted during toxinogenesis.

Indeed, previous work of our partners in CSTB showed that MVOCs can be used to predict an early fungal contamination of indoor environments. This is very useful, since in this work we showed that once visible contamination is present, it is hard to completely eliminate it. In this thesis we devoted to collect more information about possible use of same approach to predict active toxinogenesis and therefore possible presence of mycotoxins in indoors by targeting MVOCs specific to development of toxinogenic fungal strains.

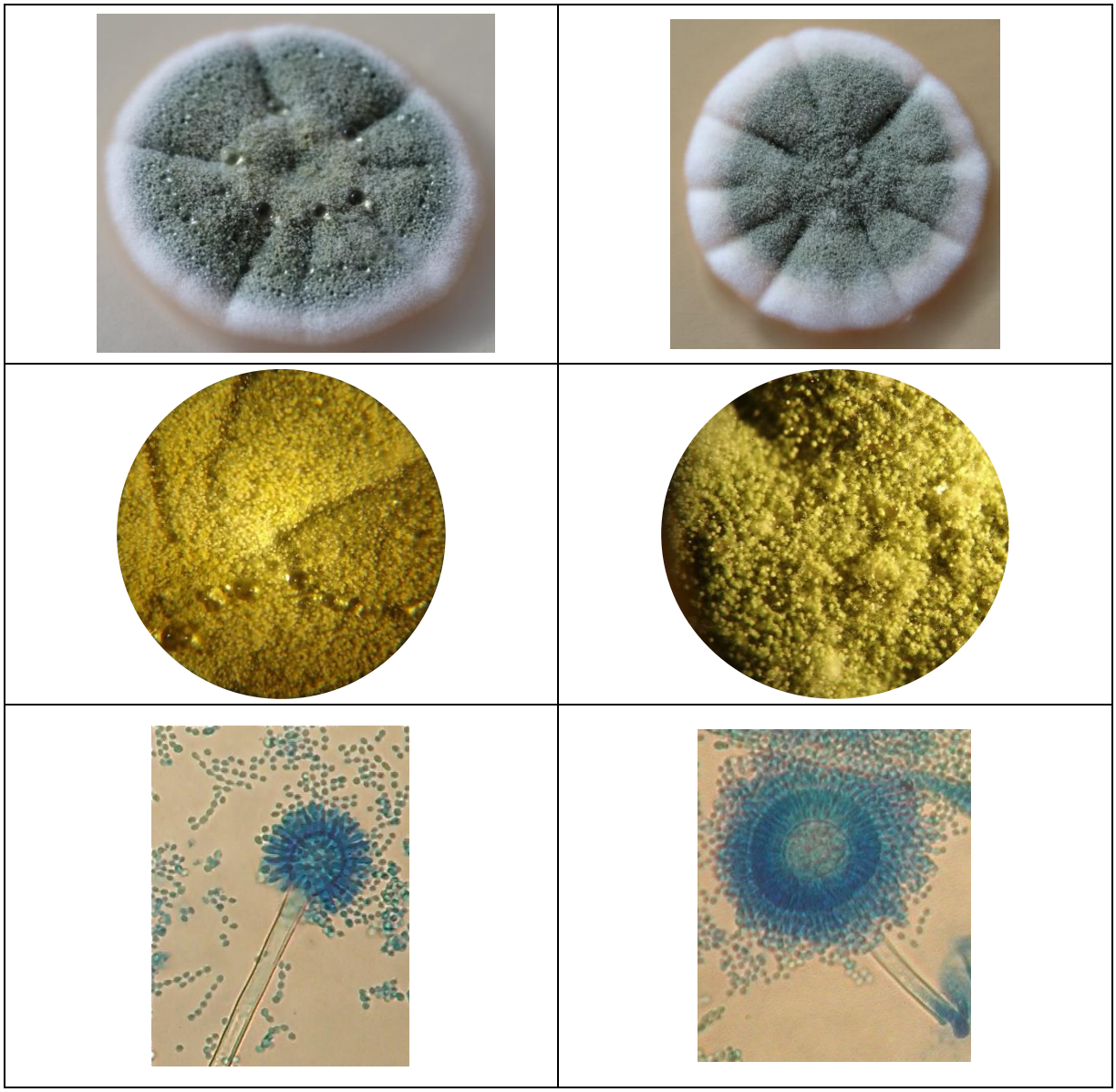
As a conclusion, variation of emitted MCOVs profiles by different fungal species and strains as well as on different materials on which they were growing didn't allow determination of MVOCS that can be specifically related to mycotoxin production by all TOX+ strains and on all materials.

Nevertheless, deepened treatment of data was performed using PCA and fragment ions analysis. Results of this approach were more encouraging. Indeed, the "fragment ions" approach has allowed the discrimination of the strains according to their toxinogenic potential for each species, but also the identification of 7 fragment ions that appeared correlated to a high level of mycotoxin production for the three tested fungal species.

In future, these results will be confirmed by testing the emission using other toxinogenic strains.
PCA results also suggest that, the identification of MVOCs that could serve as indicators of mycotoxins' synthesis in indoor environments, could require an optimization of the analytical methodology in order to allow access to molecules with higher molecular weight.
Therefore adaptations of established method will be performed in order to analyze those molecules which seems interesting as eventual searched MVOCs.

Annex 1. Macroscopic and microscopic aspects of TOX + strains of interest used in this study (Photos: Dr. S. Bailly and B. Aleksic)

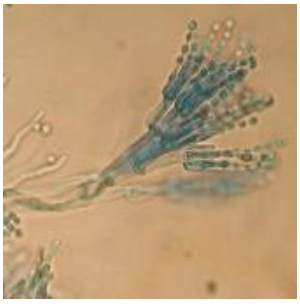
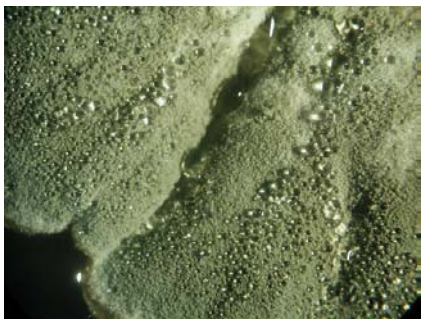
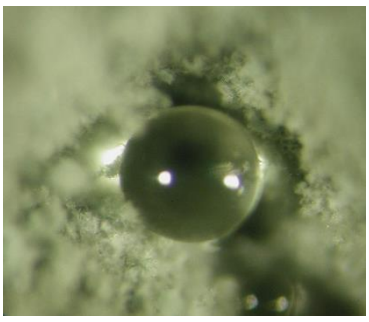
Aspergillus versicolor NCPT 54 (PDA / MEA)



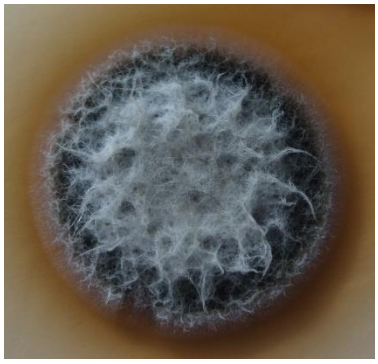
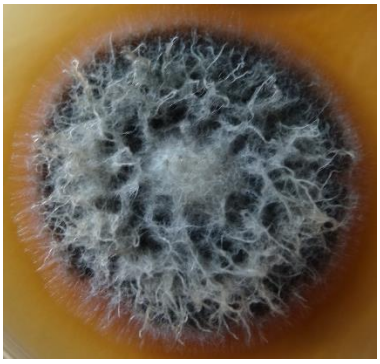
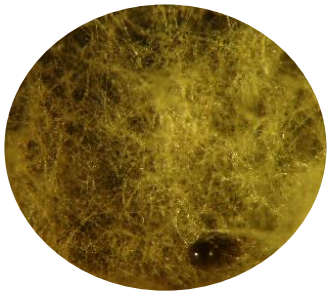
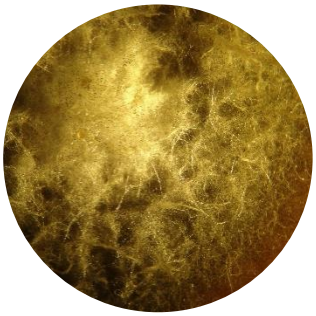
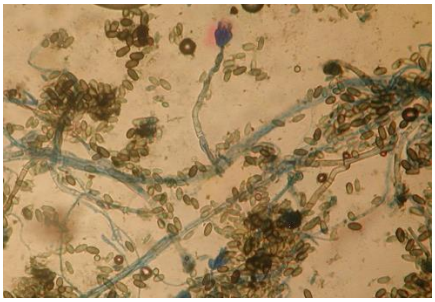
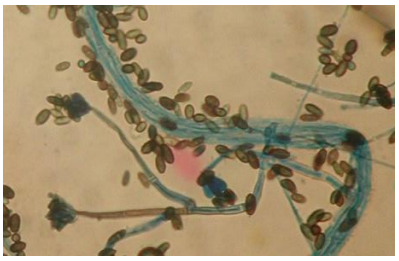
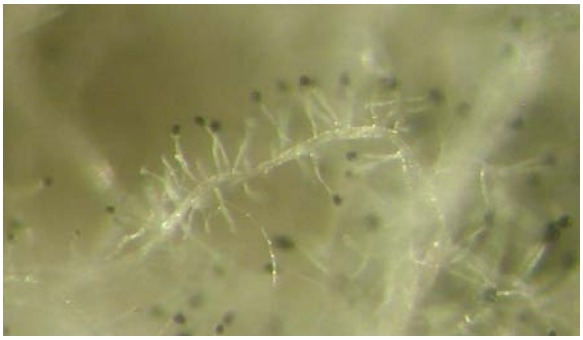
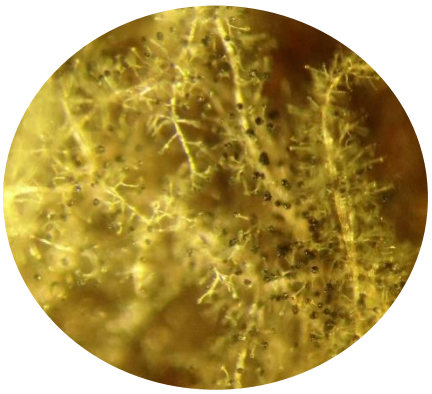
Penicillium brevicompactum IBT 23078 Parent (PDA / MEA)





Stachybotrys chartarum ST82 (PDA / MEA)

Annex 2. Use of PCA to identify chemical footprint based on ions to indicate fungal toxinogenesis

Statistical analysis: Principal component analysis (PCA)

Principal component analysis (PCA) is a statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components.

The PCA presents contents of a data table as graphical allowing simultaneous description of links between variables and similarities between individuals (projection in a small space). This representation allows therefore to highlight the existence of groups of variables or individuals, in this case the distinction between highly toxinogenic and non-toxinogenic strain. The PCA resulted in a reduction of information by grouping the variables into independent factors: the main components, which are linear combinations of variables. The PCA is described in detail in the book Statistique exploratoire multidimensionnelle (Lebart et al., 1997).

To perform this analysis we used the software for analysis of data - SPAD version 3.5 (décisia).

Determination of global footprint for production of mycophenolic acid

From the mass spectrometry results of each of the tests achieved with the TOX+ strain Pb16 and the mutant strain Pb19, the strength of signal associated with each fragment ion was summed over the entire analysis period, fixed at 80 min. A matrix fragment ions/test was then constructed, the sum of the intensities of the signals obtained for one fragment ion for a given test is reported here.

These results were then analysed using a Principal Component Analysis.

As a first approach, tests for Pb16 strain (TOX-) and Pb19 (TOX+) were analysed for their strong chemical emissions and observed constant similarities. Table 1 specifies the weight of the axes (factors) defined by the PCA.

Table 1: Specific weight of the factors, defined by the PCA, for tests performed with mutant and wild type strains of *P. brevicompactum*

NUMERO	VALEUR PROPRE	POURCENT.	POURCENT. CUMULE
1	209.3443	53.27	53.27
2	67.2828	17.12	70.39
3	59.2014	15.06	85.45
4	29.2991	7.46	92.91
5	12.7735	3.25	96.16
6	8.3491	2.12	98.28
7	6.7498	1.72	100.00
8	0.0000	0.00	100.00

The eigenvalues represent the weights of factors (variables) with respect to an axis. Therefore axis 1 is representative of 209 variables out of 393, or over 53%. Consideration of the first two axes totalize more than 70% of the overall variance. Thus, only the main plan was considered (Figure 1).

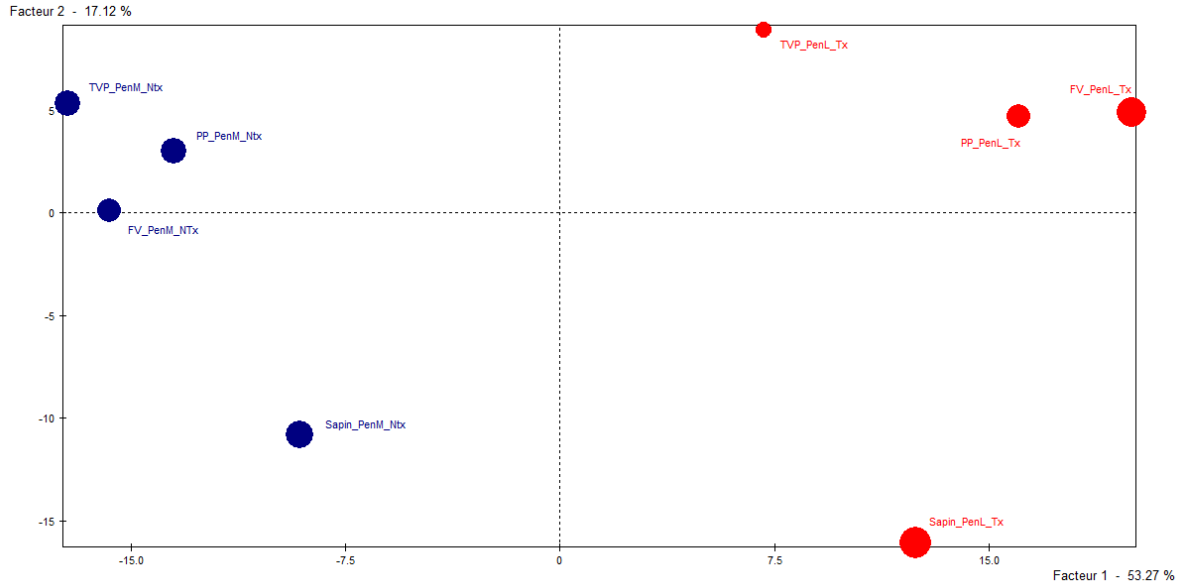


Figure 1: Plan defined by the factors 1 and 2 of the PCA for tests with strains Pb16 (PeniL_Tx) (shown in red) and Pb19 (PeniM_NTx) (shown in blue) of *P. brevicompactum* (by convention the tests are designated by "Material_strain_toxinogenic character")

Size of the dot defines the quality of their projection on the plan. Thus, the bigger the dot, the better it is represented.

It appeared that the TOX+ (Pb16) and TOX- (Pb19) strains were discriminated by the factor 1 indicating different chemical emissions. Indeed, the projection of individuals on the factor 1 shows the "toxinogenic" tests on positive part of this axis, and the "non-toxinogenic" on the negative part of this axis.

The correlation circle (Figure 2) identifies the variables that present this factor 1 as well as the fragment ions responsible for the observed separation.

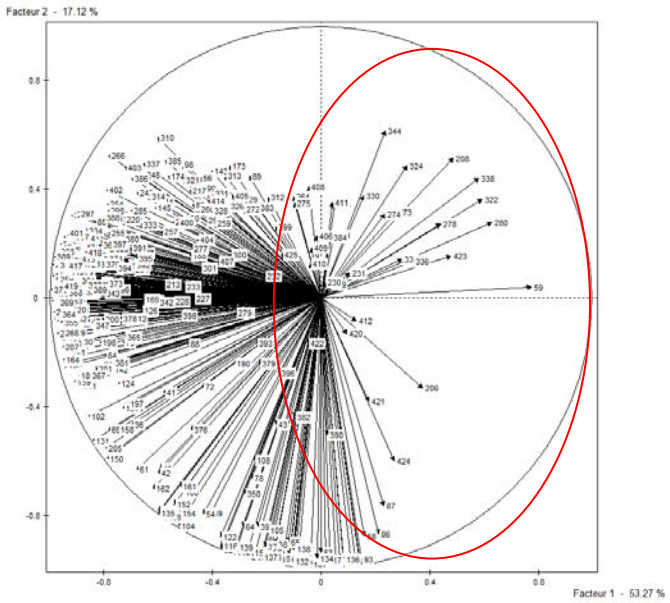


Figure 2: Representation of active variables in terms of factors 1 and 2 for the fragment ions responsible for the separation of Pb16 and Pb19 strains of *P. brevicompactum*

The ions contained in the correlation circle are constructing, by their projection on the horizontal axis, the factor 1. Only those whose contribution is on positive part of the axis 1 "sign" TOX+ strains and therefore constitute ions specific to emissions which are accompanied with toxinogenesis. Thus, 22 specific ions may be used to define a specific chemical signature for the production of MPA: 58, 59, 86, 87, 206, 208, 273, 274, 278, 280, 322, 324, 330, 336, 338, 344, 411, 412, 420, 421, 423, and 424.

To complete these data, the same statistical treatment was applied to the 3 strains: Table 2 precise weight of axes (factors) defined by the PCA.

Table 2: Specific weight of the factors, defined by the PCA, for tests performed with mutant, parent and wild type strains of *P. brevicompactum*

HISTOGRAMME DES 12 PREMIERES VALEURS PROPRES

NUMERO	VALEUR PROPRE	POURCENT.	POURCENT. CUMULE
1	192.0195	48.86	48.86
2	70.6933	17.99	66.85
3	56.1355	14.28	81.13
4	28.8164	7.33	88.46
5	12.0730	3.07	91.54
6	9.7906	2.49	94.03
7	8.3347	2.12	96.15
8	5.0576	1.29	97.44
9	4.2933	1.09	98.53
10	3.3359	0.85	99.38
11	2.4501	0.62	100.00
12	0.0000	0.00	100.00

Axis 1 is representative of 192 variables out of 393, or more than 48% of the information. The analysis of the various plans did not allow to identify one plan which discriminates toxinogenic strains from non-toxinogenic one (data not shown). The main plane, consisting of axes 1 and 2,

being most representative with more than 66% of information, was chosen for the selection of specific ions (Figure 3).

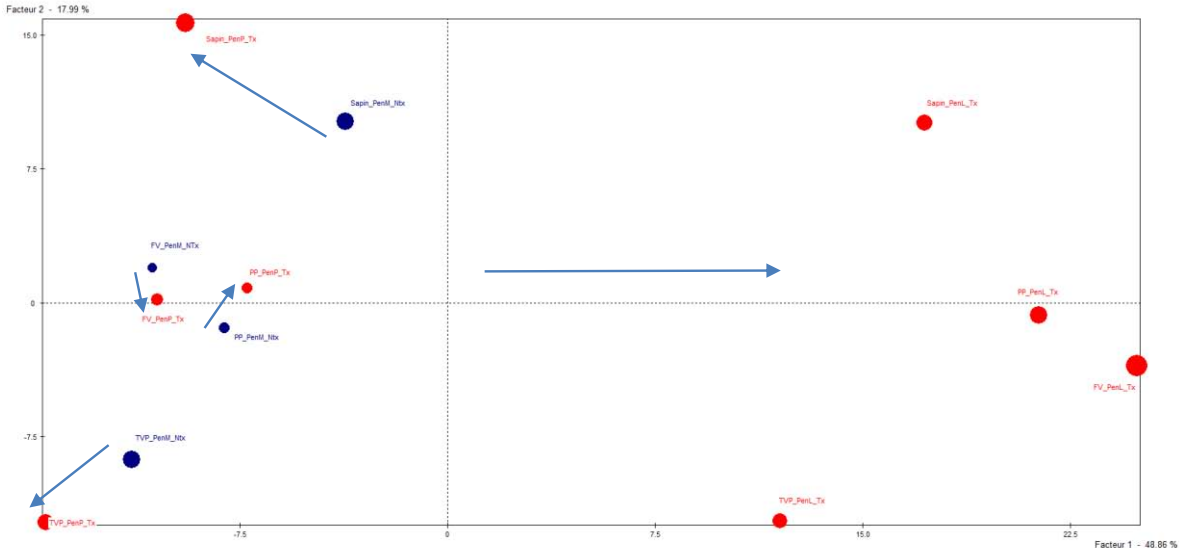


Figure 3: Plan defined by the factors 1 and 2 of the PCA for tests performed with TOC+ strains Pb16 (PeniL_Tx) and Pb25 (PeniP_Tx), shown in red; and TOX- strain Pb19 (PeniM_NTx) , shown in blue of *P. brevicompactum* (by convention the tests are designated by "Material_strain_toxinogenic character")

On this figure, the "TOX+" tests are shown in red and "TOX-" tests are blue. Dot size defines the quality of their projection on the plan. The blue arrows, inserted in Figure 3, indicate the directions associated with toxin production.

The correlations circle (Figure 4) identifies, by comparison with the directions indicated by the arrows, the variables (specific ions) responsible for the separation toxinogenic / non-toxinogenic for each test.

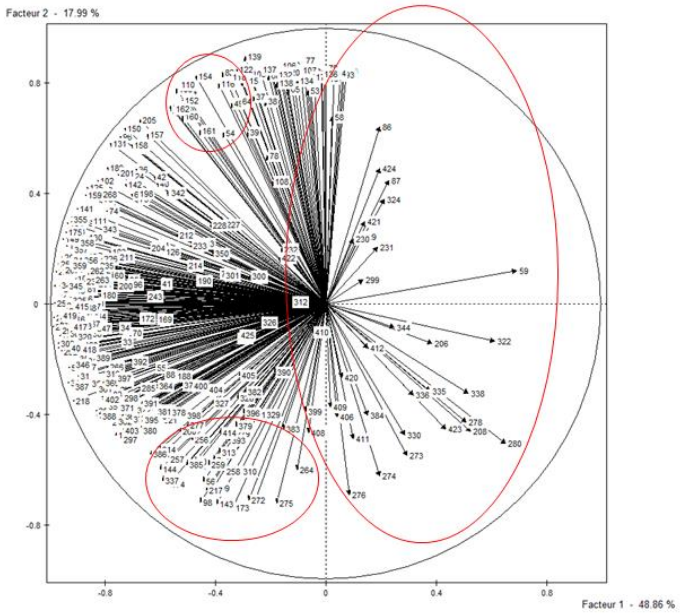


Figure 4: Representation of active variables associated with tests performed with the three strains of *P. brevicompactum* in the main plan

The ions contained in the red circles correspond to the directions identified on Figure 4 and therefore specific for TOX+ strains. Thus, 90 specific ions were retained: 54, 56, 58, 59, 71, 80, 83, 86, 87, 90, 91, 92, 93, 94, 98, 104, 109, 110, 119, 121, 135, 136, 143, 144, 145, 152, 154, 160, 161, 162, 173, 174, 199, 206, 208, 217, 229, 230, 231, 256, 257, 258, 259, 260, 264, 272, 273, 274, 275, 276, 277, 278, 279, 280, 299, 310, 313, 314, 322, 324, 327, 328, 329, 330, 331, 335, 336, 337, 338, 379, 382, 383, 384, 385, 386, 390, 393, 396, 399, 405, 406, 408, 409, 411, 412, 414, 420, 421, 423 and 424.

From this selection, a new PCA was generated. Table 3 shows the weight of the axes (factors) defined by the PCA.

Table 3: Weight of factors, defined by the PCA, after selection of 90 ions specific for tests performed with the three strains of *P. brevicompactum*

NUMERO	VALEUR PROPRE	POURCENT.	POURCENT. CUMULE
1	36.6689	40.74	40.74
2	20.0208	22.25	62.99
3	12.7075	14.12	77.11
4	8.6856	9.65	86.76
5	4.0451	4.49	91.25
6	2.7376	3.04	94.29
7	1.6187	1.80	96.09
8	1.2838	1.43	97.52
9	0.9791	1.09	98.61
10	0.7476	0.83	99.44
11	0.5054	0.56	100.00
12	0.0000	0.00	100.00

Axis 1 is representative for 36 variables out of 90, or over 40%. The analysis of the various plans revealed that one plan, formed by the axes 3 and 4, allowed to discriminate toxinogenic strains (Tx) from non-toxinogenic (NTx) (Figure 5).

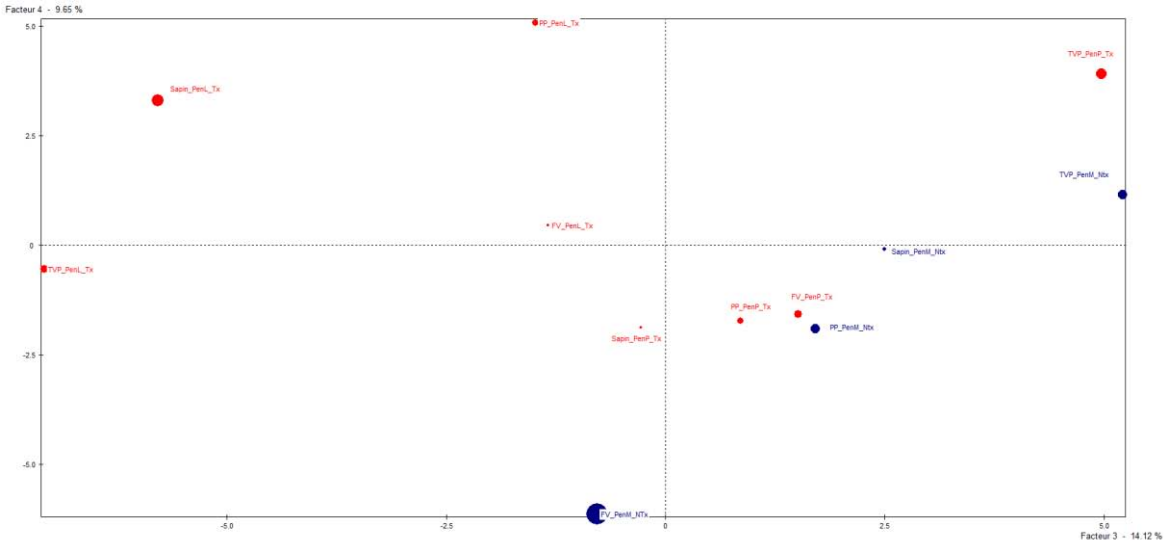


Figure 5: Plan defined by the factors 3 and 4 of the PCA for tests performed with three strains of *P. brevicompactum* after selection of the fragment ions of interest. TOX+ strains Pb16 (PeniL_Tx) and Pb25 (PeniP_Tx) are shown in red; and TOX- strain Pb19 (PeniM_NTx) is shown in blue (by convention the tests are designated by "Material_strain_toxinogenic character")

This discrimination allowed the confirmation that the 90 selected ions present good chemical footprint for production of MPA.

The new correlation circle, associated with last PCA, identifies the variables (specific ions) responsible for the separation toxinogenic / non-toxinogenic for each test (Figure 6).

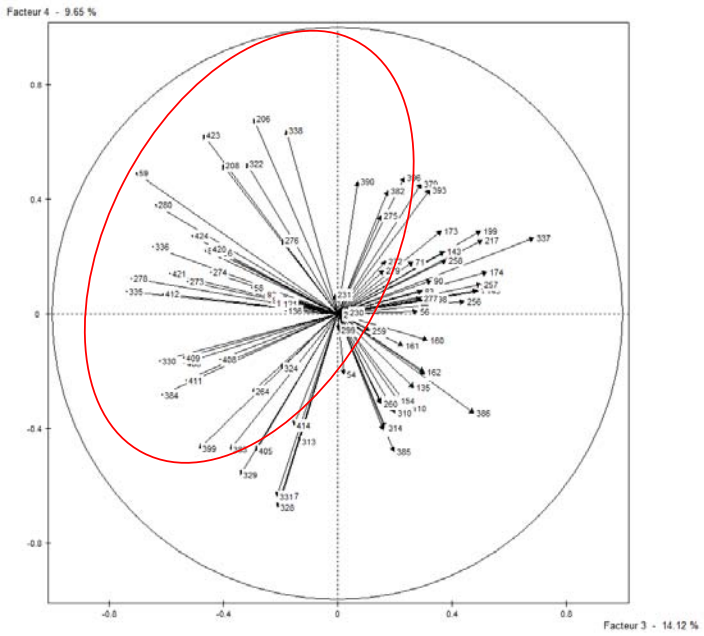


Figure 6: Representation of the active variables of tests with three strains of *P. brevicompactum* in terms of factors 3 and 4 once the ion fragments of interest are selected

The ions contained in the red circle are specifically associated with the growth of TOX+ strains. Essentially those are ions whose ratio between mass and charge is greater than 200, indicating molecules with high molecular weight, for which the used analytical method is not optimal. This finding may explain the fact that no discriminating VOCs has been identified during the first approach.

Determination of global footprint for production of mycotoxins

To search this global footprint, the same method as applied for *P. brevicompactum* was performed. Table 4 presents the specific weight of the axes (factors) defined by the PCA.

Table 4: Specific weight of the factors, defined by the PCA, for tests performed with strains of *A.versicolor* and *S. chartarum* on different materials

NUMERO	VALEUR PROPRE	POURCENT.	POURCENT. CUMULE
1	116.1302	29.55	29.55
2	99.3259	25.27	54.82
3	55.3478	14.08	68.91
4	33.1441	8.43	77.34
5	25.7588	6.55	83.89
6	20.7207	5.27	89.17
7	10.2896	2.62	91.79
8	8.5145	2.17	93.95
9	7.3905	1.88	95.83
10	5.8041	1.48	97.31
11	4.8998	1.25	98.56
12	4.0069	1.02	99.58
13	1.0605	0.27	99.85
14	0.6067	0.15	100.00
15	0.0000	0.00	100.00

Axis 1 is representative of 116 variables out of 393, or over 29%. Consideration of the first four axes (framed in red) totalize more than 77% of the overall variance. Thus, six plans were considered: plan consisting axis 1 and 3 was retained because it separated the highly toxinogenic strains (Tx) from weakly toxinogenic ones (NTx) (Figure 7).

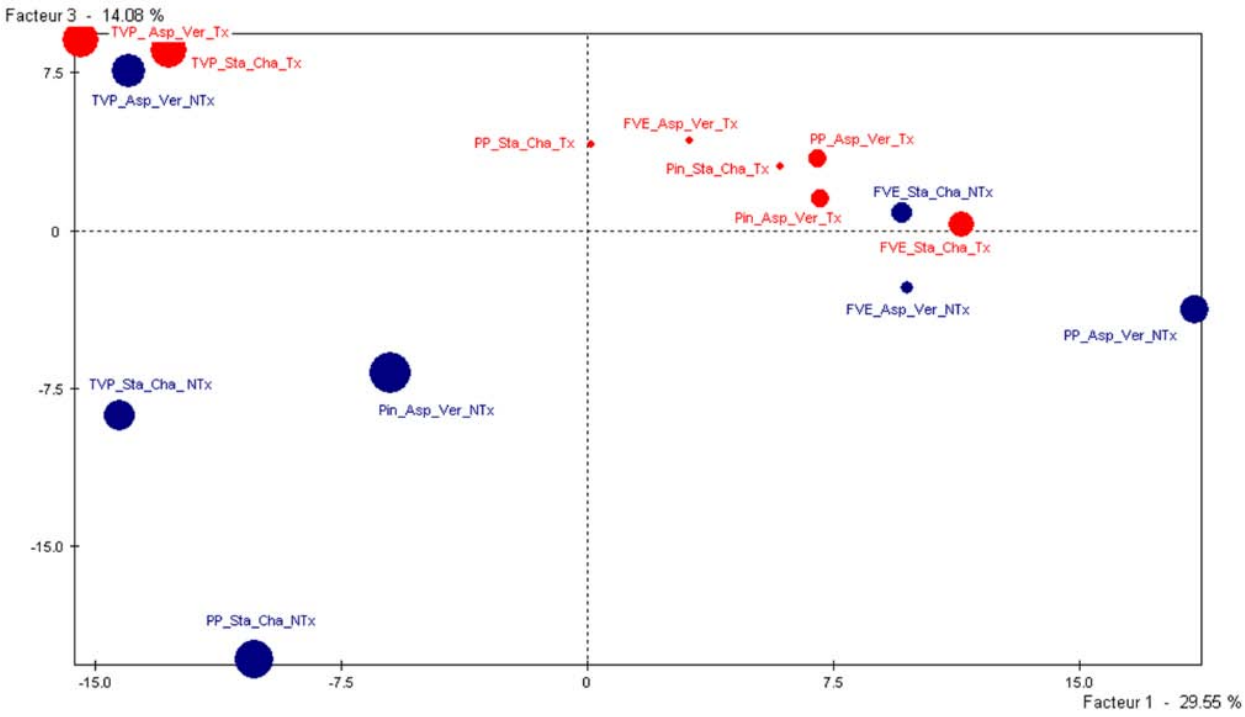


Figure 7: Plan defined by the factors 1 and 3 of the PCA, for tests performed with strains of *A.versicolor* and *S.chartarum* grown on different materials (by convention the tests are designated by "Material_toxinogenic strain character")

On this figure, the "highly toxinogenic" tests are shown in red and "weakly toxinogenic" tests are presented in blue. Dot size defines the quality of their projection on the plan.

Both populations, highly and weakly toxinogenic, were discriminated by the factor 3 indicating different chemical emissions. Indeed, the projection of individuals on the factor 3 showed the tests "highly toxinogenic strains" in positive part of this axis, where tests for "weakly toxinogenic" strains, for the majority, were projected on the negative side of this axis.

The correlations circle (Figure 8) identified the variables that present this factor 3 as well as the fragment ions responsible for the observed separation.

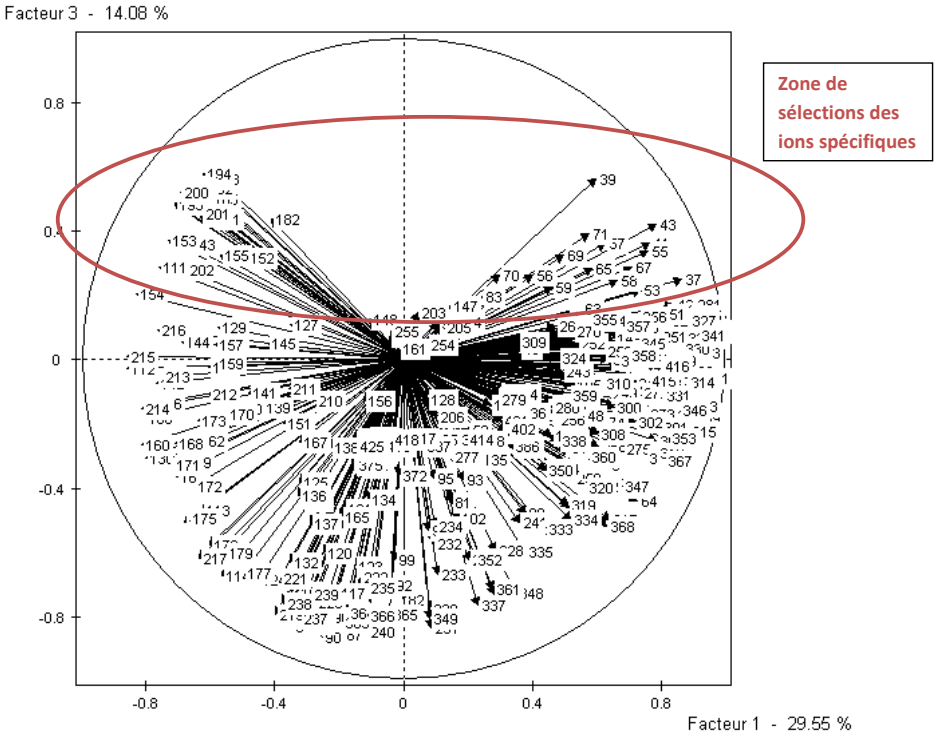


Figure 8: Representation of active variables associated with tests performed with the strains of A.versicolor and S.chartarum on different materials in the plan of factors 1 and 3

The ions contained in the correlation circle constructed, by their projection on the vertical axis, the factor 3. Only those whose contribution in the positive part of the axis was greater than 0.3 are “signing” highly toxinogenic strains and have therefore been taken into account. Thus, 41 specific ions were retained: 37, 39, 41, 43, 55, 56, 57, 58, 59, 65, 67, 69, 70, 71, 111, 143, 152, 153, 155, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201 and 202. Only ions 58 and 59 were common to the two fingerprints (obtained for *A. versicolor* and *S. chartarum*). From this selection, a new PCA was generated. Table 5 shows the weight of the axes (factors) defined by the PCA.

Table 5: Weight of factors, to narrow PCA, for the tests conducted with *A. versicolor* and *S. chartarum* strains on various growth materials

NUMERO	VALEUR PROPRE	POURCENT.	POURCENT. CUMULE
1	26.9465	65.72	65.72
2	6.5541	15.99	81.71
3	2.1601	5.27	86.98
4	1.8049	4.40	91.38
5	1.2245	2.99	94.37
6	0.6855	1.67	96.04
7	0.5541	1.35	97.39
8	0.4361	1.06	98.45
9	0.2459	0.60	99.05
10	0.1509	0.37	99.42
11	0.1125	0.27	99.70
12	0.0499	0.12	99.82
13	0.0389	0.09	99.91
14	0.0360	0.09	100.00
15	0.0000	0.00	100.00

The main axes (1 and 2), totalizing more than 80% of the overall variance, were selected to build the PCA shown on Figure 9.

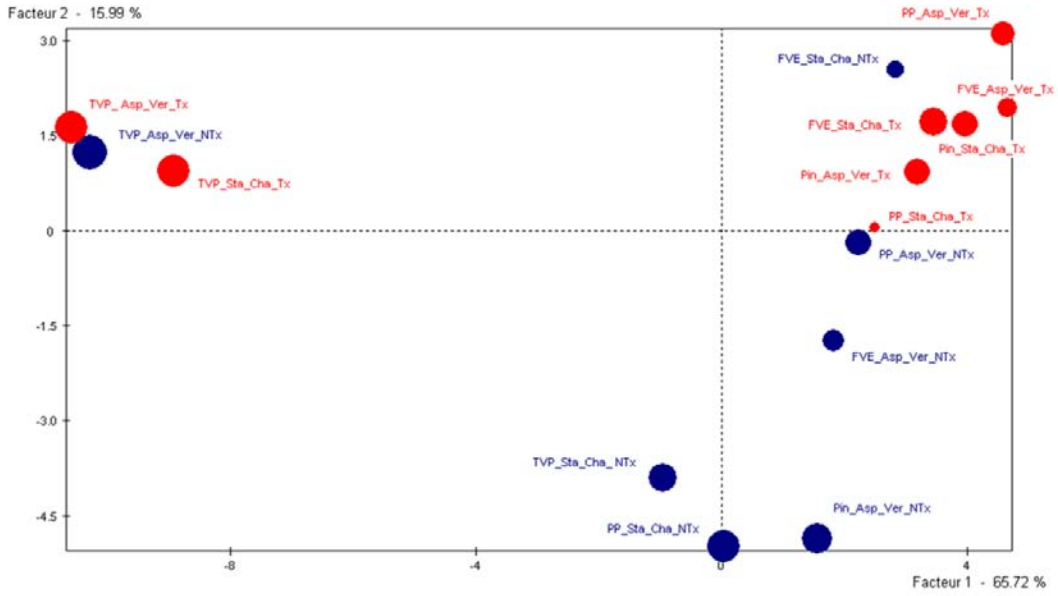


Figure 9: Representation by PCA of tests with strains of *A. versicolor* and *S. chartarum* on different growth materials in the plan of factors 1 and 2

This new analysis helped to improve the overall representativeness of individuals, the points being bigger. The tests "highly toxinogenic strains" and "weakly toxinogenic strains" were discriminated by the factor 2. Also, the fragment ions constitute a chemical fingerprint of the mycotoxinogenesis. Generated new correlation circle (Figure 10) brought up two groups of perpendicular ions therefore independent.

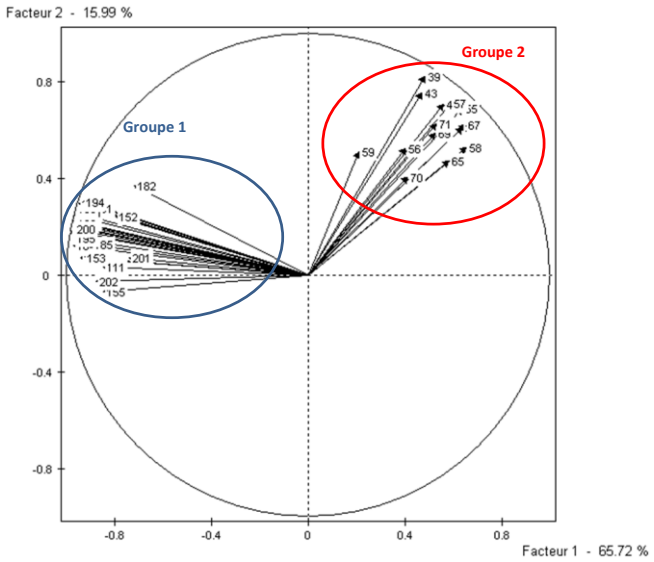


Figure 10: Representation of the active variables associated with tests with strains of *A. versicolor* and *S. chartarum* on various growth materials in the plan of the factors 1 and 2

Group 1 mainly defined the axis 1. These ions appeared associated with painted fiberglass wallpaper (FWP). Meanwhile, group 2 defined the axis 2 and allowed the separation of highly toxinogenic and weakly toxinogenic strains on other materials.

To test developed footprint, it has been applied to an independent sample. Tests were performed with the same strains grown on fiberglass and on a new medium composed of cellulose. A new PCA was generated from these new tests, keeping only the fragment ions which are forming the footprint. Table 6 shows the weight of the axes (factors) defined by the PCA.

Table 6: Weight of the factors, defined by the PCA, associated with tests of *A. versicolor* and *S. chartarum* strains on FG and cellulose material

NUMERO	VALEUR PROPRE	POURCENT.	POURCENT. CUMULE
1	24.9341	60.81	60.81
2	7.8626	19.18	79.99
3	4.6162	11.26	91.25
4	2.6253	6.40	97.65
5	0.9619	2.35	100.00
6	0.0000	0.00	100.00

The main axis (1 and 2), totalizing nearly 80% of the overall variance, were selected to construct shown PCA (Figure 11).

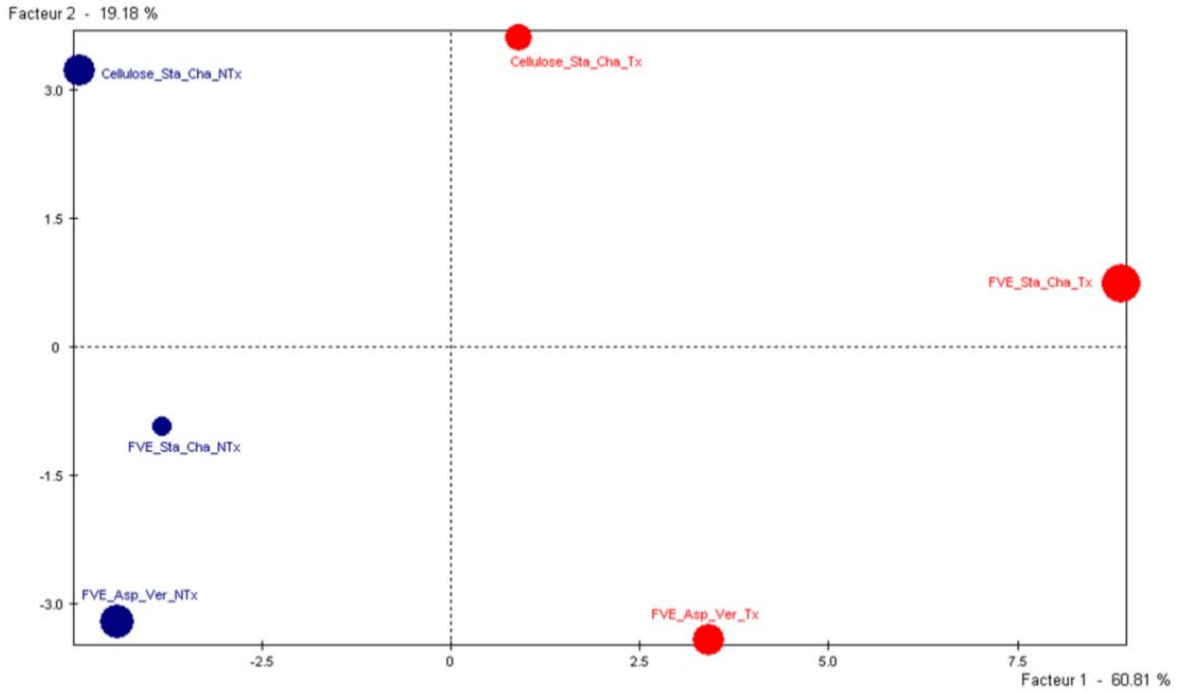


Figure 11: Representation of the tests with the *A. versicolor* and *S. chartarum* strains on FG and cellulose on the main plane of the PCA

The tests "highly toxinogenic strains" and "weakly toxinogenic strains" are discriminated by the factor 1. Also selected footprint developed from the fragment ions showed to be highly relevant.

Annex 3. Configuration of the blowing device

The blowing device placed in the closed space, contains 16 semi-circular orifices of 1 mm diameter, as showed on figure below (figure 1). It is positioned so the air stream forms an angle of 45 ° with respect to the fungal culture.

Moreover, the assembly is positioned that the distance between the bottom of the blowing nozzles and the fungal cultures was 1 cm.

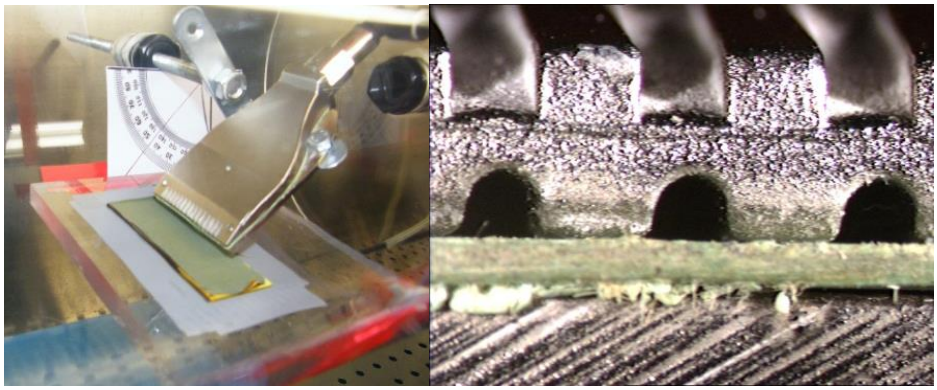


Figure 1: Blowing device and its placement

Experimental measurements were performed in the assembly according to the configuration shown on Figure 2 to further study the blowing air speed toward the culture (Figure 2).

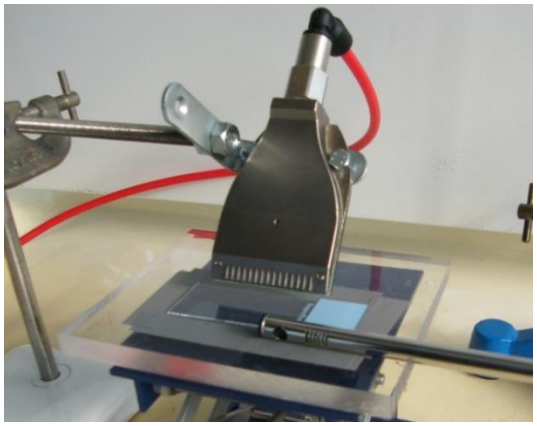


Figure 2: Configuration of the blowing air speed to the targeted material

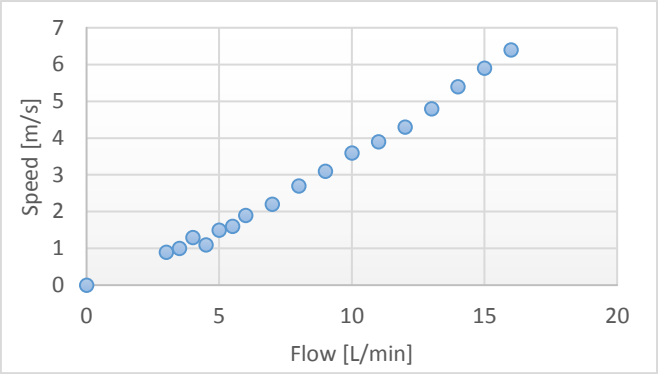


Figure 3: Air speed measured by the applied blowing airflow

The characterization of the device was specified by the modeling of airflow according to the applied blowing rate (Figure 3). The characteristics of the blowing system and its positioning relative to the surface to be aerosolized, consisting of rectangular areas of 5 cm, were used. Air flow conditions at each blowing nozzle are shown in Table 1.

Table 1: Aerodynamic conditions in each blowing nozzle

Global flow [L/min]	Flow [m^3/s]	Speed [m/s]	Reynolds
10	1,67.10-4	13,5	800
6	10-4	8,1	480
3	5.10-5	4,1	240

The turbulence is modeled by an approach Reynolds Stress Model (RSM) by solving the transport equations for each of the six components of the Reynolds tensor. The hypothesis of isotropy of the eddy viscosity is not maintained. In this model, closing averaged equations of Navier-Stokes (RANS) is obtained by solving the transport equations of the six components of the Reynolds tensor. The model RSM "Linear Pressure-Strain" was used with the options "Wall BC from equation k", "Wall reflection effects," "Wall Enhanced treatment" and "Pressure gradient effects" enabled. The boundary conditions and parameters applied to the model are presented in summary form in tables 2 and 3.

Table 2: Summary of the used model

Phenomena	**Model**	**Options**
Turbulence	*RSM_ Linear Pressure-Strain*	Enhanced Wall Treatment_Pressure gradient effects
Gravity	Considered	9,81 m^2/s
Heat equation	Deactivated	

Table 3: Summary of options of the solver

Variable	Discretization algorithm	Coefficient of under relaxation
Coupling pressure / speed	SIMPLE	
Pressure	Standard	0,2
Continuity	First order upwind	0,7
Energy turbulent kinetic	First order upwind	0,6
Rate of turbulent dissipation	First order upwind	0,6
Turbulent viscosity		1

The walls are considered as adiabatic. The air is considered as an incompressible ideal gas. The limit conditions at input and output are given in table 4.

Table 4: Limit conditions of the model

Zone	Limit conditions	Direction of speed vectors	Speed [m/s]	Turbulent intensity [%]	Hydraulic diameter [m]
Buse_jet	Velocity_inlet	Normal to limit conditions	13,5	0,07	$9{,}2.10^{-4}$
Buse_jet	Velocity_inlet	Normal to limit conditions	8,1	0,07	$9{,}2.10^{-4}$
Buse_jet	Velocity_inlet	Normal to limit conditions	4,1	0,08	$9{,}2.10^{-4}$
Limite_du_domaine	Outflow				

Figure 4 shows the velocity fields of air generated in the region of the surface for an applied air flow of 10 L/min.

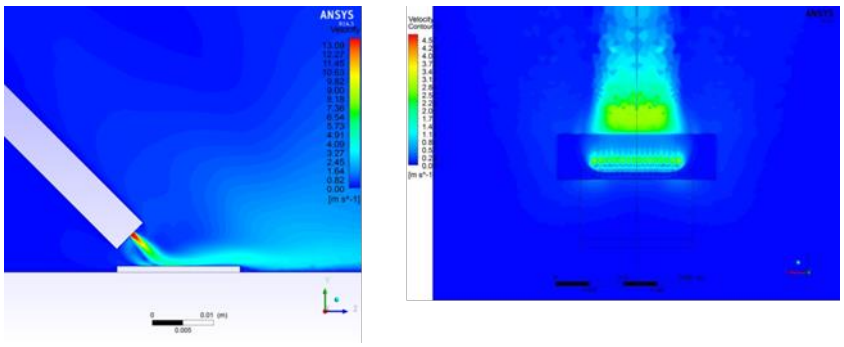


Figure 4: Representation of the velocity fields in the proximity of the surface to be aerosolized by air flow rate of 10 L/min

Near the surface, the velocity fields are distributed uniformly over a width corresponding to the width of the blowing device. The correspondence of the modeled and measured for different air flow velocities is shown on figure 5.

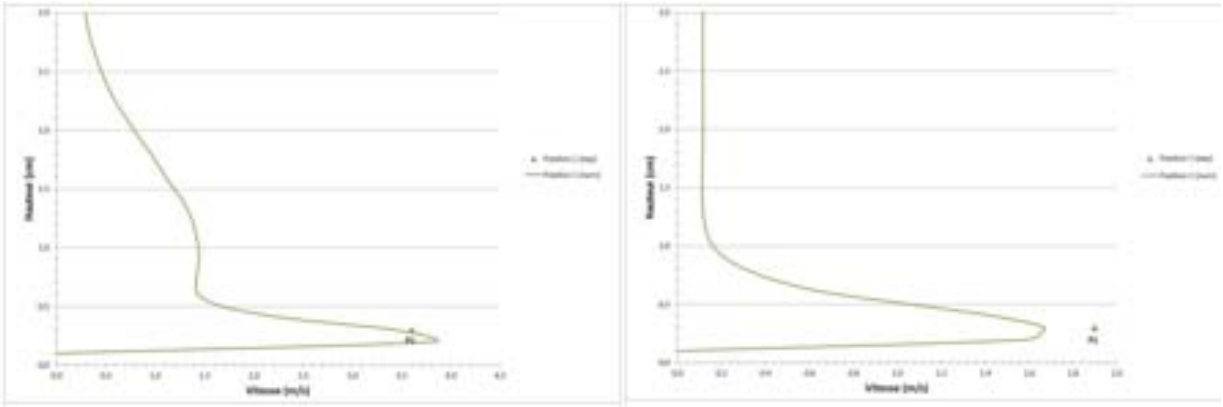


Figure 5. Representation of the modeled air velocities and flow rates measured for 6 and 10 L / min

It appears that the estimated and measured speeds, with a gap of 0.2 m/s are of the same order of magnitude for each speed and compatible with the desired airflow solicitation toward contaminated media.

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