Aerosolization of mycotoxins after growth of toxinogenic fungi on wallpaper

Brankica Aleksic[1,2], Marjorie Draghi[3], Sebastien Ritoux[3], Sylviane Bailly[1], Marlène Lacroix[1], Isabelle P. Oswald[1], Jean-Denis Bailly [1*], Enric Robine[3]

[1]Toxalim, Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, F-31000 Toulouse, France.

[2] French Environment and Energy Management Agency, Angers, France

[3] Scientific and Technical Centre for Building, Airborne Pollutants and Bioaerosol Division, Marne-la-Vallée, France

[*]Corresponding author: jd.bailly@envt.fr

Abstract

Many fungi can develop on building material in indoor environments if moisture is high enough. Among species that are frequently observed, some are known to be potent mycotoxin producers. This presence of toxinogenic fungi in indoor environments raises the question of the possible exposure of occupants to these toxic compounds by inhalation after aerosolization.

This study investigated the mycotoxin production by *Penicillium brevicompactum, Aspergillus versicolor* and *Stachybotrys chartarum* during their growth on wallpaper and the possible subsequent aerosolization of produced mycotoxins from contaminated substrates.

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. Moreover, part of the produced toxins could be aerosolized from substrate. The propensity to aerosolization differed according to the fungal

species. Thus, particles were aerosolized from wallpaper contaminated with *P. brevicompactum* when air velocity of just 0.3 m/s was applied, where *S. chartarum* required air velocity of 5.9 m/s. *A versicolor* was intermediate since aerosolization occurred under air velocity of 2 m/s.

Quantification of the toxic content revealed that toxic load was mostly associated with particles of size equal or higher of 3 µm, which may correspond to spores. However, some macrocyclic trichothecenes (especially satratoxin H and verrucarin J) can also be found on smaller particles that can penetrate deeply in the respiratory tract upon inhalation. These elements are important for risk assessment related to mouldy environments.

KEYWORDS

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

IMPORTANCE

The possible colonisation of building material by toxinogenic fungi in case of moistening raises the question of the subsequent exposure of occupants to aerosolized mycotoxins. In this study, we demonstrated that three different toxinogenic species produce mycotoxins during their development on wallpaper. These toxins can subsequently be aerosolized, at least partly, from mouldy material. This transfer to air requires air velocities that can be encountered in « real life conditions » in buildings. The most part of the aerosolized toxic load is found in particles whose size corresponds to spores or mycelium fragments. However, some toxins were also found on particles smaller than spores that are easily respirable and can deeply penetrate into human respiratory tract. All these data are important for risk assessment related to fungal contamination of indoor environments.

INTRODUCTION

In industrialized countries, people spend most of their time inside buildings (1). Many physical, chemical or microbiological pollutants can have detrimental effects for occupants, such as allergies or infections (2, 3). Among the microorganisms that are present in indoor environments, micromycetes are ubiquitous and capable of growing on most construction and decoration materials if appropriate environmental conditions are present (4–6). Thus, it is estimated that, in Northern Europe and North America, 20 to 40 % of buildings display macroscopically visible fungal growth (7, 8).

Among the fungal species commonly observed in habitats, some are known to produce toxic secondary metabolites called mycotoxins (4, 9, 10). For instance, *Aspergillus versicolor*, a potent producer of sterigmatocystin (STG), is one of the most frequent fungal contaminant of indoor environments that can be found together on building materials, in dust or in the air samples (4, 11). On the other hand, *Stachybotrys chartarum* is often isolated from building materials in homes that have suffered from water damages (12–14). This species is known to be able to produce different toxic compounds belonging to the family of macrocyclic trichothecenes (MCT) (satratoxins G (SG) and H (SH), roridin L2 (RL2), verrucarin J (VerJ)) (15, 16). On the same way, *Penicillium brevicompactum*, a species able to produce mycophenolic acid (MPA), was also frequently identified in indoor environments (17).

Such observations 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 (9) and could therefore subsequently be inhaled (18, 19).

To evaluate presence of these contaminants in indoor environments, some studies have measured mycotoxins on contaminated materials (20–23) or settled dust (24, 25). Thus, STG

could be found in more than 20 % of analyzed samples. Similarly, MCT were also found on material samples taken from water-damaged homes (13).

However the toxin quantification from material or settled dust does not predict 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 studies have focus primarily on aerosolization of conidia or fungal fragments (26–28) without associating them with mycotoxins. Only one previous work demonstrated the presence of MCT in highly respirable particles (< 1 µm) (29). In this study, the authors demonstrated that, while passing over cellulose ceiling tiles contaminated with a toxinogenic strain of *S. chartarum*, house air could be contaminated with MCT, in relation with aerosolization of fungal particles but also due to the presence of toxins on particles smaller than spores.

Within this context, the aim of this study was to quantify mycotoxins production by three fungal species often present in indoor environments that are *P. brevicompactum, A. versicolor*, and *S. chartarum,* during their growth on wallpaper. We also aimed to evaluate possible aerosolization of produced toxins as a function of both air velocity and size of released particles. Wallpaper was chosen since it has been shown that this substrate is favorable for mycotoxin production (13, 14, 21). Moreover, this material is often used in indoor furnishing and is therefore in direct contact with indoor air.

We demonstrate here that part of mycotoxins, produced on wallpaper during fungal growth, can be aerosolized following air velocities that can be encountered in buildings. Toxic load is mostly observed on particles whose size corresponds to spores but some toxins could also be found in easily respirable particles of less than 2 µm.

106 **MATERIALS AND METHODS**

107

108 **Mycotoxin standards.** Mycophenolic acid (MPA), sterigmatocystin (STG), verrucarin A
109 (VerA), o-methylsterigmatocystin (o-mSTG) and mycophenolic acid-d_3 (MPA-d_3) were
110 purchased from Sigma (Saint-Quentin Fallavier, France). Standards of satratoxin G (SG),
111 satratoxin H (SH), roridin L2 (RL2) and verrucarin J (VerJ) were gracious gift from Professor
112 J.J. Pestka (Department of Microbiology and Molecular Genetics, Michigan State University,
113 USA).

114 All standards were dissolved in methanol (MeOH) to obtain stock solutions that were
115 stored at -20 °C as recommended by manufacturer.

116

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

122 Wallpaper (WP) (Papier Peint BLAN BLA 0 INSP, Leroy Merlin) was purchased in a
123 specialized store. The material, visually clean and dry, was cut into 2×5 cm pieces and then
124 sterilized by autoclaving at 121 °C for 20 min before use(27, 30).

125

126 **Fungal strains.** *P. brevicompactum* IBT 23078 strain was a gracious gift from Dr J.B.
127 Nielsen (Assistant Professor, Technical University of Denmark, Lyngby, Denmark), *A.*
128 *versicolor* NCPT 54 was a gift from Dr O. Puel (INRA, Toulouse, France), and *S. chartarum*
129 82 (ST82) strain was previously isolated in our laboratory (31). These three strains were
130 selected for their ability to produce mycophenolic acid, sterigmatocystin and macrocyclic

131 trichothecenes, respectively. All strains were maintained in the laboratory on malt extract agar
132 (MEA, Biokar, France) at 4°C and were regularly checked for viability by culturing on MEA.
133
134 **Growth and toxinogenesis of fungi on wallpaper.** The fungal strains were grown on potato
135 dextrose agar (PDA, Biokar, France) for 14 days at 25 °C to obtain highly sporulating
136 cultures. Spores were harvested by flooding the plate with 10 mL of Tween 80 (0.05 %).
137 Spores were suspended by smoothly scraping the medium with sterile inoculating loop and
138 liquid was then collected. Spore concentration was measured by direct counting on a counting
139 cell (Malassez cell, CML, Nemours, France). Spore suspensions were then diluted to reach a
140 concentration of 2 x10^6 spores/mL and contamination was achieved by applying dropwise 500
141 µL of those suspensions (10^6 spores/sample) on the decorative side of sterile wallpaper. This
142 contamination level was previously identified as sufficient to observe a fungal development
143 within few days (31).
144 Contaminated wallpaper pieces (2 x 5 cm) were placed in flasks, on a layer of 2 cm of
145 glass beads and 8 mL of sterile water, in order to maintain moisture level at saturation
146 throughout the test, and incubated for 10 days at 25 °C in darkness. After incubation, fungal
147 growth was assessed by visual examination of samples (importance of colonized surface).
148 Both hyphae development and density of sporulated conidial heads on the whole sample
149 surface (10 cm^2) were observed by examination under stereomicroscope (magnification from
150 12 to 120) (Olympus SZX9) and under Scanning Electron Microscopy (SEM) (Jeol JSM
151 5600LV) (magnification from 40 to 30 000).
152 Some samples were used for mycotoxin determination whereas others, incubated in the
153 same conditions, were used for aerosolization as described below.
154 Initial mycotoxin baseline due to inoculum deposit on materials (= T0 value) was
155 measured using samples that were frozen immediately after spores deposit, without incubation
156 to avoid fungal growth.

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

158

159 **Aerosolization of mycotoxins from wallpaper.** To evaluate aerosolization of particles and
160 toxins from wallpaper, a specific experimental device capable of producing controlled air
161 velocities over contaminated substrates was developed. The principle of this device is shown
162 on Figure 1. To ensure the safety of the operator, the entire assembly was placed in a
163 microbiological safety cabinet.

164 The developed assembly presents cylindrical volume of 10.5 L equipped with a blowing
165 device provided with filtered humidified air (50 % RH at 22 °C) to ensure the aerodynamic
166 stresses on contaminated material. The blowing device placed in the closed space consists of
167 16 semicircular holes of 1 mm diameter (PNR industrie, Collègien, France). It was placed so
168 that the air stream forms an angle of 45 ° with respect to the contaminated material.
169 Moreover, the assembly is leveled so the distance between the bottom of the blowing nozzles
170 and the fungal cultures was 1 cm (Figure 1). Characterization of the air speed over the
171 substrate as a function of the flow from the blowing device was characterized (supplementary
172 data 1).

173 Different increasing air velocities were firstly tested to define air speeds allowing
174 significant particles' aerosolization from substrates for the three fungal species
175 (supplementary data 2). Once air velocity was defined for each fungal species, the
176 characterization of aerosol was done following air jets of 5 seconds each that were repeated
177 until the measured concentration of aerosolized particles decreased to 1 particle/L.
178 The physical characterization of the produced aerosols was carried out using an optical
179 counter (Model 3340, TSI) set at 0.1 L/min. An Andersen multi-stage impactor (Tish
180 Environmental, OH, USA)), was used for capturing particles according to 6 ranges of size and
181 aerodynamic characteristics. Each stage of the impactor was equipped with fiberglass disk to
182 collect particles and allow mycotoxin determination as described below. Filters were placed

183 on support whose thickness preserved the right distance between the orifice inlet of the
184 impactor and the filter.
185
186 **Mycotoxin determination.** Four MCT (SG, SH, VerJ and RL2), MPA and STG were
187 extracted from samples (wallpaper and fiberglass disks) by gentle mechanical agitation on an
188 agitation table (Reciprocating Shaker, IKA HS501 Digital, Grosseron, France) in
189 chloroform:methanol (2:1). Mycophenolic acid-d_3 and o-methyl sterigmatocystin were added
190 at known concentration before starting extraction in order to serve as internal standards for
191 MPA and STG respectively. For MCT, verrucarin A was chosen as internal standard as
192 already described (31).
193 After 4 hours, extracts were centrifuged for 5 min at 3500 rpm and filtered through a
194 phase separator filter (Whatman 1 PS). Filtered extracts were evaporated to dryness and
195 suspended in 1mL of methanol.
196 Quantification of mycotoxins was performed using an Acquity ultra performance liquid
197 chromatography (UPLC) system coupled to a Xevo triple quadrupole mass spectrometer
198 (Waters, Milford, MA, USA). The desolvation temperature and nitrogen flow rate were set at
199 650 °C and 800 L/h, respectively. Argon was used as the collision gas at a flow rate of 0.12
200 mL/min.
201 Mycotoxins (5 µL of samples) were eluted on an Acquity BEH C18 column (2.1 x 100
202 mm; 1.7 µm; Waters) with an AcN/H_2O gradient (*t*(0-0.5 min): 10 % AcN; *t*(0.5-4 min): 90 %
203 AcN) at a flow rate of 0.35 mL/min. Quantification was carried out by Multiple Reaction
204 Monitoring (MRM) mode in positive electrospray ionization (ESI+). MRM transitions, cone
205 voltage and collision energies used for the different toxins are listed in Table 1.
206 Chromatographic data were monitored by Masslynx 4.1 software (Waters, Milford, MA,
207 USA).

Limits of detection (LOD) were determined from 3 injections of the mycotoxins standards at the lowest concentration that could be detected with a signal to noise ≥ 3. They 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. 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 LOQs were set at 10 ng/mL for MPA, STG, RL2 and VerJ, and 100 ng/mL for SG and SH. .

For all analyzed toxins, percentage of aerosolized toxin from contaminated substrates was calculated as follow:

$$\% \text{ of airborne toxin} = \frac{\text{quantity of airborne toxin}}{\text{quantity of produced toxin on WP sample}} * 100$$

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.

225 **RESULTS**

226

227 **Growth and toxinogenesis of fungal strains on wallpaper**. After 10 days of incubation at
228 25 °C, the three tested fungal species grew and sporulated on wallpaper. Nevertheless, some
229 differences could be observed between species (Figure 2).

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

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

240 *S. chartarum* displayed an intense and regular growth with abundant hyphae colonizing
241 the whole sample's surface with many sporulated heads. Conidiophores were simple or
242 branched. Phialides, organized in clusters, bore black ellipsoidal conidia agglomerated by a
243 slimy coating.

244 Mycotoxins measurements revealed that all three species produced mycotoxin(s) during
245 their growth on wallpaper (Table 2). STG was produced in larger quantities with more than
246 110 mg/m^2. The four analysed MCT were also found. SH was the most abundant one,
247 followed by SG and RL2. Only mild amounts of VerJ were measured after growth of *S.*
248 *chartarum* ST82 strain on wallpaper.

249

Aerosolization of particles from wallpaper. To define the conditions leading to particle aerosolization from substrate as a function of the fungal species, contaminated wallpaper samples were submitted to increasing aeraulic stresses. It appeared that for *P. brevicompactum,* air velocity of 0.3 m/s was sufficient to aerosolize some particles from substrate. An increment in air velocity increased the number of aerosolized particles from wallpaper without any modification of the bioaerosol profile (Supplementary data 2). For *A. versicolor*, an air velocity of 2 m/s was required to aerosolize a significant number of particles from substrate. In order to compare aerosolization of these two species, airflow of 2 m/s was applied on contaminated wallpaper to further characterize bioaerosols and airborne mycotoxins.

By contrast, for *S. chartarum*, air speed of almost 6 m/s was needed to observe an aerosolization of particles. This air velocity was therefore applied for toxin measurement.

Characterization of bioaerosols. For *P. brevicompactum*, the application of an air velocity of 2 m/s led to the release of a total number of particles of 5.6×10^4 from mouldy wallpaper. They 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 2.3×10^3 particles/L).

For *A. versicolor*, an air velocity of 2 m/s allowed the aerosolization of a total number of 1.5×10^4 counted particles that were mostly made of fine aerosols with optical diameter about 100 nm (maximal concentration of 1.2×10^3 particles/L) and few particles with optical diameter between 2 and 8 µm (maximal concentration of 700 particles/L).

For *S. chartarum*, application of an air speed of almost 6 m/s led to the overall production of 7×10^3 counted particles from substrate with a poly-dispersed distribution of

276 particle sizes. The production of sub-micronic particles represented 77.5 % of the total
277 airborne particles.

278 The distribution of particle size in bioaerosols obtained from the three species is
279 represented in Figure 3.

280

281 **Airborne mycotoxins.** The aerosolization of mycotoxins from wallpaper was measured
282 following air velocities of 2, 2 and 6 m/s for *P. brevicompactum, A. versicolor* and *S.*
283 *chartarum*, respectively. The global mycotoxins loads of aerosols from the three fungal
284 species are reported in Table 3.

285 All analysed toxins were found in the aerosols but it appeared that the percentage of
286 airborne toxins strongly differed between them. Fifteen per cent of the MPA present on
287 wallpaper was transferred to air. It represented a total quantity of 271 ng of MPA. By
288 contrast, the percentage of aerosolized STG was only 0.2 %. However, since it was the most
289 produced toxin on wallpaper, total quantity of airborne STG reached almost 180 ng. The
290 proportion of total aerosolized MCT was 4.5 %. It has to be noted that, even if *S. chartarum*
291 required a higher air speed to be aerosolized than the two-other species, the total quantity of
292 airborne toxins was the most important for that species. Among the 4 analysed trichothecenes,
293 VerJ was the most aerosolized with 13.3 % of the initial toxic load, followed by SH, SG and
294 RL2. However, when considering the quantities that were transferred from substrate to air, SH
295 appeared predominant, representing almost 80 % of the overall toxic load.

296 In order to analyse the distribution of mycotoxins as a function of particle sizes and
297 subsequent risk of inhalation, the mycotoxin loads of each domain size of released aerosols
298 were quantified and results are presented in Table 4.

299 MPA was quantifiable on 5 of the 6 considered granulometric ranges, the maximum
300 (about 140 ng) being associated with the particles collected on the third stage of the impactor,
301 with a granulometric domain between 3.3 and 4.7 µm. For STG, no toxin was found on stages

302 corresponding to the particles with size below 2.1 µm and total mycotoxin load was
303 associated with bigger particles. Almost 95% of the toxic load was associated with particles
304 bigger than 3.3 µm. Macrocyclic trichothecenes produced by *S. chartarum* and aerosolized
305 from wallpaper were detected in all stages of Andersen collector, even on stages 5 and 6 that
306 correspond to sub-micron particles. Nevertheless, 90 % of the total toxic load (1129 ng) was
307 found on stages 1, 3 and 5.

308 The four-analysed macrocyclic trichothecenes were differently distributed within
309 Andersen collector's stages. RL2 was found on all stages. SG was exclusively found on stages
310 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
311 toxic load being associated with these two later stages whereas no toxin was measured on
312 stages 1, 2 and 4. Stage 3 that corresponds to particles ranging from 3.3 to 4.7 µm was the
313 most contaminated with 41 % of the total MCT load. It was also the only one containing all
314 four-tested macrocyclic trichothecenes.
315
316
317
318 **DISCUSSION**
319
320 The possible implication of mycotoxins in some disorders observed in occupants of mouldy
321 homes is a growing public health question, worldwide (32–34). Indeed, the risk of exposure to
322 those fungal toxic metabolites by inhalation emerged in the late 90's, when macrocyclic
323 trichothecenes produced by *S. chartarum* were implicated in the appearance of pulmonary
324 haemorrhages in infants in the USA (35). More recently, these mycotoxins were also
325 suspected to play a role in the sick building syndrome (36, 37). However, data on the direct
326 relationship between mycotoxin production on materials and their transfer to air are missing
327 and therefore do not allow precise risk assessment. That is why the present study aimed to

328 evaluate the ability of mycotoxins produced by *A. versicolor, P. brevicompactum* and *S.*
329 *chartarum* to be aerosolized from mouldy wallpaper.

330 Firstly, we investigated the ability of these three toxinogenic species to grow and
331 produce toxic compounds on wallpaper. This material, frequently used for indoor decoration,
332 allowed both mycelial growth and sporulation in conditions that can be considered as "worst
333 case" but that can be encountered in homes (25°C, humidity and darkness), especially behind
334 furnitures, during warm seasons. This is in agreement with surveys reporting a frequent
335 contamination of such materials by moulds, particularly in case of water damage (14, 21, 38,
336 39). Of note, for *P. brevicompactum* some morphological features were peculiar when this
337 species was grown on wallpaper compared to agar culture media. Indeed, usual aspect of *P.*
338 *brevicompactum* colony on agar medium is dominated by a dense felt of large and compact
339 conidiophores and a velutinous aspect of the thallus with only few trailing (40). On wallpaper,
340 the colony displayed more abundant aerial mycelium with conidiophores borne by aerial
341 hyphae. Such structure may have an important role in facilitating aerosolization of fungal
342 structures.

343 Wallpaper also allowed mycotoxin production by the tested species. Concentrations as
344 high as 112 mg/m^2, 14 mg/m^2 and 7 mg/m^2 were found for STG, SH and SG, respectively.
345 These findings are in agreement with previous studies about production on wallpaper of SG
346 and SH by Gottschalk et al. (14) and STG by Polizzi et al. (21).

347 The investigation of the aerosolization of mycotoxins produced on wallpaper firstly
348 showed that aerosolization of particles from substrate strongly differed from one species to
349 another, possibly related to mycelium organization and conidial structures. As an illustration,
350 both *A. versicolor* and *P. brevicompactum* are fungal species characterized by the presence of
351 small and light spores organized in chains at the extremity of phialides (41). For these two
352 species, air velocity of 2 m/s, which matches air speed observed due to mechanical and
353 natural ventilation in tertiary buildings (28), allowed the aerosolization of numerous particles

354 from wallpaper. These particles were distributed in two main categories: one including very
355 small particles, smaller than 0.15 µm, and the second including particles ranging from 2 to 6
356 µm. This second group may correspond to spores, groups of spores or mycelium debris (40,
357 41), in agreement with previous data on aerosolization of these fungal species (42–44). One
358 can note that for *P. brevicompactum,* the total number of particles aerosolized from substrate
359 was higher than for *A. versicolor*. This is in relation with the disposition of spores on mycelial
360 structures. In *P. brevicompactum*, long chains of spores are borne by aerial conidiophores and
361 may easily be aerosolized. For *A. versicolor*, spores' chains are shorter and located on tight
362 and compact phialides, making them mildly more difficult to aerosolize from material.

363 For *S. chartarum*, a higher air velocity was required for aerosolization from substrate.
364 An air velocity of about 6 m/s is more frequent outdoor but could also be encountered in
365 buildings due to mechanical ventilation (28). The use of fans may also generate airflows able
366 to aerosolize *S. chartarum*. Of note, the total number of airborne particles was lower than that
367 observed for other species and this may explain why this fungal species is not commonly
368 observed in air samples and is more frequently found by direct examination of building
369 materials (12-14). In case of a sufficient airflow, a poly-dispersed particle cloud was
370 generated from *S. chartarum* contaminated substrate. There was an important cluster made of
371 particles ranging from 0.4 to 1 µm, which are therefore smaller than spores. It could
372 correspond to micro-fungal particles, debris of wallpaper released from substrate due to
373 cellulolytic activity of *S. chartarum,* or exudate droplets from culture (45). Such finding is
374 important since these small particles could easily penetrate deeply in human respiratory tract
375 in case of inhalation.

376 All tested mycotoxins were found in aerosols generated from mouldy wallpaper and the
377 proportion transferred to air varied with fungal species. MPA was the most aerosolized, with
378 15 % of the produced toxin. This is related to the higher facility of *P. brevicompactum* to be
379 aerosolized from substrate compared to other species. By contrast, the proportion of airborne

380 STG was low (0.2 %). Since numerous particles can be released from substrate contaminated
381 with *A. versicolor*, this suggests that STG could be located in fungal parts that are strongly
382 adherent to the substrate and probably mainly present/located in mycelium (5, 7). However,
383 considering that STG was the major produced toxin, the quantity of airborne STG was
384 comparable to MPA.

385 For MCT, even if the required air speed for aerosolization was higher, it has to be
386 highlighted that the four analyzed toxins were found in aerosols and total aerosolized toxic
387 load was 5 times higher than that of other toxins.

388 The analysis of the toxin distribution according to the aerosol profile and size of released
389 particles also brought some important information. For MPA and STG, maximal toxic load
390 was found on particles whose size corresponds to spores, groups of spores or mycelium
391 debris. However, low proportion of MPA was also found on particle smaller than spores. It
392 could be related to the excretion of part of the toxin in exudate droplets as previously
393 demonstrated for other *Penicillia* (46). The excreted toxin could be then adsorbed on small
394 particles of dust.

395 The distribution of MCT was different. Toxins were found in all stages of Andersen
396 collector, even those collecting particles smaller than spores. This result is in agreement with
397 a study by Brasel et al. (29). As for *P. brevicompactum*, it could be the result of the excretion
398 of MCT by fungus in droplets outside the mycelium (45) and their adsorption on dust
399 particles or wallpaper debris generated by cellulolytic activity of *S. chartarum*.

400 Of note, the analyzed MCT differed regarding their distribution in the various particles
401 sizes. This result suggests that the different MCT analyzed in this study could be differently
402 distributed/excreted within fungal structures. Further studies are required to characterize the
403 distribution of macrocyclic trichothecenes in *S. chartarum* mycelium. It would help better
404 understanding the biosynthetic pathway and processing of these compounds in fungal cells.

All these results on aerosolization of mycotoxin 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. (47) 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.

CONCLUSION. This study demonstrated that during their growth on wallpaper, *P. brevicompactum*, *A. versicolor* and *S. chartarum,* that are frequently found indoor, produce mycotoxins. These toxins can subsequently be aerosolized, at least partly, from mouldy material. This transfer to air requires air velocities that can be encountered in building since they correspond to movement of people in a room (0.2 m/s), air speed in ceiling diffusers (2 m/s), slamming door,air drafts from opening the window or mechanical ventilation (6 m/s).

Most 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 found also on particles smaller than spores, that could be easily inhaled by occupants 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 the possible toxicity associated to inhalation of these toxins.

Acknowledgements

The authors would like to thank Professors JJ Pestka (Department of Microbiology and Molecular Genetics, Michigan State University, USA) for toxins' standards; Dr J.B. Nielsen

(Technical University of Denmark, Lyngby, Denmark) for strain of *P. brevicompactum* and Dr O. Puel (INRA, UMR Toxalim) for strain of *A. versicolor*.

Funding sources

This work was financed by the French Ministry of Ecology, Sustainable Development and Energy (PRIMEQUAL project DSC-BIO/2013-121) and by the French Environment and Energy Management agency (ADEME) and the Scientific and Technical Centre for Building (CSTB) (Ph.D. grant for B. Aleksic).

Author contribution statements

Brankica Aleksic performed experiments and participated to the article redaction; Marjorie Draghi and Sebastien Ritoux designed the device to study aerosolization of particles and mycotoxins from mouldy wallpaper; Sylviane Bailly did the morphological analysis of wallpaper after fungal development and participated to the development of the analytical method for mycotoxin measurement; Marlène Lacroix did the toxin measurement on both wallpaper and aerosols; Isabelle P. Oswald participated to the overall supervision of the project and to the redaction of the article; Jean-Denis Bailly and Enric Robine supervised the work and participated to the redaction of the article, Enric Robine took the SEM photos.

REFERENCES

1. Brown L. 1983. National Radiation Survey in the UK: Indoor Occupancy Factors. Radiat Prot Dosim 5:203–208.

2. Le Moullec Y, Squinazi F. 1996. Pollution atmosphérique à l'intérieur des bâtiments: sources expositions et risques sanitaires. Conseil supérieur d'hygiène publique de France - Section de l'Evaluation des risques de l'environnement sur la santé, Lavoisier - Tec & Doc., Paris.

3. Squinazi F. 2002. La pollution de l'air à l'intérieur des bâtiments (allergènes exclus). Rev française d'allergologie d'immunologie Clin 42:248–255.

4. Andersen B, Frisvad JC, Sondergaard I, Rasmussen IS, Larsen LS. 2011. Associations between fungal species and water-damaged building materials. Appl Environ Microbiol 77:4180–4188.

5. Nielsen KF, Gravesen S, Nielsen PA, Andersen B, Thrane U, Frisvad JC. 1999. Production of mycotoxins on artificially and naturally infested building materials. Mycopathologia 145:43–56.

6. Polizzi V, Adams A, De Saeger S, Van Peteghem C, Moretti A, De Kimpe N. 2012. Influence of various growth parameters on fungal growth and volatile metabolite production by indoor molds. Sci Total Environ 414:277–286.

7. Nielsen KF. 2002. Mould growth on building materials. Secondary metabolites, mycotoxins and biomarkers.

8. Moularat S, Derbez M, Kirschner S, Ramalho O, Robine E. 2008. Les moisissures dans les environnements intérieurs et leurs effets sur la santé. Pollut Atmos 197:34–37.

9. Jarvis BB, Miller JD. 2005. Mycotoxins as harmful indoor air contaminants. Appl Microbiol Biotechnol 66:367–372.

10. Verdier T, Coutand M, Bertron A, Roques C. 2014. A review of indoor microbial growth across building materials and sampling and analysis methods. Build Environ 80:136–149.

11. Lappalainen S, Kähkönen E, Loikkanen P, Palomäki E, Lindroos O, Reijula K. 2001. Evaluation of priorities for repairing in moisture-damaged school buildings in Finland.

Build Environ 36:981–986.

12. Bellanger AP, Reboux G, Roussel S, Grenouillet F, Didier-Scherer E, Dalphin JC, Millon L. 2009. Indoor fungal contamination of moisture-damaged and allergic patient housing analysed using real-time PCR. Lett Appl Microbiol 49:260–266.

13. Bloom E, Nyman E, Must A, Pehrson C, Larsson L. 2009. Molds and mycotoxins in indoor environments — a survey in water-damaged buildings. J Occup Environ Hyg 6:671–678.

14. Gottschalk C, Bauer J, Meyer K. 2006. Determination of macrocyclic trichothecenes in mouldy indoor materials by LC-MS/MS. Mycotoxin Res 22:189–192.

15. Cabral JPS. 2010. Can we use indoor fungi as bioindicators of indoor air quality? Historical perspectives and open questions. Sci Total Environ 408:4285–4295.

16. Pestka JJ, Yike I, Dearborn DG, Ward MDW, Harkema JR. 2008. Stachybotrys chartarum, trichothecene mycotoxins, and damp building-related illness: New insights into a public health enigma. Toxicol Sci 104:4–26.

17. Reboux G, Bellanger AP, Roussel S, Grenouillet F, Sornin S, Piarroux R, Dalphin JC, Millon L. 2009. Indoor mold concentration in Eastern France. Indoor Air 19:446–453.

18. Eduard W. 2009. Fungal spores: A critical review of the toxicological and epidemiological evidence as a basis for occupational exposure limit setting. Crit Rev Toxicol 39:799–864.

19. Le Bars J, Le Bars P. 1985. Etude du nuage de spores de Stachybotrys atra contaminant des pailles: risque d'inhalation. Bull la Soc Fr Mycol Medicale 2:321–324.

20. Charpin-Kadouch C, Maurel G, Felipo R, Queralt J, Ramadour M, Dumon H, Garans M, Botta A, Charpin D. 2006. Mycotoxin identification in moldy dwellings. J Appl Toxicol 26:475–479.

21. Polizzi V, Delmulle B, Adams A, Moretti A, Susca A, Picco AM, Rosseel Y, Kindt R, Van Bocxlaer J, De Kimpe N, Van Peteghem C, De Saeger S. 2009. JEM Spotlight:

Fungi, mycotoxins and microbial volatile organic compounds in mouldy interiors from water-damaged buildings. J Environ Monit 11:1849–1858.

22. Täubel M, Sulyok M, Vishwanath V, Bloom E, Turunen M, Järvi K, Kauhanen E, Krska R, Hyvärinen A, Larsson L, Nevalainen A. 2011. Co-occurrence of toxic bacterial and fungal secondary metabolites in moisture-damaged indoor environments. Indoor Air 21:368–375.

23. Tuomi T, Reijula K, Johnsson T, Hemminki K, Hintikka EL, Lindroos O, Kalso S, Koukila-Kähkölä P, Mussalo-Rauhamaa H, Haahtela T. 2000. Mycotoxins in crude building materials from water-damaged buildings. Appl Environ Microbiol 66:1899–1904.

24. Bloom E, Bal K, Nyman E, Must A, Larsson L. 2007. Mass spectrometry-based strategy for direct detection and quantification of some mycotoxins produced by Stachybotrys and Aspergillus spp. in indoor environments. Appl Environ Microbiol 73:4211–4217.

25. Engelhart S, Loock A, Skutlarek D, Sagunski H, Lommel A, Farber H, Exner M. 2002. Occurrence of toxigenic Aspergillus versicolor isolates and sterigmatocystin in carpet dust from damp indoor environments. Appl Environ Microbiol 68:3886–3890.

26. Cho SH, Seo SC, Schmechel D, Grinshpun S a., Reponen T. 2005. Aerodynamic characteristics and respiratory deposition of fungal fragments. Atmos Environ 39:5454–5465.

27. Górny RL, Reponen T, Grinshpun S, Willeke K. 2001. Source strength of fungal spore aerosolization from moldy building material. Atmos Environ 35:4853–4862.

28. Kanaani H, Hargreaves M, Ristovski Z, Morawska L. 2009. Fungal spore fragmentation as a function of airflow rates and fungal generation methods. Atmos Environ 43:3725–3735.

29. Brasel TL, Douglas DR, Wilson SC, Straus DC. 2005. Detection of airborne

Stachybotrys chartarum macrocyclic trichothecene mycotoxins on particulates smaller than conidia. Appl Environ Microbiol 71:114–122.

30. Peitzsch M, Bloom E, Haase R, Must A, Larsson L. 2012. Remediation of mould damaged building materials—efficiency of a broad spectrum of treatments. J Environ Monit 14:908–915.

31. Aleksic B, Bailly S, Draghi M, Pestka JJ, Oswald IP, Robine E, Bailly JD, Lacroix MZ. 2016. Production of four macrocyclic trichothecenes by Stachybotrys chartarum during its development on different building materials as measured by UPLC-MS/MS. Build Environ 106:265–273.

32. WHO. 2009. WHO guidelines for indoor air quality : dampness and mold. WHO Eur 277–303.

33. Hope J. 2013. A review of the mechanism of injury and treatment approaches for illness resulting from exposure to water-damaged buildings, mold, and mycotoxins. Sci World J 2013:6–10.

34. Gutarowska B, Piotrowska M. 2007. Methods of mycological analysis in buildings. Build Environ 42:1843–1850.

35. Dearborn DG, Yike I, Sorenson WG, Miller MJ, Etzel R a. 1999. Overview of investigations into pulmonary hemorrhage among infants in Cleveland, Ohio. Environ Health Perspect 107:495–499.

36. Hossain MA, Ahmed MS, Ghannoum MA. 2004. Attributes of Stachybotrys chartarum and its association with human disease. J Allergy Clin Immunol 113:200–209.

37. Kuhn DM, Ghannoum M a. 2003. Indoor Mold , Toxigenic Fungi , and Stachybotrys chartarum : Infectious Disease Perspective Fungal Organisms in Damp Buildings. Clin Microbiol Rev 16:144–172.

38. Bloom E, Grimsley LF, Pehrson C, Lewis J, Larsson L. 2009. Molds and mycotoxins in dust from water-damaged homes in New Orleans after hurricane Katrina. Indoor Air

19:153–158.

39. Nunez M, Sivertsen MS, Mattsson J. 2012. Growth preferences on substrate , construction , and room location for indoor moulds and Actinomycetes, p. Vol 1, 5H.3.. *In* Proceedings of Healthy Buildings 2012.

40. Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B. 2010. Food and Indoor fungi. CBS Laboratory Manual Series, CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.

41. Pitt JI, Hocking AD. 2009. Fungi and Food Spoilage. Springer US, Boston, MA.

42. Afanou KA, Straumfors A, Skogstad A, Nilsen T, Synnes O, Skaar I, Hjeljord L, Tronsmo A, Green BJ, Eduard W. 2014. Submicronic Fungal Bioaerosols: High-Resolution Microscopic Characterization and Quantification. Appl Environ Microbiol 80:7122–7130.

43. Gorny RL, Ławniczek-Wałczyk A. 2012. Effect of two aerosolization methods on the release of fungal propagules from a contaminated agar surface. Ann Agric Environ Med 19:279–284.

44. Madsen AM, Larsen ST, Koponen IK, Kling KI, Barooni A, Karottki DG, Tendal K, Wolkoff P. 2016. Generation and characterization of indoor fungal aerosols for inhalation studies. Appl Environ Microbiol 82:2479–2493.

45. Gareis M, Gottschalk C. 2014. Stachybotrys spp. and the guttation phenomenon. Mycotoxin Res 30:151–159.

46. Gareis M, Gareis E-M. 2007. Guttation droplets of Penicillium nordicum and Penicillium verrucosum contain high concentrations of the mycotoxins ochratoxin A and B. Mycopathologia 163:207–214.

47. Carey SA, Plopper CG, Hyde DM, Islam Z, Pestka JJ, Harkema JR. 2012. Satratoxin-G from the black mold Stachybotrys chartarum induces rhinitis and apoptosis of olfactory sensory neurons in the nasal airways of rhesus monkeys. Toxicol Pathol 40:887–898.

587

Applied and Environmental Microbiology

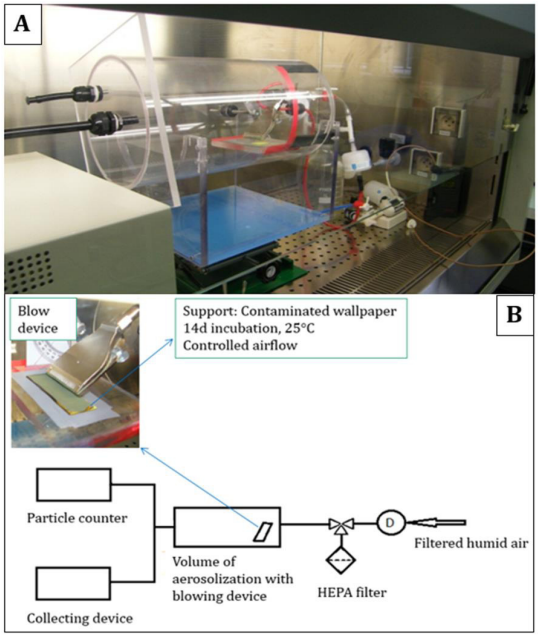


Figure 1. Experimental assembly used for aerosolization of mycotoxins from wallpaper (A)
And schema of the experimental assembly (B)

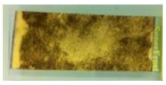
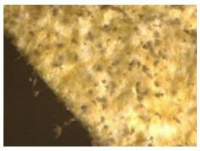
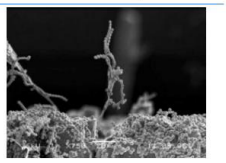
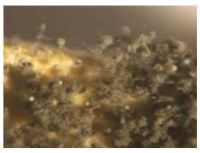
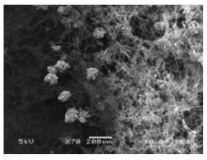
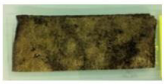
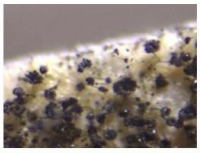
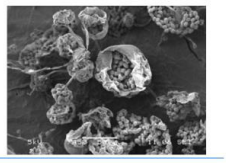
Species	Mycelial development	Macroscopic aspect of contaminated wallpaper		Microscopic aspect of contaminated wallpaper
		Observation with naked eye	Observation under stereomicroscope	Observation by SEM
Penicillium brevicompactum	+++			
Aspergillus versicolor	+++			
Stachybotrys chartarum	++++			

Figure 2. Macroscopic, under stereomicroscope and SEM observations of mycelial growth on wallpaper contaminated with different species.

++++: colonisation of whole sample; +++: development on about 4/5 of the sample

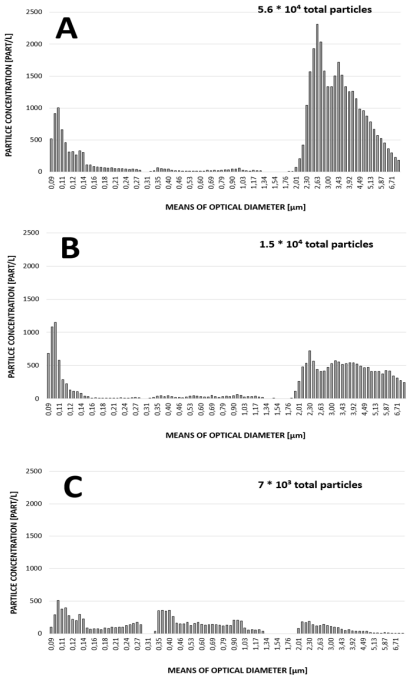


Figure 3. Granulometric profiles of aerosols from *P. brevicompactum* (A), *A. versicolor* (B) and *S. chartarum* (C) following aeraulic solicitations on contaminated wallpaper with airflows of 2, 2 and 6 m/s, respectively

Table 1. 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

Table 2. Toxin(s) production on wallpaper contaminated by three different toxigenic fungal strains

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

ND: not detected; MPA: mycophenolic acid; STG: sterigmatocystin; MCT: macrocyclic trichothecenes; RL2: Roridin L2; VerJ: Verrucarin J; SG: Satratoxin G; SH: Satratoxin H

Table 3. Global mycotoxin content of aerosols generated from wallpaper

Species	Toxin(s)	Air velocity [m/s]	Total quantity of airborne toxin [ng]	% of emitted toxin
P. brevicompactum	**MPA**	2	271	15
A.versicolor	**STG**	2	179	0.2
S.chartarum	**Total MCT**	6	1260	4.5
	RL2		64	1.1
	VerJ		80	13.3
	SG		102	1.4
	SH		1014	7.1

ND: not detected; MPA: mycophenolic acid; STG: sterigmatocystin; MCT: macrocyclic trichothecenes; RL2: Roridin L2; VerJ: Verrucarin J; SG: Satratoxin G; SH: Satratoxin H

Table 4. Quantification of mycotoxins in the different stages of the Andersen collector

		Quantity of emitted toxin [ng]						
Stage	**Size range [µm]**	**MPA[1]**	**STG[2]**	**MCT[3] (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: mycophenolic acid; STG: sterigmatocystin; MCT: macrocyclic trichothecenes; RL2: Roridin L2; VerJ: Verrucarin J; SG: Satratoxin G; SH: Satratoxin H

1 – produced by *P.brevicompactum;* 2 - produced by *A. versicolor;* 3 - produced by *S.chartarum*