ACADEMIE UNIVERSITAIRE WALLONIE-EUROPE
UNIVERSITE DE LIEGE
FACULTE DE MEDECINE VETERINAIRE

DEPARTEMENT GIGA-INFLAMMATION, INFECTION AND IMMUNITY
LABORATOIRE D'IMMUNOLOGIE CELLULAIRE ET MOLÉCULAIRE

Développement d'outils moléculaires de détection des moisissures présentes dans l'air intérieur afin de déterminer leur impact sur la santé publique

Development of molecular tools for rapid detection and quantification of indoor airborne molds to assess their impact on public health

Libert Xavier

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Résumé

Aujourd'hui, la contamination de l'environnement intérieur par des moisissures aéroportées est considérée comme un problème de santé publique. Les méthodes analytiques classiques de surveillances, basées sur la culture et l'identification microscopique, présentent des limitations liées à la dépendance vis-à-vis de la culture et du temps requis pour les analyses.
Par conséquent des biais peuvent être introduits, notamment concernant la fraction morte (champignons non-cultivables ou morts), pouvant avoir potentiellement un impact sur la santé humaine.

Dans ce contexte, les outils moléculaires semblent être d'excellentes alternatives pour la surveillance des contaminations fongiques aéroportées d'intérieurs. Ainsi, différents outils moléculaires ont été développés lors de cette thèse pour détecter et identifier les champignons dans l'air intérieur (qPCR SYBR®green, High Resolution Melting (HRM), Luminex xMAP® et NGS). L'objectif étant d'améliorer la détection des contaminants fongiques, la fraction morte inclue, par rapport aux méthodes de surveillance classiquement utilisées, mais également d'améliorer les connaissances actuelles sur la contamination fongique aéroportée d'intérieur.

Ainsi, un test PCR en temps réel (qPCR) a été développé pour la détection d'*Aspergillus versicolor*, une moisissure pathogène de l'air intérieur,et d'*Exophiala jeanselmei*, une moisissure pathogène suspectée de faire partie de la "fraction morte". Bien que validée selon des critères stricts prouvant la qualité des outils développés, des limites, notamment concernant la discrimination des espèces génétiquement proches et le multiplexage ont été observées.
Dans cette thèse, la première question a été résolue par l'utilisation d'une analyse post-PCR *High Resolution Melting* (HRM). Utilisé tel un *proof of concept*, le HRM a été testé sur 3 *Aspergillus* génétiquement proches (*A. versicolor*, *Aspergillus creber* et *Aspergillus sydowii*) démontrant que son utilisation peut améliorer le suivi de ce type de contaminants.

La problématique du multiplexage a été résolue grâce à la technologie Luminex xMAP®. Développé pour la détection simultanée de 10 moisissures fréquemment observées dans l'air intérieur. Ainsi, il a été démontré que l'introduction de cette technique dans un protocole de monitoring permettrait de réduire le temps d'analyse et, *in fine*, le temps de transmission des résultats à l'équipe médicale en charge du patient. Cependant, puisqu'une sélection des espèces à identifier est nécessaire avant l'analyse, cette technologie ne convient pas pour l'étude de la diversité fongique.
Dans ce contexte, la technologie *next generation sequencing* (NGS) semble offrir une alternative valable en tant qu'outil universel d'identification des moisissures intérieures. Dans cette étude, la fraction « morte » a été investiguée grâce à une analyse NGS métagénomique, permettant par la

même occasion de détecter pour la première fois *E. jeanselmei* dans l'air intérieur. De plus, une analyse de la diversité fongique de l'air intérieur provenant de résidences contaminées a été réalisée démontrant qu'une analyse NGS métagénomique peut contribuer à l'amélioration des données sur la diversité fongique aéroportée d'intérieur, nécessaire au développement de méthodes de détections, ainsi que de tests immunologiques représentatifs de cette diversité.

Les méthodes développées ainsi que les résultats obtenus lors de ce doctorat sont une première étape pour une meilleure compréhension du lien existant entre les champignons aéroportés présents dans l'intérieur de l'habitat et la santé publique.

Summary

Currently, contamination of the indoor environment by fungi is suggested to be a public health problem, although scientific evidence on the causal link is still limited. The monitoring of indoor airborne fungal contamination is a common tool to help understanding the link between fungi in houses and respiratory problems. Classical monitoring methods, based on cultivation and microscopic identification, have some limitations. For example, uncultivable or dead fungi ("unknown" fraction) cannot be identified, although they could have an impact on human health.

In this context, molecular tools seem to be a valuable alternative. In this PhD work, different molecular tools were developed, from simplex to multiplex, to detect and identify indoor airborne fungi. The goal was to improve the detection of fungal contaminants, including the "unknown" fraction, as compared to the currently used classical monitoring methods. The necessary air sampling and DNA extraction protocols, adapted to the downstream molecular monitoring methods have also been developed. Through the application of the developed tools to specific case studies, we aimed to improve the current knowledge on fungal contamination.

At first, we developed a specific ITS-based SYBR®green real-time PCR (qPCR) assay for *Aspergillus versicolor*, a species frequently observed in indoor air and known to be allergenic. Additionally, an ITS-based qPCR assay was developed for the specific detection of *Exophiala jeanselmei*, a pathogenic yeast suspected to be a part of the "unknown fraction". The performance of these qPCR methods was assessed. This comparison demonstrated that SYBR®green qPCR assays can be used as a molecular alternative for monitoring of contaminated samples while eliminating the need for culturing and thereby considerably decreasing the required analysis time.

However, qPCR has some limitations especially concerning the discrimination of genetically close species and multiplexing. The first issue was addressed through the use of post-qPCR high resolution melting (HRM) analysis, providing a proof-of-concept for this approach, using 3 closely related *Aspergillus*, i.e., *A. versicolor*, *Aspergillus creber* and *Aspergillus sydowii*. This HRM tool will allow a more accurate monitoring of these closely related indoor air contaminants, thereby contributing to an improved insight in the causal link between the specific presence of these species and health issues.

The multiplexing issue was overcome through a Luminex xMAP® assay, developed for the simultaneous detection of the 10 most frequently in indoor air found fungi. All the species identified with the classical method were also detected with the xMAP® assay, however in a shorter time frame, and using less sample material. This assay will improve the communication with the involved medical team and the patient.

To provide scientific evidence for the causal link between indoor airborne fungi and health

problems, the full diversity needs however to be identified. This cannot be achieved by using a targeted assay. Therefore, next generation sequencing (NGS) could offer a valuable alternative as an open approach multiplex monitoring method. An NGS-based metagenomics approach was used to investigate the "unknown" agents in air samples of offices in contact with air-conditioning reservoirs and showed the first detection of *E. jeanselmei* in indoor air. Finally, a metagenomics analysis was performed to investigate the indoor airborne fungal diversity in contaminated residences in Brussels where people with health problems were living. This demonstrated that NGS could contribute to improved data concerning the indoor airborne fungal diversity, as compared to the currently used classical methods.

The methods developed in this PhD work and the insights obtained are a first step for a better understanding of the causal link between indoor airborne fungi and public health.

Abbreviations

µl	microliter
ΔC_q	quantitative cycle difference
ΔG	Gibb's energy
A_c	Avogadro's constant
Aversi_ITS assay	qPCR SYBR®green assay developed for the detection of *Aspergillus versicolor*
BCCM/IHEM	Biomedical Fungi and Yeasts Collection
BLAST	basic local alignment search tool
BLAST	standard nucleotide BLAST
bp	base pair
CFU	colony forming unit
CFU/m³	colony forming unit per cubic meter
CFU/m³	colony forming unit per cubic meter
CFU/ml	colony forming unit per milliliter
C_n	genomic copy number
C_q	quantitative cycle
CRIPI	*Cellule Régionale d'Intervention en Pollution Intérieure* from Brussels Environment
CTAB	hexodecyltrimethylammonium bromide
ddNTP	2',3'-deoxythimidine triphosphate
DNA	desoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsDNA	double stranded desoxyribonucleic acid
E	efficiency
EDTA	ethylenediaminetetraacetic acid
Ejeanselmei_ITS assay	qPCR SYBR® green assay developed for the detection of *Exophiala jeanselmei*
ELISA	enzyme-linked immunosorbent assay
ENGL	European Network of GMO Laboratories
EPA	Environmental Protection Agency of the Unites States of America
ERMI	environmental relative moldiness index
FNR	false negative ratio
FPR	false positive ratio
FRET	fluorescence resonance energy transfer
g	unit of speed
gDNA	genomic desoxyribonucleic acid
GIGA	*Groupe Interdisciplinaire de Génoprotéomique Appliquée*
GMO	genetically modified organism
G_s	genome size
HPLC	high performance liquid chromatography
HRM analysis	high resolution melting analysis
IgE	immunoglobuline E
IgG	immunoglobuline G
ITS	internal transcribed spacer

ITS-1	internal transcribed spacer 1
ITS-2	internal transcribed spacer 2
l/min	liter per minutes
LDA	ligation dependent amplification
LNA	locked nucleic acids
LOD	limit of detection
LSU	large subunit of ribosomal RNA
m	amount of DNA
m^3	cubic meter
Mb	megabase
MEA	malt extract agar
MFI	median fluorescence intensity
MFI	median fluorescence intensity value
mg	milligrams
min	minutes
ml	milliliters
MSQPCR	mold-specific qPCR
MUSCLE	multiple sequence comparison by log-expactation
M_w	base pair mean molecular weight
NCBI	National Center of Biotechnology Information
ND	not determined
ng	nanograms
NGS	next generation sequencing
NTC	no template control
NRL	National Reference Laboratory
OTU	operational taxonomic unit
PacBio	Pacific Biosciences
PC	positive control
PCR	polymerase chain reaction
PG	pulse group of the air-conditioning system
PNA	peptide nucleotide acids
qPCR	real-time polymerase chain reaction
r	repeatability
R^2	coefficient of determination
RAST	radioallergosorbent test
RCS	Reuter Centrifugal Sampler® plus – air sampler
rDNA	ribosomal DNA
RFU	relative fluorescence unit
RNA	ribonucleic acid
RSDr	relative standard deviation of the repeatability
s	seconds
SAPE	streptavidin-R-phycoerythrin
SBS	sick building syndrome
SD	standard deviation
SMRT	sequencing single-molecule real-time sequencing
SMRT	single molecule real-time sequencing
spores/m^3	spores per cubic meter
SSU	small subunit of ribosomal RNA

SN	signal-to-noise ratio
T_m	melting temperature
Taq	*Thermus aquaticus* (DNA polymerase)
TMAC	tetramethylammonium chloride
ULg	University of Liège
VFMs	volatile fungal metabolites
VOCs	volatile organic compounds
WHO	World Health Organisation
WIV-ISP	*Wetenschappelijk Instituut Volksgezondheid – Institut Scientific de Santé Public*
xMAP	MAP: multi-analyte profiling, x: the unknown

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Chapter 1

General introduction, rationale and outline of the PhD thesis

1.1 Indoor air pollution and public health

Currently, poor indoor air quality is considered as a public health issue, this is important as people spend increasing times in indoor environment such as at home, in residential buildings or professional buildings (Asikainen et al. 2016; Bernstein et al. 2008; Bruce et al. 2000; European Environment Agency 2013; United States Environmental Protection Agency 2016; World Health Organization 2009). Three groups of airborne compounds are indexed as major indoor air pollutants i.e., smoke, volatile organic compounds (VOCs) and biological agents (European Environment Agency 2013; Revah and Morgan-Sagastume 2005). 'Smoke' groups all the products from the outdoor or indoor obtained by fuel combustion, especially from traffic, heating or cooking and cigarette smoking. The second group of indoor air pollutants includes all VOCs released in gaze by some liquids and solids such as sprays, paints, solvents, cleaners, air fresheners, plastic components, etc. VOCs are defined as all the volatile compounds with a photochemical reactivity in atmospheres which contain carbon molecules except carbon monoxide, carbon dioxide, carbonic acid, carbonates and ammonium carbonates (Revah and Morgan-Sagastume 2005). Finally, the biological agent air pollutants refer to all the compounds produced or emitted by biological organisms which could have an impact on health. So, this group includes for instance algae, bacteria, dust mites, plants, parasites, pollen, viruses but also fungi and fungal compounds such as spores, cell wall fragments, mycelium and mycotoxins.

Among those biological contaminants, indoor fungal contamination is increasingly studied in order to define the possible impact on health, especially the implication in respiratory diseases (Anses 2016; Bellanger et al. 2009; Packeu et al. 2012; Reboux et al. 2010). Indeed, a large number of studies in many geographical regions has found a consistent association between evident indoor dampness, fungal contamination and health effect in infants, children as well as adults (Fig 1.1) (Anses 2016; Bornehag et al. 2004; Douwes 2005; Douwes et al. 2003; Mendell et al. 2011). According to the World Health Organization (WHO) and the scientific literature, indoor air pollution could be implicated in some diseases including allergies, asthma exacerbation, cancer, cystic fibrosis, allergies and in some cases strokes, particularly in immune-deficient people (Amegah and Jaakkola 2016; Anses 2016; Asikainen et al. 2016; European Environment Agency 2013; Ponsoni and Gonçalves Raddi 2010; World Health Organization 2009). Poor air quality is also associated to the sick building syndrome (SBS) characterized by mucous irritation (eyes and throat especially), neurotoxic effect as fatigue and loss of concentration, chemosensory

disturbance, skin symptoms and respiratory problems such as wheezing or shortness of breath (Anses 2016; European Environment Agency 2013; United States Environmental Protection Agency 2016; World Health Organization 2009). Nevertheless, even if the impact of the air pollution on health is recognized, some scientific evidence, especially for the causal link between fungal airborne contamination and health, is still not well elaborated.

Figure 1.1: Worldwide distribution of fungal allergy (adapted from Twaroch et al. 2015).
In yellow, countries in which sensitization to fungi has been described.

1.1. Fungi: definition, reproduction, classification and ecology

1.1.1. The kingdom of Fungi

The Mycota, or Fungi kingdom brings together different groups of micro- and macroscopic multicellular and heterotrophic eukaryotes showing various forms and cellular organizations and have great economic importance (Scientific Institute for Public Health 2015; Tom and Aime 2012). They live everywhere in air, in water, on land, in soil, and on or in plants and animals. The organisms in kingdom fungi include mushrooms, yeasts, molds, rusts, smuts, puffballs, truffles, morels, and molds. The abence of cellulose as well as the wall cell composition (formed by a network of microfibrils containing chitine, β-glucans and soluble polysaccharides) distinguish the true fungi, or Eumycota (e.g. Ascomycota and Basidiomycota), from the filamentous false fungi (i.e. the Chromista group (which groups the Hyphochytriomycota, the Labyrinthulomycota, the Oomycota, diatoms or brown algae) and non-filamentous false fungi from the Protista group (i.e., the Plasmodiophoromycoyta, the Dictyosteliomycota, the Myxomycota and the Acrasiomycota) (Scientific Institute for Public Health 2015) (Fig. 1.2).

The diversity of the Mycota kingdom is still being investigated and might contain at least 1.5 10^6 species (Hawksworth 2001), but according to new estimations obtained with high-troughput sequencing methods, this diversity might reach 5.1 10^6 species (Blackwell 2011). Despite these estimations, until today only 99,000 species have been described (Carris et al. 2012). All of them are characterized by a cell wall containing chitin and are able to carry out a zygotic meiosis (Tom and Aime 2012).

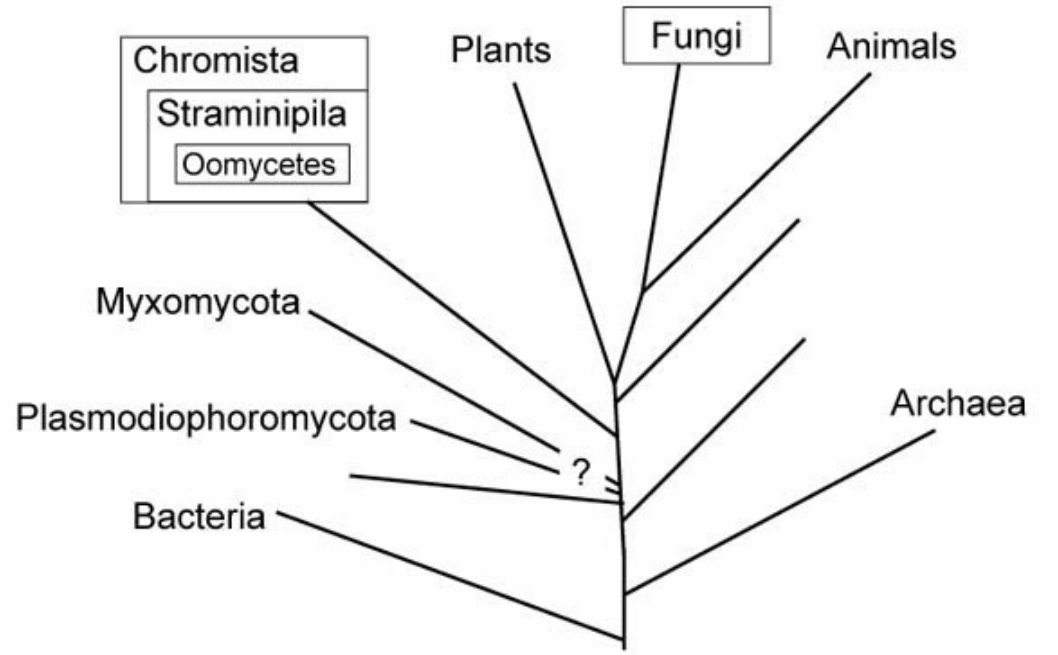

Figure 1.2 Relationship between organisms referred to as fungi (adapted from Rossman and Palm 2017). Fungi correspond to the Eumycota or true fungi and include Ascomycota and Basidiomycota. The Chromista, Myxomycota and Plasmodiophormycota correspond to false fungi.

1.1.2. Morphological characteristics of the vegetative growth

Fungi present different kinds of morphological structures i.e., vegetative structures and reproductive structures. Vegetative structures such as the thallus or rhizoids are developed for the fungal growth and nutrition.

The fungal thallus could be unicellular (i.e., yeast such as *Saccharomyctes cerevisae, Candida* sp., etc) or pluricellular (mushrooms, *Aspergillus* sp., *Alternaria* sp., *Cladosporium* sp., etc) (Scientific Institute for Public Health 2015). The thallus is composed of a network of hyphae containing protoplasm which moves from the oldest parts to the new ones. A mass of hyphae is called mycelium. The mycelium grows through nucleic divisions and could be divided by septa in regular segments having one or more nuclei. Each septum is pierced in the middle by a pore

allowing the moving of the organelles, inclusions and nucleus from a segment to another (Scientific Institute for Public Health 2015). In some cases, hyphae are aggregated forming a false tissue (sort of stacking up of cells) called pseudoparenchyma or could form a solid tissue rich in nutrients called sclerotium (Scientific Institute for Public Health 2015). The mycelium could present also some particular structures such as rhizoids, or appressoria which are branched organs developed for the fixing of the organisms on substrates. In parasite fungal species, the nutrients are taken directly in the host through a special structure called haustorium (Scientific Institute for Public Health 2015).

1.1.3. Reproduction

Fungal reproduction could be sexual or asexual (Fig. 1.3) and most of the true fungi are pleomorphic (or polymorphic), i.e., able to produce different forms of spores (i.e., sexual or asexual) having a reproductive function. The sexual reproductive stage is called teleomorph or perfect stage, while the asexual reproductive stage is called anamorph or imperfect stage. The term holomorph is used to appoint the whole fungus (i.e., including the anamorph and the teleomorph) (Scientific Institute for Public Health 2015).

Asexual reproduction

The asexual reproduction produces identical individuals through the production of asexual "spores", called conidia. Conidia are produced either through the budding of the mycelium (or a cell for unicellular thallus) or through particular cells (i.e., conidiogenous cell) set into the mycelium or on specific hyphae called conidiophores (Cyr 2009; Scientific Institute for Public Health 2015).

Sexual reproduction

The sexual spores are diploid cells (true spore) obtained through the fusion of 2 particular cells (i.e., gametangium) from a same mycelium or from 2 compatible mycelia. The gametangium is a structure which contains gametes (haploids), which could be morphologically identical (i.e., isogamy) or different (heterogamy). In the case of heterogamy, the gametangium containing the male gametes is called the antheridium (Fig. 1.3). The one containing the female gametes is called ascogonium (Fig. 1.3). In some cases, the vegetative thallus is totally converted in gametangium (or in reproductive structure). The thallus is then considered as holocarpic (Cyr 2009; Scientific Institute for Public Health 2015). In other cases, vegetative and reproductive structures coexist forming a eucarpic thallus.

The life cycle of most of the fungi (Fig. 1.3) could be summarized as the germination of spore or a

conidium, giving a haploid mycelium (Cyr 2009; Scientific Institute for Public Health 2015). Two compatible mycelia or spores form a diploid cytoplasm (fusion of cytoplasm without fusion of nuclei) (Fig. 1.3). Then, after caryogamy, the meiose occurs and the zygote is transformed in sexual spores (4, 8 or more) (Cyr 2009; Scientific Institute for Public Health 2015). Finally, after dispersion, spores grow (germination) and give rise to new mycelia (Fig. 1.3).

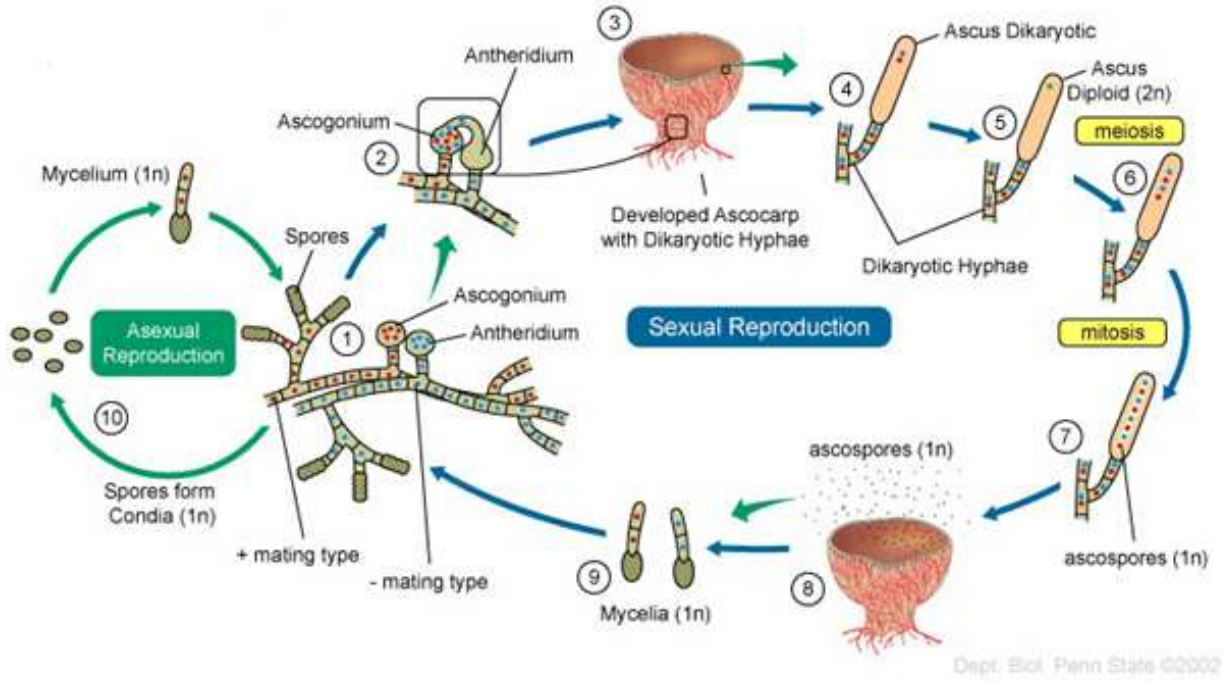

Figure 1.3 Fungal life cycle: the example of Ascomycota (Cyr 2009).
Sexual part of Ascomycota life cycle:

(1) Formation of the ascogonium and antheridium from haploid hyphae type "+" and type "-".
(2) Nuclei from antherium moved into the ascognium.
(3) Ascocarp formation with dikaryotic hyphae.
(4) Formation of ascus (dikaryotic) into an ascocarp at the tips of dikaryotic mycelium.
(5) Karyogamy and formation of diploid nucleus (diploid ascus).
(6) Meiosis of the diploid ascus, giving new genetically distinct nuclei into the ascus.
(7) Mitosis and formation of new haploid nuclei into the ascus.
(8) Formation of ascopores containing haploid nucleus (one nucleus per ascospore).
(9) Ascospore release in the environment.
(10) Development of haploid mycelia from the released ascopores.

Asexual part of Ascomycota life cycle:

(11) Segmentation of the hyphae and formation of haploid conidia (asexual spores). Conidia dispersion and germination giving a new haploid mycelium identical with the original hyphae.

Sporulation and spores dispersal

For most of the fungi, spore maturation, as well as sporulation (i.e., spores dropping into the environment), is light and circadian cycle dependent (Scientific Institute for Public Health 2015). Most of the time, the sporulation is due to an increase of pression inside the ascus which causes a membrane breakdown and then, the drop of spores into the environment. In some cases, the sporulation is caused by humidity variation which causes structural changes (turgescence, torsion…) and finally the propelling of the spores (Scientific Institute for Public Health 2015). Other factors could also induce sporulation such as rain or thermic changes as well as physical interactions (Scientific Institute for Public Health 2015).

Spore forms and sizes are variable. They could be small, large, curved, ovoid, spheric, ramified or colored. They could also present ornamentation such as wings or hooks to favour the dispersion into the air or by animals (Scientific Institute for Public Health 2015). Most of the time, spores are required to perform the species identification (Scientific Institute for Public Health 2015).

Spore viability is variable according to the taxa. Some of them are resistant to extreme conditions (desiccation, insolation,…) such as the spores of rusts, other ones have a short life time (e.g., 10 hours for the *Cronartium ribicola* spores) (Scientific Institute for Public Health 2015). The germination is also variable. Parasitic species require hosts to germinate; other ones require a specific substrate. In some cases spores could result in an infertile mycelium (i.e., a mycelium which is not able to sporulate) which do not show the morphological structures required for their identification (Scientific Institute for Public Health 2015).

1.1.4. Classification

In mycology, the classical classification is still based on the microscopic visualisation and the identification of reproductive structures from teleomorphs (e.g., spore form and size, presence of special structure on the spores, form of the reproductive structures…). However, while in environmental conditions, anamorphs and teleomorphs stages coexist, in culture, anamorphic colonies are often observed due to the culture conditions (especially due to the selected medium), the absence of a compatible partner or because the strains are not able to sporulate. In addition, some species, such as *Penicillium chrysogenum*, are also only known in the anamorphic stage (Scientific Institute for Public Health 2015). Therefore, before the use of molecular tools, the anamorphic forms were included with a specific nomenclature into a group called Fungi imperfecti, till the observation of the teleomorphs. But this classification system is complex, because a double nomenclature occurs (i.e., one name for the anamorph, one for the teleomorph), e.g. *Aspergillus glaucus* (anamorphe) and *Eurotium herbariorum* (teleomorphe) (Scientific Institute for Public Health 2015). Moreover, in some cases the classical identification does not

give the same species for the 2 stages due to the absence of the anamorphic stage on plate, or because the strain cannot produce conidia and asexual spores (Scientific Institute for Public Health 2015). In this case, the closely related species are grouped into a complex of species. However, since 2011, new fungal taxonomy rules were defined and it was decided to use only one name to identify the anamorph and the corresponding teleomorph fungus. This decision has had an impact on the whole nomenclature and induced some bias or loss of information. Indeed, most of the species were determined according to their physiology and some species are known only under their anamorphic stage. For example *Penicillium marneffei* (anamorph) is now named *Talaromyces marneffei* (because the genus *Talaromyces* groups together the sexual stage of *Penicillium*) (Scientific Institute for Public Health 2015; Yilmaz et al 2014).

The more recent classification is based on the use of molecular tools and the DNA sequence of the fungi. Because the use of new molecular tools improves the knowledge on fungal phylogeny, the fungal classification is continuously adapted. However, it is now considered that Fungi are divided in 7 phyla, two of which, the *Ascomycota* and the *Basidiomycota*, are contained within a branch representing subkingdom Dikarya (Fig. 1.4) (Hibbett et al. 2007; Scientific Institute for Public Health 2015). The *Ascomycota* and the *Basidiomycota* phyla are characterized by the production of dicaryons (i.e., cells containing two nuclei during the dicryotic stage of the sexual reproduction) and non-mobile spores. The *Basidiomycota* phylum contains the most familiar fungi such as mushrooms, toadstools, stink-horns, puffballs, shelf fungi and plant pathogens such as rust or smuts (Scientific Institute for Public Health 2015). The *Ascomycota* contains taxa such as yeasts, *Aspergillus*, *Penicillium*, *Cladosporium*, but also edible morels or truffles (Scientific Institute for Public Health 2015). In scientific literature, the term “molds” is often used to show anamorphic fungi, as well as some microscopic teleomorphic fungi from the *Ascomycota* or *Basidimycota* phyla (Scientific Institute for Public Health 2015).

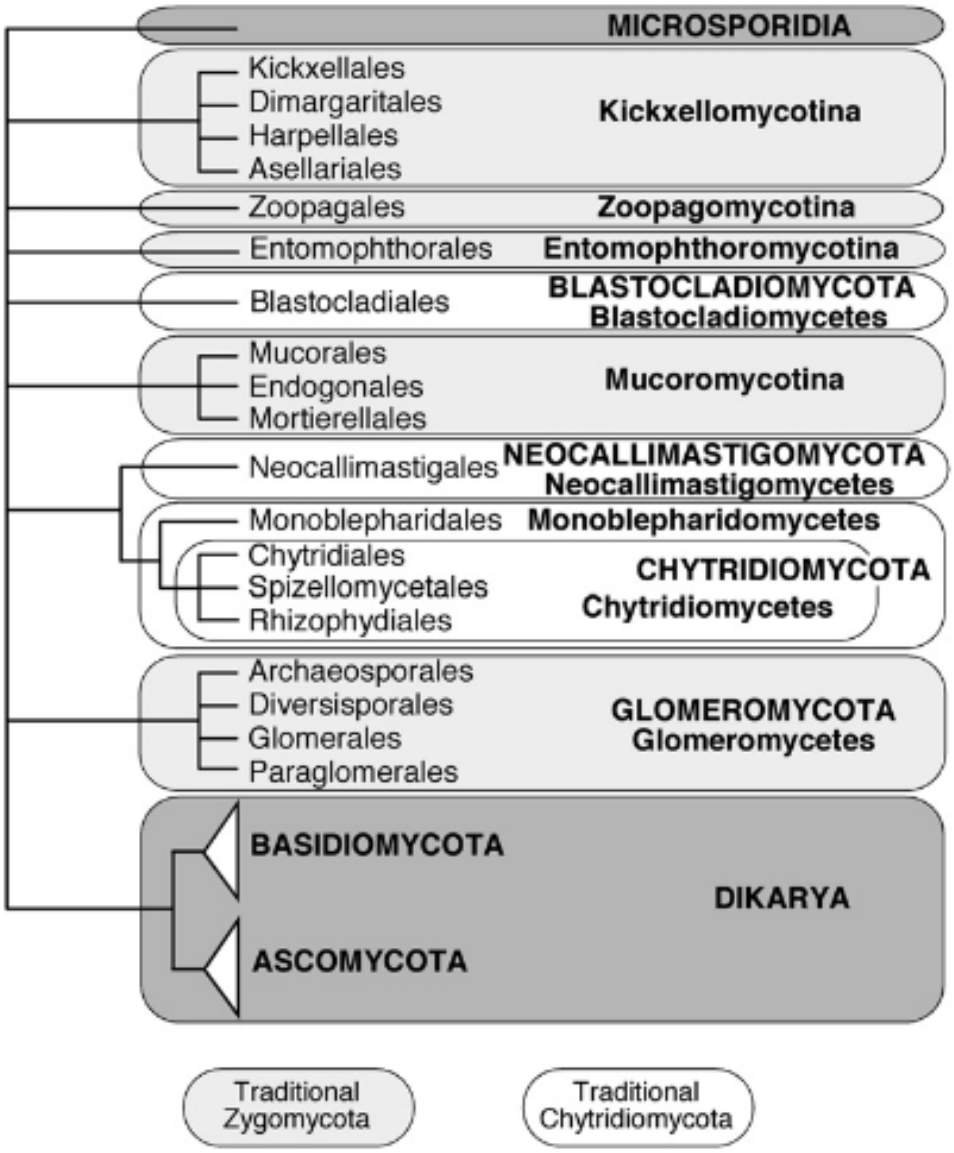

Figure 1.4 Phylogeny and classification of Fungi (Hibbett et al. 2007).
Branch lengths and genetic distances are not proportional (Hibbett et al. 2007).

1.1.5. Ecology

Adapted to various ecological niches, fungi are found in several habitats from the sea to the air as well as on soil, living organism as hosts (plant or animals) or rocks. Most of the fungi grow between 15°C and 30 °C, but some could grow in extreme environments (below 10 °C or up to 50 °C) (Scientific Institute for Public Health 2015). Based on this, fungi could be classified as

- thermophilic if the organism could growth till 50 °C, but not below 20 °C
- thermotolerant if the organism has a minimum temperature of growth at or above 20 °C, and a maximum temperature of growth extending up to 60 to 62 °C

- mesophilic if the organism has an optimal temperature of growth around 25 °C
- psychrophilic if the organism has an optimal temperature of growth lower than 10 °C.

The humidity level is also an important factor for the fungal growth. Actually, two groups of fungi are observed (Scientific Institute for Public Health 2015) i.e., the osmophilic species which require a water saturation of the substrate lower than 80 % and the hygrophilic species which require a water saturation of the substrate higher than 90 %.

Fungi are generally aerobic taxa. Some species, such as *Mucor* species, consume a high level of oxygen and so, could only grow in upper area. *A contrario*, *Stachybotrys* is able to grow at depths where the level of oxygen is very low (Scientific Institute for Public Health 2015).

Fungi are key species for the ecosystem. These heterotrophic organisms (no chlorophyll) are able to digest the biologic matter externally and absorbing the nutrient through their cell wall. Some of them are biotrophs (i.e., obtain nutrients from a living host), other are saprotrophs (i.e., nutrients are taken from dead organisms) or necrotrophs (i.e., species growing on living host and causing the death of the cell) (Carris et al. 2012).

In the ecosystem, fungi play the role of decomposer and so, are important for the recycling of nutrients. The source of carbon used by fungi comes from glucose and fructose especially. But because only a low number of fungi can produce the enzyme required for the hydrolysis of starch and cellulose, only a few species are able to grow in indoor environment (Scientific Institute for Public Health 2015). However, even if the number of fungal species able to grow in indoor environment is limited in comparison to the diversity observed in outdoor environment, their impact on health could be important due to their ability to produce toxins and allergens, but also due to their strong association to non-healthy environments (water-damadged and damp habitats).

1.2 Fungal contamination of indoor environment

Contamination of indoor environment by fungi is commonly observed in industrialized countries (European Environment Agency 2013; United States Environmental Protection Agency 2016; World Health Organization 2009). For example, in 1994 the prevalence of fungal contamination inside houses was estimated at 50 % in the United States of America (USA) (Mudarri and Fisk 2007). In 2013, Moularat and colleagues sampled 94 houses located in the urban and rural Auvergne region (France), and they showed that 59 % of these houses were contaminated by fungi and 19 % by visible moisture (Moularat et al. 2011). In Brussels (Belgium), 42.2 % of houses sampled between 2000 and 2006 in the framework of the regional intervention unit for indoor pollution, called *Cellule Régionale d'Intervention en Pollution Intérieure* or CRIPI (Brussels

Environment, Belgium), showed fungal contamination (visible or not) (Brussels Environment 2007). In 2011, 20 % of by the CRIPI investigated housings in Brussels showed more than 3 m² of visible fungal colonies in a single room (Brussels Environment 2015).

Fungal contamination could consist of active compounds (spores or mycelium) or of spores in dormancy. They penetrate into buildings via different ways of contamination. Most of them come from the direct outdoor environment and are transported in buildings via aeration and ventilation systems (i.e., windows, air conditioning, etc). Others are carried on build materials or on cloths (Nevalainen et al. 2015). Because they are ubiquitous, fungi grow on various substrates such as building materials (e.g., gypsum, paste, wood) or finishing materials (e.g., paints, linoleum, wall paper), mattress, pipes, and even on fabric or on compost of indoor plants (Andersen et al. 2011; Kelley and Gilbert 2013; Horner et al. 2004; Nevalainen et al. 2015; Nishimura et al. 1987; World Health Organization 2009; World Health Organization Regional Office for Europe 2004). Additionally, energy saving measures not correctly implemented could also favor fungal development (World Health Organization Regional Office for Europe 2004). Indeed, moisture, poor ventilation and darkness could stimulate the development of fungal contamination (World Health Organization Regional Office for Europe 2004). According to the WHO, in 2009, among 10 to 50 % of buildings around the world were affected by water-damage and 15 to 40 % of them showed fungal contamination (World Health Organization 2009). In Brussels, in 1994, it was estimated that 90 % of water-damaged dwellings showed also fungal contamination (Beguin and Nolard 1994).

Even if numerous data on indoor fungal diversity are available in scientific literature, the community of indoor fungi is complex to study due to the variability of the indoor environment and the fungal ecology (Nevalainen et al. 2015). Furthermore, climate changes and urbanization could also affect the diversity of the fungal community observed in indoor environment with the detection of species not yet observed in our industrialized countries (Vardoulakis et al. 2015; World Health Organization Regional Office for Europe 2004). However, in our industrialized countries, it is commonly accepted that indoor fungal communities are dominated by 6 genera i.e., *Alternaria*, *Aspergillus*, *Cladiosporium*, *Penicillium*, *Stachybotrys* and *Ulocladium* (Andersen et al. 2011; Beguin and Nolard 1994; Chew et al. 2003; Gots et al. 2003; Jones et al. 2011; Shelton et al. 2002). More specifically among these genera, the most common species are *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Cladosporium cladosporioides*, *Cladosporium herbarum*, *Cladosporium sphaerospermum*, *Penicillium chrysogenum*, *Stachybotrys chartarum* and *Ulocladium botritys* (Andersen et al. 2011; Beguin and Nolard 1994; Chew et al. 2003; Jones et al. 2011; Shelton et al. 2002). *A. versicolor* and *P. chrysogenum* are considered as the major species in terms of prevalence and public health effects among the species

in indoor environment especially in water-damaged buildings (Andersen et al. 2011; Andersson et al. 1997; Beguin and Nolard 1994) and to a lesser extent, *S. chartarum* which is particularly observed on humid substrata containing cellulose such as wood or wall paper (Andersen et al. 2011). As for *A. versicolor*, *U. botrytis*, *S. chartarum* is commonly observed on gypsum and other mural coatings (Scientific Institute for Public Health 2015). These species are typically considered as "real indoor fungi" because their occurrence in indoor environments is higher than that observed outdoors (Fradkin et al. 1987). Conversely, some species are observed with a lower occurrence in indoor area than in outdoor, such as *A. alternata*, *A. fumigatus* and the 3 *Cladosporium* species. Their levels of detection are closely linked to the level of their contamination observed in outdoor air (Beguin and Nolard 1994). Present in the air from May to October, these species present a seasonality with an important peak of sporulation during summer. During this period, their spores could reach numbers of more than 10.000 spores/m³ (Sautour et al. 2009; Scientific Institute for Public Health 2015) and could correspond to more than 50 % of the total diversity of fungal spores observed in the outdoor air (Sautour et al. 2009; Scientific Institute for Public Health 2015). In Belgium, it was estimated that the contamination of indoor air by *Alternaria* sp. spores correspond to 1.5 % of the total amount of spores yearly observed in outdoor air (i.e., minimum 500 spores/m³) and that around 50 % of dust samples contain *A. alternata* (Scientific Institute for Public Health 2015).

1.2.1 Indoor airborne fungi and public health

Because fungi are ubiquitous, but also because many of them are found all around the world, fungal contamination of indoor environment, by spores, mycelia or other fungal particles, is increasingly assimilated into a public health question.

Fungi cause adverse human health effects through 3 specific mechanisms: direct infection by the organism, generation of a harmful immune response (e.g., allergy or hypersensitivity pneumonitis) and toxic-irritant effects from fungi by-products (Bush et al. 2006). The world-wide prevalence of fungal allergy among atopic subjects is estimated to be from 3 to 10 % depending on the allergic population studied, the test system and extract used and fungi species tested (Horner et al. 1995). Additionally, it was reported that 80 % of the asthmatic patients are sensitized to fungi (Simon-Nobbe et al. 2007). Indeed, today, the exposure to fungal components is considered as a potential source for allergic diseases like allergic asthma, allergic rhinitis, allergic sinusitis and hypersensitivity (Pieckova and Wilkins 2004). Allergens could be found in food and feed but also in air. The fungal allergens group mycotoxins (< 1kDa), volatile fungal metabolites (VFMs), spores (viable or not), hyphae and fungal fragments containing especially (1-3)-β-D glucans, intracellular proteins, secreted proteins and many glycopeptides which have enzymatic activities (Douwes 2005; Khan and Karuppayil 2012; Horner et al. 1995; Portnoy et al. 2008; World Health

Organization 2009). Most of the these allergens are type I allergens (Fig. 1.5) (Crameri et al. 2014; Green et al. 2006; Khan and Karuppayil 2012). The fungal allergens' aerosolization could occur by differents mechanisms i.e., the sporulation and associated mechanisms of spore dispersion, fungal fragment dispersion by air, physical disturbances and suspension of dust containing fungal allergens (Gorny et al. 2002). However, the exact mechanism of aerosolization and inhalation is still not well understood (World Health Organization 2009).

It is considered that the airborne fungal genera mostly implicated in allergies are *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicilium* which are all common in indoor environments and which are characterized by their capacity to excrete allergens.

These allergens could induce IgE-mediated hypersensitivity (Portnoy et al. 2008) which could cause systemic anaphylaxis and localized anaphylaxis such as asthma, eczema and wheeze (Janeway et al. 2001). But, fungi are also regarded as type III (IgG-inducing) allergens producers, especially species from the *Aspergillus* and *Penicillium* genera (World Health Organization Regional Office for Europe 2004, Khan and Karuppayil 2012). In some case, fungal contamination could also be implicated in the combination of type III and IV allergic reactions (Fig. 1.5), such as is the case for hypersensitivity pneumonitis (World Health Organization Regional Office for Europe 2004).

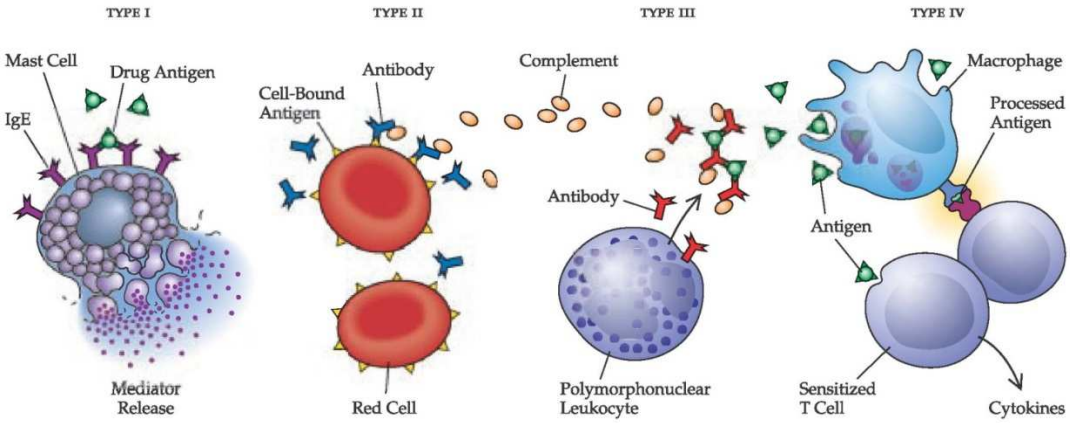

Figure 1.5: Schematic representation of the allergenic reaction types I, II, III and IV (http://what-when-how.com)

Indeed, as mentioned above, fungi are able to produce compounds such as spores, proteins, mycotoxins, (1-3)-β-D glucan and volatile fungal metabolites (VFMs) which could induce illness by inhalation or ingestion. Some scientific studies showed a correlation between some diseases such as allergies, asthma exacerbation, SBS or rhinitis and fungal contamination (de Ana et al. 2006; Gots et al. 2003; Horner et al. 1995; Jarvis and Miller 2005; Jones et al. 2011; Meheust et al. 2014; Mendell et al. 2011; Packeu et al. 2012a,b; Piecková and Wilkins 2004; Portnoy and Jara 2015; Rosenbaum et al. 2010; United States Environmental Protection Agency 2016; Vardoulakis

et al. 2015; Verhoeff and Burge 1997; Vesper et al. 2013; World Health Organization 2009). The following paragraphs discuss the most important fungal compounds which could be implicated in health problems.

Proteins

Most of the time fungal allergens are proteins from the cell wall of airborne spores or inserted in the mycelium membrane (Horner et al. 1995; Kurup 2003). Produced by several species, these proteins are frequently observed in indoor environments. Indeed allergenic proteins are observed in several genera and species commonly observed in indoor environment as for example, in *Alternaria* such as the Alt a1 from *A. alternata;* in *Aspergillus* such as Asp f1 and Asp f2 from *A. fumigatus*, in *Cladosporium* such as Cla c9 form *C. cladosporioides* or Cla h1 and Cla h2 from *C. herbarum*; in *Penicillium* such as Pen ch13 from *P. chrysogenum*; and in *Ulocladium* such as Ulo C1 (Horner et al. 1995; Fukutomi and Taniguchi 2015).

Among all proteins currently found, Alt a1 from *A. alternata* (Kustrzeba-Wójcika et al. 2014; Gabriel et al. 2016), Asp f2 from *A. fumigatus* (Banerjee et al. 1998) and Cla c9 from *C. cladosporioides* (Chou et al. 2008) are considered as major allergens, involved in IgE mediated allergy.

Mycotoxins

Mycotoxins are toxic secondary metabolites produced by several fungal species and which could contaminate food and feed. Generally not volatile, these toxics compounds could be found in air because of their attachment to some small biological particles (Portnoy et al. 2008). Mycotoxin inhalation could have an impact on health inducing mucous irritations, skin rash, immune system suppression and immunotoxic effects, liver damage, damage of the central nervous system and the endocrine system (Fromme et al. 2016; Jarvis and Miller 2005; World Health Organization 2009). Some mycotoxins are also known to be cytotoxic and carcinogenic (De Ruyck et al. 2015; Jarvis and Miller 2005).

Today, around 400 different mycotoxins have been identified and they are produced especially by *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp. and *Stachybotrys* spp. (Fromme et al. 2016; World Health Organization 2009). These toxins are found in the indoor air of water-damaged, air-conditioned or ventilated buildings and agricultural buildings such as cereals depots, stables, compost or manure depots (Fromme et al. 2016; World Health Organization Regional Office for Europe 2004). In indoor environment, the most often detected mycotoxins are the aflavotoxins produced especially by *Aspergillus* species; the ochratoxins produced for the most part by *Aspergillus* and *Penicillium*; cladosporin from *Cladosporium* species; and alternariol and alternariol methyl esther from *Alternaria* species (Fromme et al. 2016). As shown in Table 1.1,

more than one mycotoxin could be observed simultaneously in indoor environments. Polizzi and colleagues investigated 99 samples from 7 Belgian houses and showed that 62 samples contained at least one mycotoxin. Using liquid chromatography-tandem massive spectrometry (LC-MS/MS), they have identified 7 major mycotoxins i.e., roquefortine C, chaetoglobosin A, sterigmatocystin, roridin E, ochadrine A, aflavotoxin B(1) and aflavotoxin B(2) (Polizzi et al. 2009). The mycotoxin production depends largely on the growth conditions both in terms of quantity and of number of toxins produced by species (Jarvis and Miller 2005; World Health Organization Regional Office for Europe 2004). For example, *A. versicolor*, one of the most important indoor fungal contaminants, produces sterigmatocystin only under a high level of humidity (Jarvis and Miller 2005; World Health Organization Regional Office for Europe 2004).

It should be noted that chromatographic techniques, such as LC-MS/MS, are not the only ones used to detect these toxins, but MALDI-TOF (matrix assisted laser desorption/ionization - time of flight massive spectrometry) and immunochemical-based methods such as ELISA (enzyme linked immunosorbent assay) are also largely exploited and have contributed to improve mycotoxins monitoring (Anfossi et al. 2015). However, today the causal link between exposure to indoor mycotoxins and specific diseases is still poorly documented (Khan and Karuppayil 2012).

Table 1.1: Frequently found indoor fungal species and their mycotoxins

Species	Mycotoxins	Reference
Alternaria alternata	Alternariol, alternariol monomethyl esther, Fumonisin B1	Ren et al. 1998; Jarvis and Miller 2005
Aspegillus creber	Sterigmatocystins	Jurjevic et al. 2013
Aspergillus flavus	Aflavotoxin B1, Aspergillic acid, Kojic acid, cyclopiazonic acids, 3-nitropropionic acid	Nielsen 2003
Aspergillus fumigatus	Gliotoxins, fumigaclavines, fumitoxins, fumitremorgens, tryptoquivalins, verruculogen	Nieminen et al. 2002; Nielsen 2003
Aspergillus niger	Ochratoxin A, naphtho-c-pyrones, tetracyclic compounds, nigragillin, kotanin, orlandin, malformin A, B, and C	Nielsen 2003
Aspergillus sydowii	Aspermutarubrol, sydowinins, sydowic acid	Jarvis and Miller 2005
Aspergillus versicolor	versicolorins, Sterigmatocystins, 5-methoxysterigmatocystin	Jarvis and Miller 2005
Cladosporium cladosporioides	Asperentin, cladosporic acids	Jarvis and Miller 2005
Penicillium chrysogenum	Roquefortine C, meleagrin, chrysogine, x-hydroxyemodine, pyrovoylamin-obenzamides, xanthocillin X	Jarvis and Miller 2005; Nielsen 2003
Stachybotrys chartarum	Macrocyclic trichothecenes, atranones, dolabellanes stachybotrylactones and lactams, stachybotrydials	Jarvis and Miller 2005

(1-3)-β-D glucans

(1-3)-β-D glucan is a polymer of glucose, found in most of the fungal species, and it has a structural function in the cell. Inside the cell wall, this polymer is bound to carbohydrates, lipids, proteins and other polymers such as (1-6)-β-D glucans (World Health Organization Regional Office for Europe 2004). According to the scientific literature, bioaerosols containing (1-3)-β-D glucans could be implicated in respiratory problems and non-specific inflammation (Douwes et al. 2003; Douwes 2005).

Volatile Fungal metabolites (VFMs)

Besides glucans and mycotoxins, indoor air could also contain volatile fungal metabolites (VFMs). VFMs are secondary metabolites such as alcohols, aldehydes or ketones produced during the exponential growth of several fungal species (Wilkins et al. 2003). The impact of VFMs on health is poorly documented, but some studies consider that VMFs inhalation could have some respiratory irritation (Weinhold 2007).

1.2.2 Fungal immunological testing

Today, fungal sensitivity is detected through cutaneous tests. Briefly, these tests consist of putting some drops of standardized allergens from different fungal species, on the skin of the patient. After incubation, a patient is considered as sensible to the allergen when immunological signs such as red patches, inflammation or rashes are visible.

A second approach is based on allergen-specific serum IgE antibodies such as in the radioallergosorbent test (RAST) and enzyme-linked immunosorbent assay (ELISA) (Horner et al. 1995; Kurup et al. 2003). The RAST diagnosis consists of an *in-vitro* detection of IgE antibodies in blood, through the use of allergens coated to an insoluble substrate and a radiolabeled anti-human IgE (Fig. 1.6). Briefly, some specific antigens coated to an insoluble substrate are dropped into the patient serum. If the patient is sensitive to the allergens, specific IgE will bind the allergens. After a wash step, radiolabeled anti-human IgE are added into the solution and bind the antigens-antibodies complexes. The presence of IgE antibodies are detected by the radioactivity measurement. The radioactivity level is proportional to the level of IgE anti-allergens binding in the serum (Negrini et al. 1985).

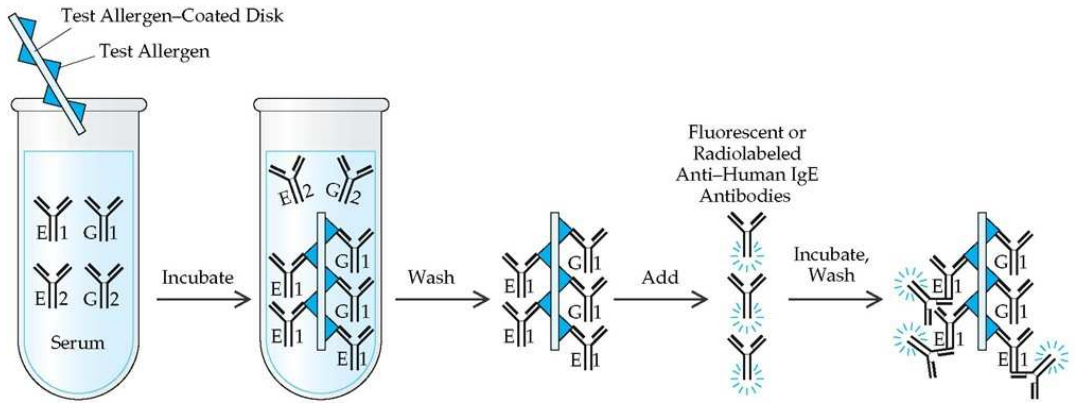

Figure 1.6: RAST test - schematic principle (http://what-when-how.com)

With the ELISA test (Fig. 1.7), antigens of interest are coated to the bottom of a polystyrene plate with proteins blocking the free plastic sites. When the serum of patients is added, if they are present in the serum, specific IgE (primary antibodies) bind the antigens. After a wash of the non-binding IgE, enzyme labeled antibodies (secondary antibodies) bind primary antibodies. Finally, a specific substrate is added and consumed by the enzyme which changes the color of the liquid.

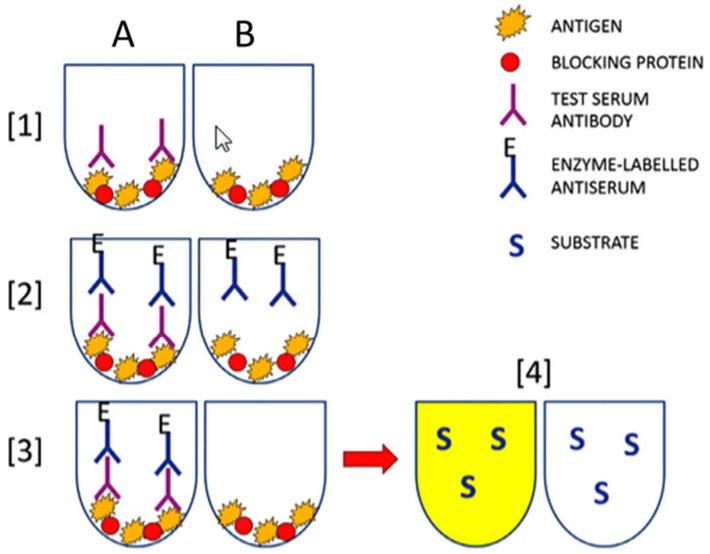

Figure 1.7: ELISA test – schematic principle (Adapted from Day 2015)

A: Positive test. B. corresponding to the negative control

[1] If they are present in the serum, specific IgE (primary antibodies) recognize the coated antigens.

[2] Addition of secondary antibodies labeled with an enzyme (E) which bind primary antibodies.

[3] After a wash, free secondary antibodies are removed.

[4] Addition of a substrate which is used by the enzyme and changes the substrate color.

Even though the allergenicity of several fungal species is well known, the health impacts of a large part of the diversity are unknown. Indeed, today, numerous fungal monitoring protocols, still culture-based, are not able to detect dead or uncultivable species (also called dead fraction or unknown agent). But, because these unknown agents could contain allergenic compounds, they could be allergenic and could have an impact on health.

Moreover, the lack of data concerning the unknown agents as well as the difficulties to produce and to standardize allergens required for clinical tests has an impact on the representativeness of the fungal species tested during immunological tests (Portnoy et al. 2008; World Health Organization 2009). Thus, today, representative fungal extracts available for immunological tests are focused on allergens from fungi only frequently found indoor. However, to understand the causal link between fungal contamination and health problems such as respiratory diseases and allergies, the representativeness of immunological tests is required to demonstrate that a direct exposure of an affected person to the detected fungal species occurred.

1.2.3 Some important fungal species found indoor and their clinical relevance

This paragrah describes the fungal genera and species targeted in this doctoral research and their clinical relevance.

Alternaria

The genus *Alternaria* is found all around the world and contains about fifty species. *Alternaria* species are plant pathogens, but are also decomposers of organic matter from vegetables, food, and soil.

At the clinical level, *Alternaria* is associated with allergies. In outdoor air, it is considered that a concentration of 100 spores/m³ is a clinical threshold. In Europe, between June and October, this concentration is commonly reached (Scientific Institute for Public Health 2015). In addition, a concentration of 500 spores/m³ is considered as a threshold for asthmatic reactions. Also this level of outdoor air contamination is often reached during the year.

In indoor environment, *Alternaria* species are also frequently observed, but most of their spores found inside are coming from outdoor air. The total of indoor *Alternaria* spores corresponds to 1.5 % of the *Alternaria* spores observed outdoor (Scientific Institute for Public Health 2015). However, *Alternaria* colonies are also observed inside buildings on walls or mattresses. In Belgium, 50 % of dust samples contain *Alternaria* species (Scientific Institute for Public Health 2015).

In indoor environment, *Alternaria alternata* is the most abundant species of the genus. This species is frequently associated with respiratory deseases such as allergies, allergic rhinitis or asthma. In Europe, sensitization to *A. alternata* allergens is ranging between 3 to 30 % of allergenic patients (Katotomichelakis et al. 2012; Kustrzeba-Wójcicka et al. 2014). In the United States, 3 % of children between 12 to 17 years and 7.5 % of young adults (18-24 years) have been sensitized to *A. alternata* allergens (Sanchez and Bush, 2001).

Aspergillus

Aspergillus is a large fungal genus containing more than 200 species. Although this genus is common in tropical regions (Scientific Institute for Public Health 2015), some of the species are present all around the world and found in soil, compost, rooting organic matter, spices, seed depots,… *Aspergillus* colonies are colored and the color is considered as a taxonomic criterion. *Aspergillus* spores are small, generally rounded, without any specific characteristics, and especially observed in indoor environments. In outdoor air, *Aspergillus* spores are frequently found and the concentration could reach 30.000 spores/m³. While *Aspergillus fumigatus* is considered as the most common species of the genus in outdoor environments, *Aspergillus versicolor* is one of the most frequently observed fungi in indoor environments (Beguin and Nolard 1994; Packeu et al. 2012a). In indoor environment, *A. fumigatus* is less frequently observed than in outdoor habitats and is found especially on ornamental plants.

At the clinical level, *Aspergillus* species (especially *A.fumigatus, Aspergillus flavus, Aspergillus terreus, Aspergillus nidulans, Aspergillus niger and A.versicolor*) are known to induce invasive aspergillosis infection which could impact the patient's survival (Hope et al. 2005). Species from this genus, such as *A. fumigatus* and *A. versicolor,* are associated with respiratory deseases (asthma, allergies, pulmonary and broncopulmonary infections) especially due to their production of allergens and toxins (Benndorf et al. 2008; Scientific Institute for Public Health 2015; Kousha et al. 2011). Therefore, although *A. fumigatus* is less observed in indoor environment, its presence could have an important impact on health.

In the *Aspergillus* genus, species from the complex *Versicolores* are commonly observed in buildings. The *Versicolores* complex groups together 9 species (i.e., *Aspergillus amoenus, Aspergillus austroafricanus, Aspergillus cvjetkovicii, Aspergillus fructus, Aspergillus jensenii, Aspergillus protuberus, Aspergillus puulaauensis, A. subversicolor, Aspergillus tabacinus, Aspergillus tennesseensis* and *Aspergillus venenatus*), which are closely related based on morphological and genetic characteristics, and therefore difficult to discriminate with classical and molecular tools of determination.

Among them, *A. versicolor* is frequently observed in indoor environment, especially in water

damaged and humid buildings (Andersen et al. 2011; Beguin and Nolard 1994; Packeu et al. 2012a; Pitkaranta et al. 2011). In Belgium, this fungus is observed in 76 % of sampled houses and is found in all the rooms of houses; the contamination is frequently observed above 375 CFU/m³ but in some case it could pass 3.750 CFU/m³ (Scientific Institute for Public Health 2015).
A. versicolor is known to produce carcinogenic toxins, but also to be associated with lung infections and exacerbation of asthma or allergies (Benndorf et al. 2008; Fischer and Doot 2002).
Aspergillus creber and *Aspergillus sydowii* are both species from the *Versicolores* group, as is also *A. versicolor*. Genetically close, these 2 species are difficult to be morphologically discriminated from *A. versicolor* (Visagie et al. 2014), as well as to be discriminated based on results obtained with molecular tools (Haugland et al. 2004; United States Environmental Protection Agency 2015). Found in the indoor environment, *A. creber* and *A. sydowii* are less common than *A.versicolor*. However, it may seem that their impact on human health could be different. Indeed, as *A. versicolor*, *A. creber* produces sterigmatocystin, a precursor of the aflatoxin B1 which is a carcinogenic mycotoxin, but in identical environmental conditions at a lower concentration (Jurjeciv et al. 2013). In contrast, *A. sydowii* is a non-sterigmatocystin producer (Rank et al. 2011), but is known to be an agent of human mycosis (Visagie et al. 2014). Nevertheless, health data associated with *A. creber* and *A. sydowii* are poorly documented. This might be due to the fact that they are difficult to be discriminated amongst each other.

Cladosporium

Species from the *Cladosporium* genus are found in several outdoor and indoor habitats such as plants, organic waste, soil… Several *Cladosporium* species are plant parasites, but most of them are saprotrophs. Forming dark colonies, *Cladosporium* species produce ramified spores which can be easily scattered by the wind. Spores are round or cylindrical, presenting septa or not, but characterized by scars at the ends. In outdoor air, *Cladosporium* spores could reach 56 % of the total of fungal airborne spores per year (Scientific Institute for Public Health 2015). Among the *Cladosporium* genus, 3 species are mostly observed in air i.e., *Cladosporium cladosporioides*, *Cladosporium herbarum* and *Cladosporium sphaerospermum.* During the winter, the spores are present at low concentration. Although the spore concentrations of these three species are comparable during winter, the spore concentration of *C. herbarum* increases during the summer and could be above 10.000 spores/m³ (Scientific Institute for Public Health 2015). It should be noted that the scientific community considers a level of 3000 of *Cladosporium* spores/m³ as a threshold provoking respiratory diseases (Scientific Institute for Public Health 2015).

In the indoor environment, the most abundant *Cladosporium* is *C. sphaerospermum.* In Belgium, 6 % of the houses present an important contamination (i.e., over 375 CFU/m³) of *C. sphaerospermum* (Scientific Institute for Public Health 2015). This fungus is especially observed

on walls and the ceiling of bathrooms or bedrooms but also in dust of mattresses. Although its optimal temperature of growth is 24 °C, *C. herbarum* is also observed in fridges and cold rooms (Scientific Institute for Public Health 2015).

At the clinical level, *Cladosporium* are known to produce allergens and mycotxins. This genus is also known to be an opportunistic pathogen which could cause solid-organ transplant recipient infection, but which could also be implicated in pulmonary infection such as pulmonary phaeohyhpomycosis (Castro et al. 2013).

Exophiala

The *Exophiala* genus contains saprophytic fungi living in oligotrophic environments rich in hydrocarbons (Woo et al. 2012). These species are also found in hot, humid and oligotrophic environments such as sanitary pipes, bathrooms, steam baths, swimming pools, water reservoirs… *Exophiala* colonies are black and therefore this species is often named "black yeast". Most of the *Exophiala* species are known to be agents of skin and subcutaneous infections as well as to be associated with sytemic infections, especially in immunodeficient patients. Among the 30 species of the *Exophiala* genus, *Exophiala dermatitidis* is the best known, which is related to airway and respiratory infections such as cystic fibrosis ((Nucci et al. 2001, 2002; Packeu et al. 2012b; Woo et al. 2012).

Exophiala jeanselmei is an ubiquitous black yeast found, as other *Exophiala*, in warm and humid environments and on diverse substrates such as sludge, soil or water (Dixon et al. 1980; Nishimura et al. 1987; Nucci et al. 2002). *E. jeanselmei* is difficult to grow on plate as it requires specific culture conditions such as a minimum temperature of 25°C, a high humidity and a long time of incubation (till 21 days). As other *Exophiala* species, *E. jeanselmei* is considered as a causal agent of cutaneous or subcutaneous infections or systemic infections (Nucci et al. 2001, 2002). Nevertheless, limited data on the number of cases occurring are available and the epidemiology of airway infections caused by *E. jeanselmei* is poorly documented.

Inside buildings, the way of contamination by this species is presumably linked to water reservoirs of air-conditioning units or pipings (Badali et al. 2012; Wang et al. 2001). Indeed, *E. jeanselmei* is frequently observed in water from humidifiers or air-conditioning systems in which it could reach 100 CFU/ml. During a study performed in 2004 on the microbiological contamination of 417 humidifier systems, *E. jeanselmei* was observed with an occurrence of 15.51 % (26.51 % for *Exophiala* spp.) (Nolard et al. 2004). It should be mentioned that *E. jeanselmei* has never been detected in indoor air with culture-based tools of detection, probably due to the sensitivity of this species to desiccation. However, because other species from this genus seem to be implicated in

respiratory diseases (e.g., *E. dermatitidis*), *E. jeanselmei* is suspected to be an agent of respiratory infection. Alternative tools are needed to investigate its presence in the indoor air and to make the link of its presence with health problems.

Penicillium

Penicillium is the more ubiquitous fungal genus containing more than 200 species found in each part of the world in soil, organic matter, organisms alive or not. Most of the *Penicillium* colonies show a blue-green color. Spores are produced in large amounts. Chains of spores are produced by special structures called phialides and are inserted in the conidiophores.

In indoor environments, *Penicillium* is found in humid habitats on walls, furniture, mattresses and dust. In these habitats, the most common species are *Penicillium aurantiogriseum*, *Penicillium brevicompactum*, *Penicillium corylophilum* and *Penicillium spinulosum*. However the most common, which is also the most ubiquitous, is *Penicillium chrysogenum* which is particularly known by the medical community due its ability to produce and β-lactam antibiotics and penicillin, an antibiotic which inhibits the biosynthesis of bacterial cell walls (Flemming 1929).

However, several *Penicillium* species produce spores containing mycotoxins, such as *Penicillium brevicompactum* spores containing mycophenolic acid which may have some implication in immune deseases (Scientific Institute for Public Health 2015). Moreover, *Penicillium* mycotoxins may be associated chronically with respiratory diseases, especially asthma (Scientific Institute for Public Health 2015). *P. chrysogenum* is also known to have some implication on health. In the United States, it was estimated that 68 % of asthmatic patientis (on a total of 100) was sensitive to *P. chrysogenum* (Santilli et al. 1985). This particular impact on health is especially due to the emission of allergenic proteins such as the vacuolar serine protease Pen ch 13 and 18 (Shen et al. 2003). The emissions of these proteins in indoor air could induce or enhance allergenic reactions and in some cases asthmatic reactions (Fisher and Dott 2002). Moreover, some studies showed associations between *P. chrysogenum* spores in indoor air and asthmatic reaction or children rhinitis (De Ana et al. 2005; Fisher and Dott 2002), probably due to the presence of proteases on conidia (Schwab et al. 2003).

Ulocladium

Ulocladium is a genus commonly observed in the indoor environment. Species from this genus form dark colonies and produce short chains of spores characterised by a sympodial growth. *Ulocladium* spores are morphologically close to the *Alternaria* spores, but without beaks. In the indoor environment, *Ulocladium botrytis* is the common species of this genus. This species grows on gypsum and protective coating of walls. At the clinical level, *Ulocladium* species produce

allergens and some association between allergenic patients and *Ulocladium* contamination has been shown (Scientific Institute for Public Health 2015).

Stachybotrys

Stachybotrys groups less than 100 species among which some of them are anamorphic stages of species from the *Chaetosphaeria*, *Codryceps* and *Melanopsamma* genera. Colonies are dark green which grow slowly. The most known species is *Stachybotrys chartarum* which is a species observed all around the world, especially found in soil and cellulose-rich media (e.g., hay, seeds, wood chips, papers, clothes, tobacco…). In indoor environments, it is found on building materials (e.g., wall paper, wood, insulation material, gypsum boards…). *S. chartarum*, as other species from this genus, are hygrophilic and so are mostly found in rooms showing long term condensation such as the bathroom and water damaged materials (INSPQ 2016).

Because spores are aggregated and covered by mucilage, they are not easily carried by air. However, most of the time, these spores are mechanically moved (*via* animals or human) or attached on dust (INSPQ 2016). However, *S. charatum* detection, associated with poor air quality, is considered as clinically relevant and dangerous for health. Indeed. *S. chartarum* is known to produce tricholthecens mycotoxins and to be associated to chronic diseases and building-related illness such as headache, muscular pain, cough or upper respiratory complaints (INSPQ 2016; Khun and Ghannoum 2003; Scientific Institute for Public Health 2015).

1.3 Fungal monitoring

As the fungal contamination is considered as a source of indoor air pollution and because it could have some effect on health, different approaches have been developed to detect and identify this indoor airborne pollution. In our industrialized countries, these monitoring activities are performed by governmental laboratories, academic laboratories and some private companies. In Brussels for example, since 2009, a partnership has been organized between the CRIPI team from the Brussels Environment (Belgium) and the Environment and Health unit from the Scientific Institute of Public Health (WIV-ISP) (Belgium). The main goal is to detect indoor pollution, such as chemical pollution (aldehydes, carbon monoxide, nitrogen oxides and hydrocarbons) and biological pollutions (acarids, dust mites and fungi) of air and surfaces (dust, mattress or wall), which could cause some health problems. Briefly, when a patient is suffering from some health problem which could be due to indoor air pollution, the patient's habitation is sampled by the CRIPI team to give environmental data for the medical diagnosis. The following samples are collected by the team: dust from mattresses, swabs from visible mold colonies and air samples. These samples are then transferred to the laboratory of the Environment and Health unit from the WIV-ISP. There, the samples are put into culture, followed by fungal identification and quantification performed by

mycologists.

It should be noted that air and dust are not the only substratum which could be collected in the framework of fungal monitoring. In some cases, water samples are also collected in order to verify that no fungal contamination is present. This is the case for the Environment and Health unit from the WIV-ISP who analyzes water samples collected by private companies in charge of quality control of air-conditioning systems of client's buildings.

However, although fungal contamination appears to lead to public health issues, international guidelines are poorly documented to define harmonized protocols for fungal monitoring including sampling methods and identification and quantification protocols. Therefore, quantitative standard limits are ranging from 100 CFU/m³ to 1000 CFU/m³ (all fungal species mixed) to define a non-contaminated indoor environment (Rao et al. 1996). According to the recommendations made by the WHO in 1988, if the total fungal contamination is lower than 150 CFU/m³, no investigation should be made. This limit is allowed to reach 500 CFU/m³, if *Cladosporium* species are present in the mix (Rao et al. 1996). In contrast, if one species is present at a concentration higher than 50 CFU/m³ or if a toxigenic and/or pathogenic species is observed, the WHO recommends organizing investigations in order to confine the contamination (Rao et al. 1996). However, in 2009, the WHO communicated that "*As the relations between dampness, microbial exposure and health effects cannot be quantified precisely, no quantitative health-based guideline values or thresholds can be recommended for acceptable levels of contamination with microorganisms. Instead, it is recommended that dampness and mould-related problems be prevented. When they occur, they should be remediated because they increase the risk of hazardous exposure to microbes and chemicals*" (World Health Organisation 2009). Based on this recommendation, the interpretation of fungal monitoring results must be carefully formulated.

1.3.1 Fungal sampling

1.3.1.1 Surface sampling – Tape and Swab

Fungi can be found in diverse substrata. Therefore, samples could be directly collected from the substratum and transferred to the sampling surfaces with sterile swabs or tapes (Anses 2016; Urzi and De Leo 2001). Swab samples or tape samples are then analyzed directly with microscopic identification. Swab samples could also be transferred onto a culture media and incubated before identification.

The direct sampling offers the advantage to be cheap, fast and to give a picture of the fungal diversity present on the sampled surface at the moment of the sampling. Nevertheless, this direct approach has some disadvantages. Firstly, the sampling size is limited and so it is not

representative of the real diversity existing in the total sampling place. Secondly, these techniques could cause some damage to the fungal structure, thereby making the taxa unidentifiable.

1.3.1.2 Air sampling

If the direct sampling gives an idea of the fungal species present on surfaces or dust, this approach is not helpful to detect airborne fungi. To collect airborne particles such as spores or other aerosolized fungal compounds, 2 types of air samplers exist i.e., a sampler collecting air particles into growth media contained in petri dishes (e.g., Six stages Andersen cascade impactor) or strips (e.g., Reuter centrifugal sampler® (RCS® plus)), and those collecting air particles not into growth media but onto filters (e.g., Sartorius™ airport MD8) or into sampling liquid (Coriolis® μ).

Six stages Andersen cascade impactor: The six stages Andersen cascade impactor is a common sampler used in aerobiology commercialized by ThermoFisher Scientific (Waltham, USA). This sampler is designed to collect airborne particles according to their size and their aerodynamic characters into 6 stages of petri dishes (Fig. 1.8). Through a constant air flow of 28.3 L/min, from the top to the bottom, airborne particles, according to their size, are pulled down across the stages (ThermoFisher Scientific). This special design mimics the deposit of airborne particles in the human respiratory tract. Indeed, the particles larger than 7 µm are deposited in the two first stages which correspond to the size of particles deposited in the nose. Particles with a size ranged between 3 and 7 µm are collected in the 2 following stages and correspond to the particles able to reach the trachea to the bronchioles. Finally, fine particles, inferior to 3 µm, are collected into the last petri dishes and correspond to particles detected in alveoli (ThermoFisher Scientific).

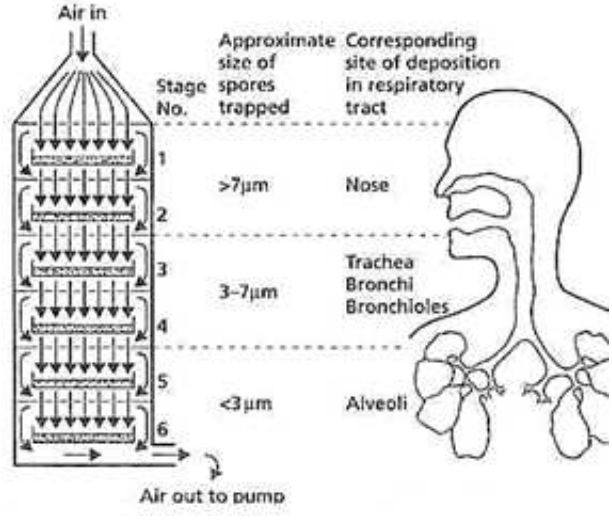

Figure 1.8 6 stages Andersen cascade impactor mimicking the deposition in the respiratory tract (adapted from http://archive.bio.ed.ac.uk)

Reuter Centrifugal Sampler® (RCS) plus: The Reuter Centrifugal Sampler® (RCS®) plus from Merck Millipore is a portable air sampler (Fig. 1.9). In this system airborne particles are impacted directly into Hycon agar strips (Merk Millipor, Darmstadt, Germany) containing growth media. With this system, the air flow can reach 100 L/min.

Figure 1.9 RCS air sampler (a) and (b) Hycon Agar strips (adapted from Merckmillipore)

Sartorius™ Airport MD8: The Sartorius™ Airport MD8 (Sartorius, Göttingen, Germany) is a portable air sampler (Fig. 1.10) commercialized by Sartorius which collects airborne particles into soluble gelatin membrane filters. The use of filters instead of growth media gives the possibility to analyze the airborne diversity independently of the micro-organisms viability. However, if culturing is required during the analysis, the micro-organisms' viability could be impacted by desiccation occurring during the air flowing through the filter. This air flow could rise till 1000 L of air with a flow rate of 125 L/min (Sartorius).

Figure 1.10 The Sartorius™ Airport MD8 (adapted from Sartorius)

Coriolis ®μ: The Coriolis®μ is an air sampler recently developed by Bertin Technologies (Montigny-le-Bretonneux, France). With this technology, air particles are collected into a sampling liquid consisting of Tween® 20 or Triton x-100 solution. Using the centrifugal force, the sampler collects the biological particles and separates them from the air into the liquid as shown in Fig. 1.11. Compatible with culture as well as with molecular analysis, the use of liquid guaranties also the integrity of viable organisms. This sampler is fast and efficient, and able to develop an air

flow of 300 L/min.

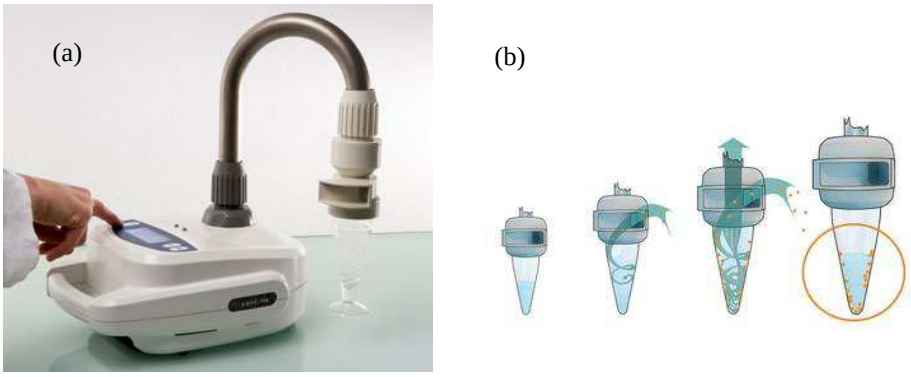

Figure 1.11 (a) Coriolis® μ sampler (b) concentration of particles into the liquid sample (adapted from Bertin Technologies)

1.3.2 Classical method for fungal identification: culture and microscopic determination

After the sampling, the next step is the identification of the fungal species contained in the sample. Today, in aerobiology, most of the techniques used to detect, identify and quantify the fungal contaminants are based on culture, isolation, microscopic and macroscopic visualization of fungal colonies on plate and fungal structures such as spores. Culturing protocols are used for different purposes such as allergen studies, clinical testing, antifungal resistance, or strain isolation.

Briefly, samples form air or dust are deposited on a plate containing a culture media and incubated at a specific temperature and during a determined incubation time. Temperature, humidity rate, incubation time and substrata are chosen in accordance with the characteristics of the species or group of species which is targeted (World Health Organization Regional Office for Europe 2004). So, during a routine analysis of indoor air samples, mesophilic hydrophilic fungal species such as *A. versicolor*, *C. herbarum*, *C. sphaerospermum* or *S. chartarum*, are cultivated on Rose Bengal chloramphenicol agar or Sabouraud chloramphenicol agar at 25 °C during 5 days (Scientific Institute for Public Health 2015), whereas mesophilic xerophilic species such as some *Penicillium* sp. or the *Aspergillus glaucus* are incubated as well at 25 °C during 5 days, but on dichloran glycerol characterized by a low water content. Some other species, such as *A. fumigatus*, are thermophilic and are isolated at 45 °C on Yeast Mold agar medium (Scientific Institute for Public Health 2015). Other species grow slower than others and so, are requiring a longer incubation time such a se.g., *Exophiala jeanselmei* which needs 21 days of incubation at 25 °C (Scientific Institute for Public Health 2015; Libert et al. 2016). So, in routine analysis, several protocols with defined culture conditions (i.e., medium composition, temperature, humidity and time of growth) are used depending on the species targeted by the analysis or monitoring.

Subsequently, fungal identification is performed by microscopic determination of colonies present on plate. The identification of species by microscopic visualization is based on their

morphological characteristics (e.g., color of the colony, spore size or form, mycelium characteristics, presence of sexual structures, form of sexual structure, etc) but also the medium used and/or the time and temperature of incubation (which could affect phenotype expression) (World Health Organization Regional Office for Europe 2004; Vesper 2011; Scientific Institute for Public Health; 2015). This is a complex discipline which requires a high expertise, especially as many classifications exist as well as many synonyms for the same species.

An advantage that is frequently cited is that culturing gives a picture of the fungal diversity existing at the time of the sampling. Nevertheless, this argument does not take into account the bias of fungal diversity observed on plate which could be impacted by inhibitors or other competition factors, the difference in growth speed, the selection induced by the used media and the nutrients availability (Vesper 2011). Moreover, some species are not able to reproduce or produce reproductive structures on culture media and so, cannot be correctly identified (Vesper 2011). Others are not viable or not cultivable on plate and therefore are not detectable on plate (Vesper 2011). Thus, due to these drawbacks and despite the common resort to culture, the culture-based techniques seem to be not sufficiently exhaustive to monitor or study the indoor fungal community in order to understand their impact on public health. Indeed, as well as viable and cultivable species, not detected or not cultivable and/or dead species could have also an impact on health.

1.3.3 Molecular tools for fungal detection

Molecular approaches are increasingly used for the investigation of indoor airborne fungi. Being fast, sensitive and accurate, molecular tools offer the possibility to reduce the time needed to perform the analysis and to be independent of the viability/cultivability of biological materials which is in contrast with the detection protocols based on the culture.

1.3.3.1 Use of polymerase chain reaction and quantitative polymerase chain reaction

Developed in the 80's by K. Mullis (Mullis et al. 1986), the polymerase chain reaction (PCR) is the most common tool of molecular biology. Briefly, PCR consists of an enzymatic *in vitro* amplification of a part of DNA by a thermostable DNA polymerase using specific nucleotides (i.e., the primers) complementary to the DNA region to be amplified. The PCR reaction is a thermal cycling process, commonly composed of 30-40 cycles, each of these cycle constituted of 3 steps i.e., the DNA denaturation at a temperature ranging between 90-95 °C, the primer hybridization, also called annealing which occurs at a temperature between 50 and 65 °C and the DNA synthesis, also called the DNA polymerase elongation. Because one copy of each targeted sequence is produced at the end of each cycle, the PCR reaction allows the exponential

amplification of the sequence of interest.

PCR reactions are used since many years in the fungal domain, especially for the detection of fungal pathogens in biological samples such as sputum, blood or biopsies (Polzehl et al. 2005), but also for the identification of isolates from environmental samples such as indoor air samples (Martin and Rygiewicz 2005; Wu et al. 2002; Zhou et al. 2000). Often these molecular tools are targeted to the internal transcribed spacer region (ITS region). This region is flanking the18S rRNA gene encoding the small subunit of the ribosomal RNA (SSU RNA), and the 28S rRNA gene encoding the large subunit of the ribosomal RNA (LSU RNA). The ITS region contains 2 internal transcribed spacers separated by the 5.8S rRNA gene (Fig. 1.12), which are both polycistronic rRNA precursor transcripts, i.e., the internal transcribed spacer 1 (ITS-1) and the internal transcribed spacer 2 (ITS-2) (Schoch et al. 2012). Most of the PCR tools used for fungal identification are based on universal primers able to amplify these ITS-1 and ITS-2 regions, i.e. primers ITS1, ITS2, ITS3 and ITS4 (White et al. 1990) (Fig.1.12). The ITS region is usually used for phylogenetic analysis of fungal taxa and is considered as a barcode marker for fungal species identification. This is because the ITS region has the advantage to be, on the one hand, highly variable at the interspecies level and on the other hand species-specific (Schoch et al. 2012; Chemidlin Prevost-Boure et al. 2011; Costa et al. 2001; Iwen et al. 2002).

The high number of copies of this ITS region in the fungal genome explains also why these ribosomal regions are targeted by molecular approaches (Black et al. 2013). The more copies of the targeted sequence are present, the more easily the detection by molecular approaches. This observation is especially true for quantitative PCR (qPCR), which is faster, more accurate and sensitive than PCR.

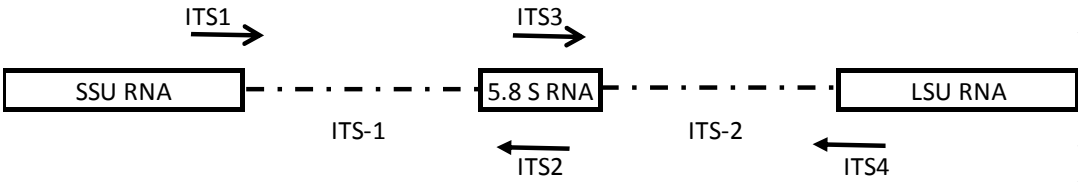

Figure 1.12: Internal transcribed spacer region and ITS primers
The arrows present the primers and their orientation. ITS1 and ITS3 are forward primers. ITS2 and ITS4 are reverse primers. ITS-1 and ITS-2 correspond to the internal transcribed spacer 1 and 2.

qPCR is another classical tool used in molecular biology. Developed in the 90's as an improvement of the classical PCR (Holland et al. 1991), qPCR gives the advantage to measure the DNA polymerization along the process of amplification. The visualization of the classical PCR amplification requires a post-amplification analysis process such as e.g., agarose gel electrophoresis and Ethidium bromide as intercalating, fluorescent dye. qPCR allows to follow in

real-time the amplification process by the detection and the measurement of a fluorescence level from a specific qPCR chemistry (Navarro et al. 2015; Tsé and Capeau 2003). Today, 2 types of chemistries are available (Navarro et al. 2015) i.e., the first one is based on nucleotides which are labeled with fluorophores, i.e. hybridization probes such as the fluorescence resonance energy transfer (FRET) probes or molecular beacons; hydrolysis probes such as TaqMan® probes; primer-probes such as scorpion primer probes or hairpin primers-probes; and finally fluorescently labeled analogues of nucleotides acids such as peptide nucleotide acids (PNA) or locked nucleic acids (LNA®). The second type of chemistry is based on the use of fluorescent double stranded DNA (dsDNA) intercalating agents such as SYBR®green or EvaGreen.

For each qPCR run, the results are expressed in quantification cycle values (C_q) (Bustin et al. 2009). The C_q corresponds to the cycle where the fluorescence level from the amplification rises above the fluorescence background (Navarro et al. 2015; Tsé and Capeau 2003). As such, a measurable amplification is observed, only when the fluorescence level is higher than the fluorescence from the background.

Currently, in the fungal domain, qPCR is largely used for the detection of fungal species in clinical samples (Costa et al. 2001; Khot and Fredricks 2009; Schalbereiter-Gurtner et al. 2007), but also for the detection of environmental contamination (Hospodsky et al. 2010; Lignell et al. 2008; Pitkaranta et al. 2011).

Many qPCR assays are available for fungal detection in indoor environment. Most of them are based on the TaqMan® chemistry (Bellanger et al. 2009; Haugland et al. 2004; Melkin et al. 2004) which consists of the hybridization and the hydrolysis of a short nucleotide sequence containing at the 5'-end a fluorescent dye reporter inhibited by a quencher located at the 3'-end (Fig. 1.13). The fluorescent signal is emitted only when the quencher is physically separated from the reporter, which will occur during the elongation step, when the probe is hydrolyzed by the DNA polymerase by its 5'-3' exonuclease activity (Navarro et al. 2015). For this technology the specificity is determined by two factors i.e., the primers and the probe, the latter which is designed to bind to a region contained in the amplicon defined by the primers. Another advantage resides on the possibility to perform multiplex analysis (i.e., analysis of more than one target at the same time) by combining different probes labeled with different dyes. The number of multiplexing is limited to 4 or 5, as there are only a limited amount of different dyes available.

Besides the TaqMan® technology, the SYBR®green chemistry (Fig. 1.13) is also used, although less than the TaqMan®, especially for fungal detection, despite its lower cost than probe-based qPCR tools. The SYBR®green chemistry is based on an asymmetric cyanine dye intercalated into dsDNA. The fluorescence signal is emitted when the dye is released during the DNA extension step and its intensity increases with the DNA polymerization (Navarro et al. 2015). Besides the C_q value, the SYBR®green chemistry provides a second value based on the fluorescence i.e., the

melting temperature (T_m). This value is obtained through a post-amplification analysis of all amplicons produced during the process and is defined as the temperature at which 50 % of dsDNA template is denaturated. Because this temperature is directly linked to the nucleotide composition of each amplicon, the T_m value from SYBR®green qPCR assays gives an evaluation of the specificity of each amplicon obtained at the end of the assay.

Even though multiplexing is more difficult to be performed with SYBR®green than multiplexing with probe-based methods, the discrimination could be made if the T_m of each amplicon is sufficiently distinct (i.e., 1 °C minimum). However, sometimes the nucleotide variation between 2 amplicons from different targets is too small to allow discrimination based on the T_m value, i.e. no discrimination can be made and false positives would be recorded.

To avoid this problem, since 2003, another qPCR tool is available for the analysis of sequences containing small differences i.e., the high resolution melting (HRM) analysis (Wittwer et al. 2003). The HRM analysis is an improvement of SYBR®green qPCR assays using the Evagreen dye instead of SYBR®green. Evagreen is a new generation of intercalating dyes which is a saturating dye, unlike SYBR®green. Consequently, all amplicons obtained after the DNA template amplification are saturated by the dye, improving the detection of nucleotide variation. In combination with a small-stepwise increase of temperature during the DNA denaturation step (commonly 0.2 °C) and a high resolution qPCR instrument, the use of Evagreen allows a very accurate T_m analysis (Wittwer et al. 2003). Therefore, closely similar targets not distinguishable with classical SYBR®green analysis will be able to be discriminated with HRM analysis (Lengerova et al. 2014; Nemcova et al. 2015; Somogyvari et al. 2012). Unlike SYBR®green qPCR assays, the results form HRM analysis are not only based on the C_q and T_m values, but also the similarities of the shape of the melting curves are analysed and samples with similar shapes of the melting curves are grouped together within a cluster. By including a positive control for each of the targets, the discrimination and identification of each cluster can be done.

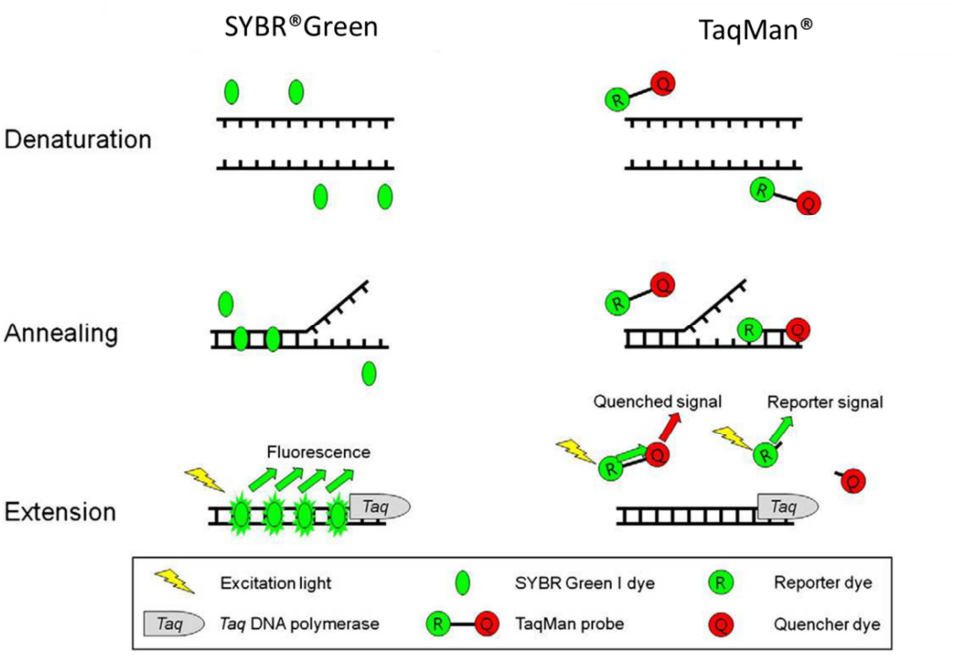

Figure 1.13: SYBR®green and TaqMan® assays (adapted from Kim et al. 2013)

Because environmental samples could contain more than one species, the detection tool must be able to identify all taxa present in the sample. However, most of the molecular tools, such as PCR and qPCR, are designed to detect only one species at once. These tools are called simplex assays. In order to reduce the time needed for a complete analysis, and therefore the time before interventions can start, and in order to save sometimes very scarce, but precious sample material, more and more assays are designed to detect multiple targets in a single run. These tools are called multiplex assays.

As explained above, TaqMan® probe- based assays are the most suited qPCR tool for multiplexing. However, due to the number of fluorophores and quenchers available for multiplexing, probe detection is limited to 4 or 5 different ones (Gurvich and Skoblov 2011). Therefore, if more targets are to be detected simultaneously, alternatives are needed.

1.3.3.2 Multiplex analysis: Luminex® technology

At the end of the 90's, Luminex Corporation developed a detection system specifically dedicated for multiplexing. Based on the principle of flow cytometry, the Luminex technology consists of the fluorescent detection of multiple sets of polystyrene magnetic microspheres (beads) characterized by a specific spectral emission (Fig. 1.14), i.e. a liquid bead suspension array. The power of this technology is that each set of beads has a unique combination of red, infra-red and orange dyes which allows their specific recognition by the Luminex instrument used for read-out

of the assay. This means that in a mixture of sets of beads, each bead could be identified individually by the machine (Dunbar 2006). Depending on the type of Luminex® instrument, the number of sets which can be detected during a single run ranges from 50 for the MAGPIX instrument (read out with a CCD camera) to 500 for the FLEXMAP 3D (read out with a laser). The emission of a red fluorescence is the signature of the beads, the green fluorescence indicates the ligation of a target to the beads. Indeed, to each bead set a biological molecule such as proteins or oligonucleotides could be coupled (Dunbar 2006) which will bind or hybridize to a specific target in the sample. In the case of oligonucleotides, which are called 'probes', different technologies exist, e.g. ligation dependent amplification (LDA) and the direct hybridization.

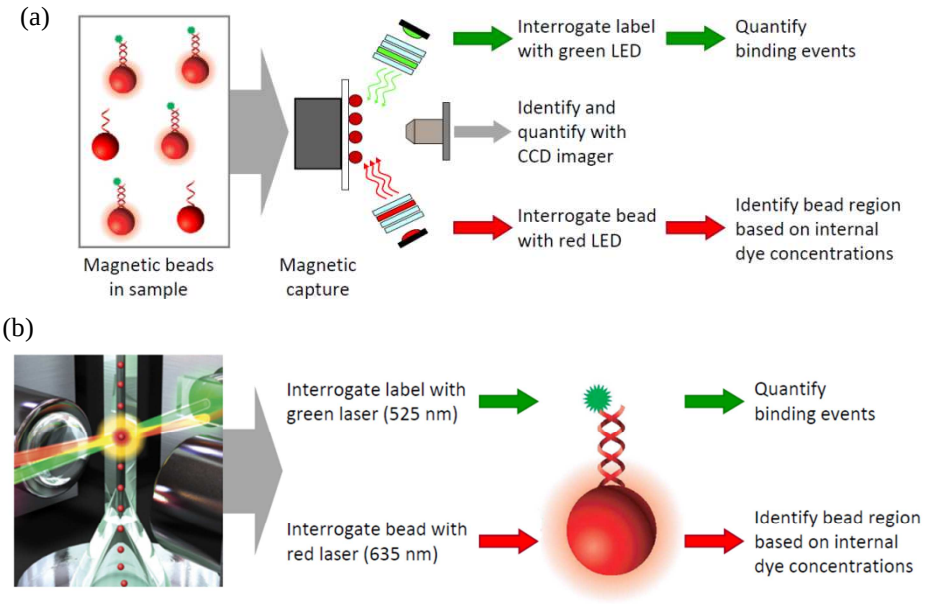

Figure 1.14: (a) Bead-suspension array detection by CCD camera imager (adapted from Luminex) (b) read-out of liquid bead suspension array with a laser read-out (adapted from Luminex)

LDA is based on the Luminex xTAG® technology and MagPlex-TAG® magnetic microspheres (xTAG® beads). Briefly, each set of xTAG® beads is pre-coupled to a specific anti-TAG which can hybridize to a complementary TAG. In parallel, each TAG is coupled to the target during a primer extension step performed in the presence of biotin. Subsequently, the TAG hybridization to the anti-TAG takes place and the resulting product is incubated with streptavidin-R-phycoerythrin (SAPE) as a reporter. The use of biotin and SAPE is aimed to produce the green fluorescence which is used during the analysis on the Luminex instrument. So, if the target is present in the sample, it will be coupled to the TAG and marked with biotin. After hybridizing to the anti-TAG and SAPE treatment, a green fluorescence will be produced, that can be measured. If the target is absent, the biotin will not be able to be added to the TAG and so, no green fluorescence will eventually be detected.

The direct hybridization approach is based on the Luminex xMAP® technology. In this case, the detection is made through target-specific probes. The probes are firstly coupled to Magplex microspheres (beads). Then, the probe-beads couple can hybridize to a specific PCR amplicon, previously obtained with biotinylated primers using the DNA of the sample as template. Finally, after the addition of SAPE, the read-out on the Luminex® instrument is performed. Once again, if the target is present in the sample, a green fluorescence signal will be measured on the instrument.

Mainly used for the study of proteins (including cytokines), microRNA, gene expression or enzyme immunoassays, the Luminex technology is also used in a public health context especially in microbiology for the detection and characterization of food-borne pathogens (Wuyts et al. 2015), but also for fungal detection in a clinical setting. Indeed, some Luminex® assays exist for the detection and characterization of fungal pathogens in biological samples such as blood, biopsies or biological secretion (Etienne et al. 2009; Landlinger et al. 2009).

Because the Luminex xMAP® technology is based on a probe detection system, the selection of species to be detected should be a priori made during the development of the assay, to be able to include the specific probes. So, if a non-targeted (i.e. initially not included) species is present in a sample, and for which no probes are present in the assay, it will not be detected during this analysis. Therefore, the Luminex technology could be considered as targeted multiplex approach. The identification of initially not included taxa requires an open approach where all the species can be detected in a single run.

1.3.3.3 From Sanger to next generation and third generation sequencing

For years, fungal identification as well as fungal monitoring has been studied with culture-based protocols (Pitkaranta et al. 2011; Vesper 2011). However, due to the long turn-around-time and the expertise required for the identification, molecular tools such as sequencing have been used. Even though sequencing is not yet used as a full detection or monitoring system, this technology is strongly linked to the improvement of knowledge on biological systems such as the fungal community (Cuadros-Orellana et al. 2013; Dudhagara et al. 2015; Tonge et al. 2014).

Considered to be a standard technique for DNA sequencing, the Sanger sequencing is largely used in the mycology research domain (Kawasaki et al. 1990; Nilsson et al. 2008; Wang et al. 2001). Sanger sequencing consists of a dideoxy reaction termination sequencing using the inhibitory activity of the 2',3'-deoxythimidine triphosphates (ddNTPs) on the DNA polymerase enzyme (Sanger et al. 1977). During the reaction, a single stranded DNA template is copied by a DNA polymerase enzyme in the presence of a specific primer. The production of the amplicon occurs in

5'-3' direction by the incorporation of complementary nucleotide bases (dNTPs). When a ddNTP (i.e., ddATP, ddTTP, ddCTP or ddGTP) is incorporated instead of a dNTP, the extension is stopped (Sanger et al. 1977). Each ddNTP is labelled with a specific fluorescent marker. Therefore each product will be specifically labelled (Fig. 1.15). Through a high resolution electrophoresis, the products are then separated by size and identified by laser detection (Fig. 1.15). Based on the detection of the specific fluorescent marker, the order of the nucleotides, i.e. the sequence, can be determined. Sanger sequencing is limited to a single strand from only one target at a time.

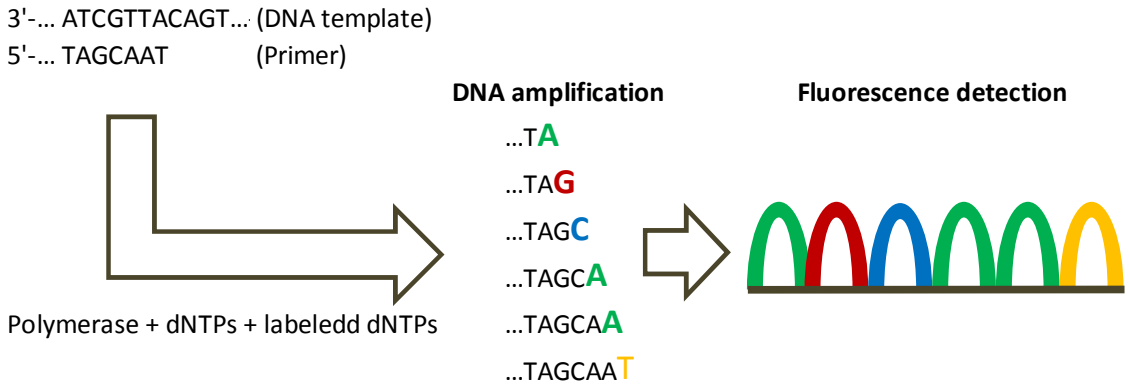

Figure 1.15: Sanger sequencing – schematic principle
Colored letters represent the fluorescent labeled ddNTPs. The colored bridges illustrate the fluorescence emitted by the ddNTPs.

Some years ago, a second generation of sequencing method appeared, referred to as next generation sequencing or NGS. These new methods are widespread in all the fields of biological research from cancer research to ecology, virology, bacteriology, food-pathogen subtyping or GMO identification, but also mycology and aerobiology. In contrast to Sanger sequencing which is limited to one single strand (one DNA sequence), NGS tools are able to sequence multiple strands during a single run.

Among the different NGS technologies available, the Illumina platform is the most used (Fig. 1.16). Developed in 2006, Illumina sequencing is based on the "sequencing by synthesis" chemistry which is characterized by the incorporation of reversible terminator nucleotides labeled with fluorescent markers (one marker per nucleotide) (Ansorge 2009; Loman et al. 2012). The reaction occurs on a support covered densely by adapters on which the DNA is linked after the denaturation step. The free extremity of the DNA is hybridized to a complementary adapter forcing the DNA to form a bridge on the support. Then, the single strand DNA fragment is polymerized by the DNA polymerase. During the PCR amplification, the nucleotide incorporation releases a fluorescent signal which is detected and identified by a camera. The signal is emitted and recorded, the 3'-end terminator group coated to the nucleotide is removed and the DNA

polymerization begins again (Ansorge 2009; Loman et al. 2012).

Illumina sequencing platforms are also characterized by the large amount of reads which could be produced per run i.e., between 12,000,000 and 3,000,000,000 per reads depending on the instrument and the sequencing kit used for analysis. The sequencing error rate is estimated to be 1 per 1000 sequenced bases (Roos et al. 2013).

Because the Illumina platform, such as other NGS tools, could analyze multiple targets in a single run, this tool could be considered as a multiplex open approach in terms of sample analysis. This approach gives the opportunity to perform environmental research based on metagenomics approaches which consist of the analysis of all DNA (or specifically generated PCR amplicons) available in a sample (Thomas et al. 2012; Venter et al. 2004). This platform was also used for the investigation targeting the ITS region of the soil fungal community (Schmidt et al. 2013), of the cutaneous mycobiota in cats (Meason-Smith et al. 2016), but also for metagenomic studies applied on indoor airborne fungal community (Tedersoo et al. 2015; Uroz et al. 2013). In 2016, Lee et al. performed a high –throughput Illumina sequencing in order to analyze the composition and the diversity of *Aspergillus* species in outdoor air (Lee et al. 2016).

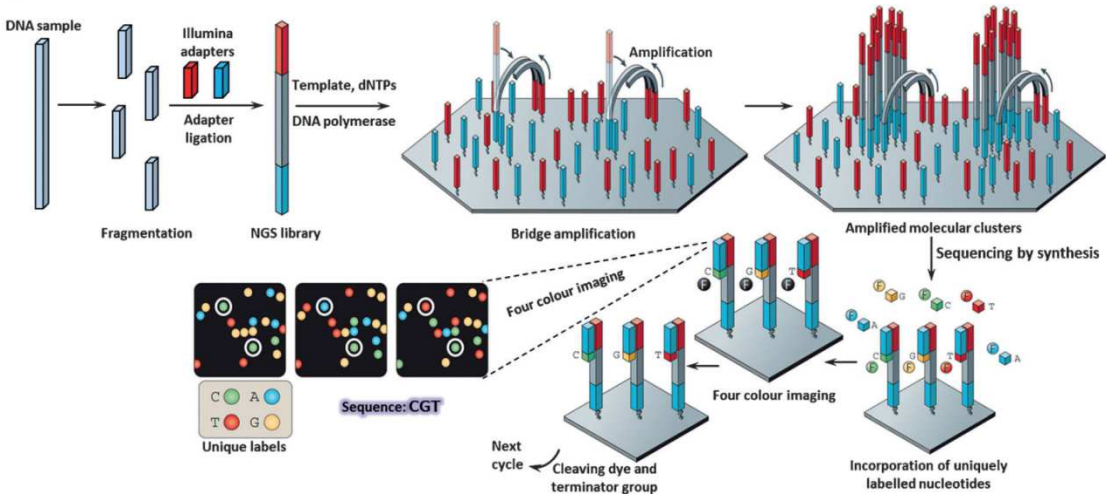

Figure 1.16: Illumina® sequencing technology – schematic principle (adapted from Anandhakumar et al. 2015)

Although NGS technologies provide a large amount of information on biological systems, these tools are limited to short read lengths i.e., between 36-100 bp for the Illumina HiSeq 2000 and HiSeq 2500 and 36-300 for the Illumina MiSeq 300 (Illumina 2016; Robert 2013). Read size restriction is eliminated by the third generation sequencing tools such as the single molecule real-time sequencing (SMRT) performed by Pacific Biosciences (PacBio), initiated in 2011, and which

could reach more than 14 kb per read. Another improvement can be found in the sensitivity of the technology, which is able to detect only one molecule without any pre-amplification step before. Due to these features, the SMRT sequencing is a helpful tool for full genome sequencing. However, the error rate is estimated to be 1 per 10 sequenced bases. This is one reason to explain why SMRT PacBio is yet currently used mainly in research, especially for whole genome sequencing applications (Di Bella et al 2013). SMRT sequencing is also a "sequencing by synthesis" technology but, in this case, an ultra-efficient DNA polymerase enzyme is coated to the surface of a SMRT cell (Fig. 1.17). Briefly, at first, the DNA fragment to identify is ligated at the 3'- and 5'- ends to adaptors. Then, this polymerization complex is annealed to the DNA polymerase. During the reaction, a constant flow of fluorescently labeled nucleotides is captured by the polymerization complex and the fluorescence from the incorporation of nucleotides is recorded in real-time all along the DNA amplification. When a nucleotide is incorporated and the signal emitted, the fluorophore is released and extension can go on (Loman et al. 2012).

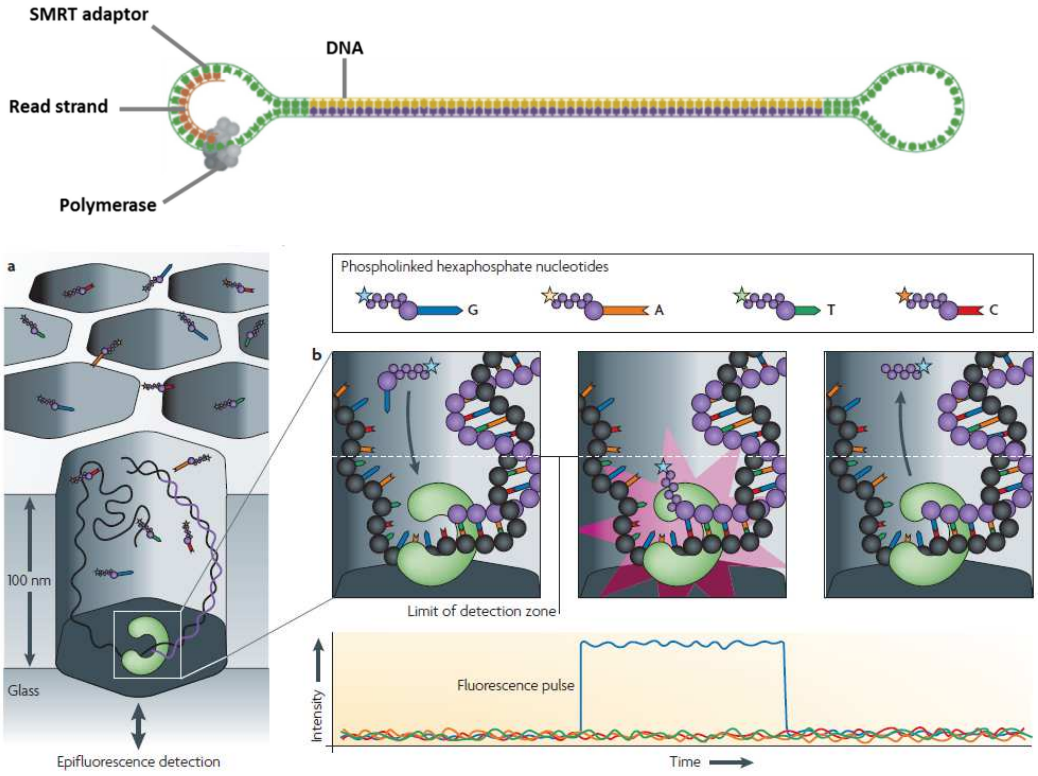

Figure 1.17: Pacific Biosciences® technology- schematic principle (adapted from Metzker 2010 and Rhoads and Au 2015)

1.3.4 Quantitation of the indoor air fungal contamination

Besides identification, quantification is also required to determine in its entirety the impact of a contaminant on health. Quantitative data on the fungal charge in indoor air is an important element in understanding the causal link between airborne fungal contamination and health effects. Because no norms or guidelines exist to harmonize the fungal quantification (as elaborated above), several approaches are used to quantify the fungal charge in air.

A lot of protocols used for fungal load quantification are culture-based as it is done for the fungal identification (Anses 2016; Rosembaum et al. 2010; Simoni et al. 2011). Using microscopic or direct visualization, these techniques consist of counting of colony forming units (CFU) present on an agar plate after a determined time of growth/incubation. In this case the results are expressed in CFU (or CFU/L air, CFU/m³ air). However, as it is observed for the identification, culture-based protocols could induce some bias, due to inhibition or competition, and so, the results are not representative of the complete fungal charge present in the sample. Moreover, some colonies are aggregated and hence are counted as a single colony.

Some protocols determine the fungal contamination through the charge of spores present in air, using spectrophotometry measurements, a hymocytometer or a flow cytometry or molecular tools such as qPCR (Anses 2016; Morris and Nicholls 1978; Vanhee et al. 2009). Although these spore-based approaches are fast, easy and useful to determine the total charge of fungal spores in air, the disadvantage is that no information is given about the other compounds (such as fragments of mycelium, parts of cell wall…) which could be present in air and which could have some impact on health. Other quantification methods are based on the detection of chitin, fatty acids or ergosterol *via* biochemical methods or high performance liquid chromatography (HPLC) (Davey et al. 2009). Nevertheless, because these compounds are not present in all fungal lineages, the measurements obtained with these tools are biased (Olsson et al. 2003).

Many qPCR assays are also developed for fungal quantification (Filofteia Diguta et al. 2010; Haugland et al. 2002; Liu et al. 2012; Zeng et al. 2006). In 2002, Haugland and Vesper proposed a metric called relative moldiness index (EMRI) to quantify molds contamination in U.S. homes. This metric is obtained through a mold-specific qPCR (MSQPCR), targeting rRNA genes (ITS regions, LSU) which measures the concentration of 36 fungi possibly present in American houses (Haugland and Vesper 2002). Although commonly used, this technique could be impacted by PCR inhibitors present in air (Melkin et al. 2007; Täubel et al. 2016). The use of standard curves is also largely used to estimate the amount of DNA amplified in a samples according to the C_q values obtained during the analysis of a serial DNA dilution containing a determined amount of DNA.

However, the observation of the lack of usable exposure assessment methods for unknown agents is also valid for the quantification methods. The absence of data concerning quantification of these "unknown" contaminants hampers the understanding of their impact on human health.

Rationale and outline of the thesis

Currently, there still exists controversy on the causal relationship between exposure to indoor airborne fungi and adverse health effects. The understanding of this relationship is key to reduce the burden of fungal exposure in favor of public health. However, scientific evidence is still lacking due to the absence of uniformly environmental sampling and detection methods to assess the indoor fungal contamination. Most of the commonly developed and used detection methods are culture-based. However, although conventional methods have proven their effectiveness, some problems are encountered such as the expertise required for morphological identification, the extended time needed to perform the analysis and a loss of representativeness of fungal diversity. Indeed, in addition to the problems related to competition, difference in speed of growth, dependence on growth media selected, culture-based protocols are not able to detect the non-viable taxa or non-cultivable species in the samples (called unknown agents or uncultivable and/or dead fraction). As a result, there is a lack of data on the fungal diversity in indoor air. In parallel, immunological tests available to assess the impact of indoor fungal contamination and health effects are still limited to the most commonly culture-based detected species. Therefore, increasing the data available on indoor airborne fungal diversity will contribute to improve the data available for the development of immunological tests and eventually to fill the gap between fungal contamination and public health.

In this context, this PhD thesis aimed to develop molecular approaches for specific, sensitive, fast, accurate and non-culture based detection of indoor airborne fungi, which could be potentially allergenic. Besides this technological objective, this work aimed at providing a better insight into fungal contamination and especially into the fraction not yet detected by conventional methods, such as the uncultivable or dead fraction.

In order to deal with this challenge (Fig. 1.18), a first step of this study was to dispose of an appropriate workflow, including sampling, DNA extraction and purification, compatible with molecular analyses. This part of the work was performed for two study cases i.e. *Aspergillus versicolor*, one of the most common indoor fungal species (chapter 2) and *Exophiala jeanselmei*, a species suspected to be a species belonging to the dead fraction and not yet detected in indoor air by the classical monitoring approaches (chapter 3). For these two study cases, a simplex qPCR

SYBR®green detection tool was developed and its performance assessed. Subsequently, the molecular tools developed in this work focused on multiplexing in order to reduce the turn-around-time of the fungal monitoring, and to reduce the amount of sample used for a single analysis. The multiplexing was applied using two different approaches i.e., a multiplex targeted approach focused on selected fungal species (3 genetically closely related species and the 10 most common species found in indoor air) and a multiplex open approach focused on the whole fungal airborne community. Chapter 4 describes the development of a post-qPCR high resolution melting analysis (HRM) used for the discrimination of 3 genetically closely related species i.e. *A. creber, A. sydowii* and *A. versicolor*. This chapter could be considered as a study case demonstrating that HRM could be useful in the context of closely related species discrimination, based on previously developed qPCR assays. While the HRM presented in the chapter 4 is focused on the detection of closely related species, the Luminex xMAP® tool elaborated in chapter 5 was developed to improve the detection, during a single run, of 10 species commonly observed in indoor air. Both chapters 4 and 5 show that multiplex targeted approaches could improve the detection and the monitoring of indoor airborne contaminations, closely related species included.

To gain a better insight into the fungal diversity, multiplex tools should however not be restricted to the most common or the most commonly observed species in indoor air but must include all species that can potentially impact health. NGS could be such an open approach multiplex tool. Chapter 6 presents a study case to demonstrate that NGS can be used to collect data on the dead fraction for which the classical detection culture-dependent tools are not adapted, applied to *E. jeanselmei*. Chapter 7 reports the metagenomic analysis of the indoor airborne fungal diversity in contaminated houses, as well as a comparison between data obtained with classical methods and those obtained by NGS.

Chapter	Technologies	Cases studies	Number of targets
	qPCR SYBR®Green		
2		*A. versicolor*	1
3		*E. jeanselmei*	1
	Post-qPCR HRM		
4		*A. creber* , *A. sydowii* and *A. versicolor*	3
	Luminex® xMAP		
5		10 most commonly found fungal species in indoor air	10
	Massive parallel sequencing		
6		First detection of *E. jeanselmei* in indoor air	1
7		Metagenomic analysis of indoor airborne fungi	Unlimited

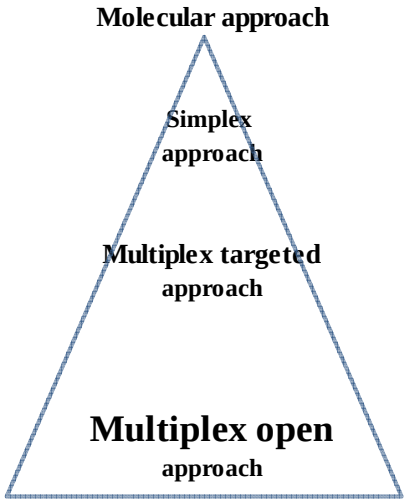

Figure 1.18: Schematic outline of the thesis

Chapter 2

Development and performance assessment of a qualitative SYBR®green real-time PCR assay for the detection of *Aspergillus versicolor* in indoor air

This chapter was previously published as Libert, X.; Chasseur, C.; Bladt, S.; Packeu, A.; Bureau, F.; Roosens, N.H., De Keersmaecker, S.C.J. (2015). Development and performance assessment of a qualitative SYBR®green real-time PCR assay for the detection of *Aspergillus versicolor* in indoor air. Appl Microbiol Biotechnol 99(17):7267-7282. DOI:10.1007/s00253-015-6785-9

Abstract

Currently, contamination of indoor environment by fungi and molds is considered as a public health problem. The monitoring of indoor airborne fungal contamination is a common tool to help understanding the link between fungi in houses and respiratory problems. Classical analytical monitoring methods, based on cultivation and microscopic identification, depend on the growth of the fungi. Consequently, they are biased by difficulties to grow some species on certain culture media and under certain conditions or by non-cultivable or dead fungi that can consequently not be identified. However, they could have an impact on human health as they might be allergenic. Since molecular methods do not require a culture step, they seem an excellent alternative for the monitoring of indoor fungal contaminations. As a case study, we developed a SYBR®green real-time PCR based assay for the specific detection and identification of *Aspergillus versicolor*, which is frequently observed in indoor environment and known to be allergenic. The developed primers amplify a short region of the internal transcribed spacer 1 from the 18S ribosomal DNA complex. Subsequently, the performance of this qPCR method was assessed using specific criteria, including an evaluation of the selectivity, PCR efficiency, dynamic range and repeatability. The limit of detection was determined to be 1 or 2 copies of genomic DNA of *A. versicolor*. In order to demonstrate that this SYBR®green qPCR assay is a valuable alternative for monitoring indoor fungal contamination with *A. versicolor*, environmental samples collected in contaminated houses were analyzed and the results were compared to the ones obtained with the traditional methods.

Key words

Aspergillus versicolor, indoor air, public health, real time PCR, SYBR®green, performance assessment

2.1 Introduction

The effects of allergenic molds on public health are well documented (Bellanger et al. 2009; Packeu et al. 2012a; Reboux et al. 2010). Indeed, molds can produce mycotoxins, spores, hyphae and wall fragments containing (1→ 3)-β-D-glucans and proteins which could induce allergic reactions of types I, III and IV (Douwes et al. 2003; Seo et al. 2008). Some studies revealed an association between the fungal levels in the air and the occurrence of allergy (Horner et al. 1995; Meheust et al. 2014; Mendell et al. 2011; Reboux et al. 2010) and between water-damaged buildings and human exposure to fungal contamination present in indoor air (Jones et al. 2011; Vesper et al. 2013). Presently, with the climate change, the new energy conservation measures, the development of the urbanization, and the considerable amount of time spent inside buildings, people are increasingly exposed and might be more susceptible to developing a respiratory problem caused by a fungal contamination (de Ana et al. 2006; Mendell et al. 2011; Sharpe et al. 2014). More specifically, *Aspergillus versicolor* is one of the most important fungal contaminants of houses (Beguin and Nolard 1994; Packeu et al. 2012a) and this species is known to produce allergenic compounds (Benndorf et al. 2008), which can have an implication in the development of asthma (de Ana et al. 2006).

Usually in routine analysis, the detection and identification of indoor airborne fungi are based on culture, microscopic visualization and visual counts. However, this classical approach requires an important level of expertise and is time-consuming (Vesper 2011). Another drawback of this approach is that it depends on the growth of the culture, which is known to be affected by the growth media chosen, the culture conditions (especially temperature and humidity), the incubation time (some species grow faster than others) or competition between species. Furthermore, a part of the sampled biological fraction is dead and non-cultivable, and therefore is not always detected by traditional analysis methods, although it could have an impact on human health as it might be allergenic. Therefore, this could lead to an underestimation of the total fungal airborne community and level of the contamination classically determined by colony forming units (CFU) on an agar plate (Pitkaranta et al. 2011; Vesper 2011).

Molecular techniques like the real-time polymerase chain reaction (qPCR), amplifying a specific DNA sequence in the fungal genome with a real-time detection of the amplification products, have been proposed as an alternative to these classical detection methods (Black et al. 2013; Haugland et al. 2004; Timothy et al. 2004). Besides being fast, sensitive and specific, qPCR also gives the advantage of being independent from culture.

Most often qPCR methods designed for fungal detection are based on the ribosomal gene complex

and particularly on its internal transcribed spacers (ITS) (Bellanger et al. 2009; Chemidlin Prevost-Boure et al. 2011; Costa et al. 2001; Johnson et al. 2012; Melkin et al. 2004; Michealsen et al. 2006; Roussel et al. 2013), which are non-coding regions of the fungal rDNA (ITS-1 and ITS-2) flanked by the small subunit (SSU) rRNA and by the large subunit (LSU) rRNA genes. The sequence variation of ITS regions has led to their use in phylogenetic studies of many different organisms (Nilsson et al. 2008). These ITS regions are selected for qPCR assays due to their good conservation and their weak level of polymorphism amongst DNA sequences of the same genus (Chemidlin Prevost-Boure et al. 2011; Costa et al. 2001; Iwen et al. 2002). Therefore they offer the possibility to develop specific molecular methods for detection at the genus or even at the species level (Hinrikson et al. 2005; Nilsson et al. 2008; Schoch et al. 2012).

The qPCR methods are started to be more frequently used for environmental investigations and monitoring of the most common fungal contaminants present inside buildings (Bellanger et al. 2009; Roussel et al. 2013), such as performed by the United States Environmental Protection Agency (EPA). Indeed, the EPA has developed a large set of qPCR methods based on the TaqMan® chemistry, as real-time detection system of the amplified products, aimed for indoor fungi monitoring (*A. versicolor* included) (Haugland et al. 2004; United States Environmental Protection Agency 2014). These labeled probe-based qPCR assays are highly specific. An alternative to TaqMan® method is the SYBR®green chemistry. Here, the detection of amplification is based on an intercalating fluorescent dye, independent of the use of a labeled probe. The specificity of this method is determined by the primers' specificity and by the melting temperature (T_m) of the amplicon. This approach is also sensitive and fast, but cheaper than the probe-based one. However, despite these advantages, not many SYBR®green assays exist yet for the detection and identification of airborne indoor molds. To the best of our knowledge, no SYBR®green based detection and identification method for *A. versicolor* has been reported yet.

Additionally, currently, even if many qPCR methods exist for the fungal detection, no specific guidelines are proposed concerning the assessment of their performance. This is in contrast to other domains where real-time PCR methods are already used during many years for the detection and identification of specific targets, such as for the detection of genetically modified organisms (GMO) in food and feed (Broeders et al. 2014; ENGL 2008; ENGL 2015). Recently, the guidelines established for the validation of real-time PCR methods for GMO detection have been used to evaluate qPCR assays for the detection and identification of bacterial pathogens (Barbau-Piednoir et al. 2013b). However, for fungal qPCR detection methods, this has not yet been done.

The present study reports on the development of a SYBR®green qPCR method for *A. versicolor* detection. The primers designed in this study are selected in the ITS region. The performance of the developed qPCR assay was subsequently assessed, using the guidelines for validation of

qualitative real-time PCR methods based on criteria defined for GMO. The PCR efficiency, dynamic range, sensitivity, selectivity and repeatability of the developed *A. versicolor* SYBR®green qPCR were evaluated and discussed. Finally, a proof of concept for the developed qPCR method was delivered using air samples collected in two contaminated houses, by comparing the qPCR results to the results obtained with traditional analysis.

2.2 Materials and methods

2.2.1 Fungal strains

All the fungal species (*A. versicolor, Aspergillus creber, Aspergillus sydowii, Aspergillus fumigatus, Alternaria alternata, Cladosporium cladosporoïdes, Cladosporium herbarum, Cladosporium sphaerospermum, Penicillium chrysogenum, Stachybotrys charatum, Ulocladium botrytis*) and strains used in this study are listed in Table 2.1. All of them were purchased from the BCCM/IHEM collection (Scientific Institute of Public Health in Brussels, Belgium).

2.2.2 Culture conditions and DNA extraction

The fungal strains were grown in a S10 Sabouraud liquid medium (Biorad, Temse, Belgium) at 25 °C with constant agitation between 3 to 10 days according to the species' growth conditions.

After this incubation time, 300 mg of wet sample were transferred to cryotubes containing 0.25 ml of acid washed glass beads (Sigma Aldrich, Diegem, Belgium) put at -80 °C during 40 minutes and freeze-dried overnight with a freeze-dryer Epsilon 1-6D (Martin Christ, Osterode am Harz, Germany). Freeze-dried fungi were subsequently beat-beaten with a Mini bead beater (Biospec Products, OK, USA) during 1 minute at maximal speed.

The total DNA was extracted with an adapted phenol chloroform (24:1) protocol (Ashktorab and Cohen 1992) and purified with the Qiagen CTAB genomic Tip-20 kit (Qiagen Benelux – B.V., KJ Venlo, the Netherlands) according to the manufacturer's recommendation. DNA was eluted with 100 µl Gibco® DNase, RNase, protease free water (Life Technologies, Gent, Belgium). The DNA integrity was verified on an 2 % agarose gel. The DNA amount and purity was evaluated with a Nanodrop® 2000 (Thermo Scientific, Wilmington, USA).

Table 2.1: Selectivity evaluation of SYBR®green qPCR *Aversi_ITS* assay

Genus	Species	Reference BCCM/IHEM*	Positive signal	C_q mean ± SD	T_m mean ± SD (°C)
Aspergillus	*versicolor*	IHEM 18884	*yes*	26.13 ± 0.06	76.25 ± 0.35
Aspergillus	*versicolor*	IHEM 2023	*yes*	26.73 ± 0.06	76.25 ± 0.35
Aspergillus	*versicolor*	IHEM 2157	*yes*	27.2 ± 0.11	76.50 ± 0.00
Aspergillus	*versicolor*	IHEM 10351	*yes*	26.74 ± 0.08	76.25 ± 0.35
Aspergillus	*versicolor*	IHEM 22014	*yes*	28.10 ± 0.10	76.50 ± 0.00
Aspergillus	*versicolor*	IHEM 22975	*yes*	26.54 ± 0.07	76.50 ± 0.00
Aspergillus	*versicolor*	IHEM 24424	*yes*	28.05 ± 0.99	76.25± 0.29
Aspergillus	*versicolor*	IHEM 29832	*yes*	28.18 ± 0.16	76.50 ± 0.00
Aspergillus	*creber*	IHEM 2646	*yes*	30.70 ± 0.70	76.50 ± 0.00
Aspergillus	*sydowii*	IHEM 895	*yes*	30.18 ± 0.13	76.25 ± 0.35
Aspergillus	*sydowii*	IHEM 1360	*yes*	37.34 ± 1.84	76.25 ± 0.35
Aspergillus	*sydowii*	IHEM 20347	*yes*	33.34 ± 0.88	76.50 ± 0.00
Aspergillus	*fumigatus*	IHEM 1365	*no*	/	/
Aspergillus	*fumigatus*	IHEM 3562	*no*	/	/
Alternaria	*alternate*	IHEM 4969	*no*	/	/
Cladosporium	*cladosporoïdes*	IHEM 0859	*no*	/	/
Cladosporium	*herbarum*	IHEM 2268	*no*	/	/
Cladosporium	*sphaerospermum*	IHEM 1011	*no*	/	/
Penicillium	*chrysogenum*	IHEM 4151	*no*	/	
Penicillium	*chrysogenum*	IHEM 20859	*no*	/	/
Stachybotrys	*charatum*	IHEM 0359	*no*	/	/
Ulocladium	*botrytis*	IHEM 0328	*no*	/	/

yes defined as a positive signal *i.e.* amplification with a $C_q \leq 40$, and T_m value (°C) as expected ; *no* defined as no amplification. C_q mean ± SD and T_m mean ± SD were based on two runs per extract from two independent DNA extracts for each strain which has given a positive signal in qPCR using 1,000 theoretical genomic copies. The strain in bold is the reference used for the validation, and was fully characterised as *A. versicolor*.

* IHEM/BCCM collection, Mycology and Aerobiology, Scientific Institute for Public Health, rue Juliette Wytsman 14, 1050 Brussels, Belgium.

2.2.3 Design of primers

All the at the time of primer design in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) available 18S rDNA sequences from *A. versicolor* strains as well as from other closely related species (namely: *A. creber, A. fumigatus, A. sydowii, P. chrysogenum*), were collected and aligned with the "MegAlign" software V10.0.1 (Lasergene, Madison, USA) to identify the sequence region of interest. The publicly available sequences of 18S rDNA used were for *A. versicolor*: AJ937751.1/ AJ937753.1/ AJ937754.1/ AJ937755.1/ AM883155.1/ AM883156.1/ AY728196.1/ EF125026.1/ EU042148.1/ FJ878627.1/ FJ878625.1/ FJ461692.1/ FJ904814.1/ KJ466864.1/ JN205048.1; for *A. creber*: KJ775474.1; for *A. fumigatus*: KC411924.1/ KC237295.1/ KC237291.1/ KC237292.1/ KC142152.1/ HE864321.1/ KC119199.1/ KC119200.1/ JX944178.1/ JX944118.1; for *A. sydowii*: DQ114468.1/ FJ807779.1/ HQ625522.1/ JN94914.1/ KJ775568.1/ KJ775569.1/ KJ775570.1/ KJ775571.1/ KJ775574.1, for *P. chrysogenum*: JN903544.1/ JN798499.1/ JX535315.1/ AF033465.1/ HQ336383.1/ GU325676.1/ EU709771.1/ JX996985.1/ JF834167.1/ FJ004280.1.

Then, different primer pairs were designed in the regions of ITS-1 and ITS-2 with the "Primer 3 V.0.4" software (http://bioinfo.ut.ee/primer3-0.4.0/) (Untergasser et al. 2007). Primer dimers and secondary structure formation were evaluated during the design with Primer 3. An in silico specificity test was performed with the "wprimersearch" software (https://wemboss.uio.no/wEMBOSS/) (Sarachu and Colet 2005). This in silico PCR simulation allows selecting the primer pairs that only amplify the targeted sequences. The specificity of the primers was also verified using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In total 7 primer pairs have been designed (Table 2.2).

Table 2.2: Primer sequences developed *in silico*

Name	Purpose	Sequence 5' to 3'
*Aversi_ITS*_r	Reverse primer	AGTTCGCTGCGTTCTTCATC
*Aversi_ITS*_r2	Reverse primer	CTGCATCACTCTCAGGCATG
*Aversi_ITS*_f1	Forward primer	CTGAGAGTGATGCAGTCTGAG
*Aversi_ITS*_f2	Forward primer	CCCACCCGTGACTACCTAA
*Aversi_ITS*_f	Forward primer	CTGAGAGTGATGCAGTCTGAGTCTG
*Aversi_ITS*_f2	Forward primer	TGCCTGAGAGTGATGCAGTCTGAGTCTGA
*Aversi_ITS*_f3	Forward primer	CTGAGAGTGATGCAGTCTGAGTCAG
*Aversi_ITS*_f4	Forward primer	GAGTGATGCAGTCTGAGTCTG
*Aversi_ITS*_f5	Forward primer	CTTCATGCCTGAGAGTGATGCAGTCTGC

In bold: primers selected as the most specific for the selected region of *A. versicolor*, yielding an amplicon of 53 base pairs.

2.2.4 Qualitative SYBR®green qPCR assay

The qPCR assay (*Aversi_ITS* qPCR method) was performed with the SYBR®green chemistry using a real-time PCR IQ5 ™ system from Biorad (Temse, Belgium).

The standard reaction mix (25 µl final volume) contained 12.5 µl of 2x SYBR®green PCR Mastermix (Diagenode, Liège, Belgium), 0.25 µl of *Aversi_ITS* forward and reverse primers (0.2 µM) and 7 µl of Gibco® DNase, RNase, protease free water (Life Technologies, Gent, Belgium). To this mix, 5 µl of genomic DNA (gDNA) at 200 theoretical genomic copy numbers per µl was added. The number of genomic DNA copies was calculated according to the formula presented below:

$$C_n = \frac{m \times A_c}{M_w \times G_s}$$

with C_n = genomic copy number; m = the amount of gDNA (grams) and determined by Nanodrop®2000 (Thermo Scientific, Wilmington, USA); *Ac* the Avogadro's constant (Mohr et al. 2008); M_w = base pair mean molecular weight (649 Da) and G_s = Genome size (expressed in bp) of *A. versicolor* = 33,130,000 bp (Joint Genome Institute 2014). There is only publicly available information on the size of the genome of one specific *A. versicolor* strain. This size was taken to

calculate the number of genomic DNA copies for all *A. versicolor* strains, although some strain-dependent deviations may exist.

All the runs were performed using following thermal cycling conditions: 1 cycle of 95°C for 10 minutes (Taq activation), followed by 40 amplification cycles of 15s at 95°C (denaturing step) and the annealing and extension step at 60°c for 1 min. Afterwards, a melting curve was performed with a gradual increase of temperature of 0.5 °C/6s from 55 to 95 °C during 15 min. The threshold level for the reaction was automatically determined by the Biorad IQ 5 software V. 2 (Biorad, Temse, Belgium).

In each reaction, a "no template control" (NTC) was included for the analysis i.e. the DNA template was replaced by ultrapure water in the reaction mix. This NTC allowed verifying that no contamination occurred and that no primer dimers were formed.

2.2.5 Strain confirmation: Sequencing and theoretical T_m calculation

In order to confirm their identity, the ITS-1 and ITS-2 regions of *A. versicolor* (IHEM 18884, IHEM 1323, IHEM 1355, IHEM 2023, IHEM 2157, IHEM 2788, IHEM 6598, IHEM 9674, IHEM 10351, IHEM 19014, IHEM 19210, IHEM 19256, IHEM 29832, IHEM 22014, IHEM 2757, IHEM 24424), *A. creber* (IHEM 2646) and *A. sydowii* (IHEM 895, IHEM 1360, IHEM 20347) was verified by Sanger sequencing analysis on an ABI3130xl Genetic Analyzer apparatus (Applied Biosystems, Life Technologies, Gent, Belgium) with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Life Technologies, Gent, Belgium) according to the manufacturer's recommendations. The ITS-1 and ITS-2 regions were firstly amplified with the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns 1993) and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') (White et al. 1990) and then sequenced with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (White et al. 1990). The consensus sequence of each of the targeted regions was made based on the forward and reverse sequence. These sequences were aligned with the MEGA v6.06 (http://www.megasoftware.net/) software and visualized with the CLC sequence viewer v7.0.2 (Qiagen Benelux – B.V., KJ Venlo, the Netherlands). The consensus sequences were also compared to the sequences available in the NCBI database by using BLASTn (http://blast.ncbi.nlm.nih.gov/) in order to confirm the amplification of the targeted DNA regions and their identity.

Based on these sequences, the theoretical T_m of the amplicon obtained in the *Aversi_ITS* assay was calculated in silico with the online tool OligoAnalyzer 3.1 from IDT (http://eu.idtdna.com/calc/analyzer) (IDT, Leuven, Belgium) under the PCR conditions described above (White et al. 1990).

2.2.6 *Aversi_ITS* assay: Performance assessment

Selectivity test

The selectivity test was composed of 2 steps as previously reported for the validation of qPCR methods for food pathogens (Barbau-Piednoir et al. 2013a, b). Firstly, a preliminary selectivity test was carried out on the target species (*A. versicolor* IHEM 18884) and 2 non-target species (i.e. *A. fumigatus* IHEM 3562 and *P. chrysogenum* IHEM 20859), using 15,000 theoretical copies of gDNA.

Secondly, a larger selectivity test was performed, evaluating the inclusivity (the selected primers should amplify DNA of each tested strain from the tested target species) and the exclusivity (DNA of non-target species close to the target or described to frequently occur in the same environment with the target species should not be amplified by the selected primers) of the *Aversi_ITS* qPCR method.

The experimental design of the full selectivity test was adapted from Barbau-Piednoir et al. (2013a), i.e. 16 target strains (*A. versicolor*) and 14 non-target strains were included, i.e. 4 from the *Aspergillus* section *Versicolores* (Jurjevic et al. 2012) (*A. creber* IHEM 2646, *A. sydowii* IHEM 895, IHEM 1360, IHEM 20347) and 10 of the most common indoor airborne fungi (Beguin et al. 1994) (Table 2.1).

Each qPCR was performed with the SYBR®green technology under the conditions described above using a total of 1,000 theoretical copies of gDNA per reaction (evaluated for each target with its own corresponding genome size).

Dynamic range and efficiency estimation

The linearity of this SYBR®green qPCR assay was assessed based on the qPCR analysis of a serial dilution, in duplicate, of gDNA (1000, 500, 100, 50, 10, 5, 2 and 1 theoretical copy number of gDNA) obtained by 2 independent extractions of *A. versicolor* IHEM 18884. This analysis gives the possibility to assess 2 parameters i.e. the coefficient of determination (R^2) and the PCR efficiency. R^2 is an indicator of the correlation of the data regarding the linear regression curve. The PCR efficiency (E) calculation was previously described in Rutledge and Cote (2003). According to the most recent guidelines developed for GMO detection with qPCR SYBR®green (ENGL 2015), the R^2 and amplification efficiency are not applicable to qualitative methods. However, a $R^2 \geq 0.98$ and a PCR efficiency ranging between 80% and 120 % have previously been indicated as performance criteria for the validation of qualitative qPCR methods (Broeders et al. 2014).

Sensitivity test: Limit of detection

To evaluate the sensitivity of the *Aversi_ITS* assay, a serial dilution of gDNA of *A. versicolor*

IHEM 18884 was performed to determine the limit of detection (LOD). The LOD is defined as the lowest concentration of an analyte which is detected with a probability of 95% (Barbau-Piednoir et al. 2013b).

To estimate this LOD, seven dilutions of gDNA of *A. versicolor* IHEM 18884 (i.e. 10, 5, 2, 1, 0.5, 0.2 and 0.1 theoretical copies of gDNA) were tested in 6 independent runs, each with 6 repetitions. The qPCR conditions applied here were the same as those described above. The LOD should be below 25 copies according to definition of minimum performance requirements for analytical methods of GMO testing (ENGL 2015).

Repeatability calculation

As previously described (Barbau-Piednoir et al. 2013b), the repeatability limit (*r*) of the *Aversi_ITS* qPCR method was evaluated with the same experimental design than that applied for the LOD evaluation (see section Sensitivity test: Limit of detection). This *r* value is defined as the maximal difference of 2 results obtained under identical experimental conditions with a probability of 95 % (Barbau-Piednoir et al. 2013b).

The relative standard deviation of the repeatability (*RSDr*) was calculated as the absolute value of the coefficient variation and expressed in percentage. For these criteria, there is no limit fixed for qualitative qPCR methods (ENGL 2015). The *RSDr*, evaluated for the C_q values, should be $\leq$ 25 % for all dilutions above the LOD for quantitative methods (ENGL 2015).

2.2.7 Environmental testing: Inhibition test

Before qPCR analysis of the environmental samples, an inhibition test was performed in order to verify that no inhibition from the collection liquid, used for air sampling with the Coriolis® μ air sampler (Bertin Technology, Montigny-le-Bretonneux, France), occurred during the qPCR. To this end, 300 mg of an *A. versicolor* IHEM 18884 culture was spiked, in duplicate, into 15 ml of collection liquid, i.e. ultra-pure water containing Tween® 20 0.01 % (Sigma-Aldrich, St Louis, USA). Then, the spiked solutions were centrifuged at 5000 *g* during 15 min. The supernatant was removed, the pellet was suspended in 1.5 ml of Gibco® DNase, RNase, protease free water (Life Technologies, Gent, Belgium) and the DNA was extracted according to the protocol described above. Afterwards, each undiluted DNA extract and a 10-fold dilution thereof were analyzed with the *Aversi_ITS* qPCR assay. Theoretically, the C_q difference (ΔC_q) between the C_q value of the 10-fold diluted and the undiluted sample is 3.3 when the PCR efficiency is 100%. The experimental ΔC_q is calculated based on the obtained C_q for both samples (difference between the obtained C_q for the 10-fold diluted and the undiluted sample). Taking into account the PCR efficiency of each run, we considered that there are no PCR inhibitors in the DNA extract if the experimental ΔC_q value for the 10-fold dilution equals 3.3 ±0.5.

2.2.8 Environmental testing: Proof of concept

To assess the performance of the *Aversi_ITS assay* to detect *A. versicolor* in real-life samples, 2 houses contaminated by fungi were studied in the framework of the activities of the CRIPI (Cellule Régionale d'Intervention en Pollution Intérieure from Brussels Environment, Brussels, Belgium). In each house, two sets of air samples were independently collected for classical and molecular analysis respectively, in minimum four different rooms, i.e. the bedroom, bathroom, living and kitchen. In each procedure, air samples were taken at the height of a seated person. To avoid any contamination by air flow, each room was as much as possible isolated from outside by closing windows and doors.

A first set of samples of air (0.08 m³ for each sample) were collected and analyzed according to the procedure previously defined (Nolard et al. 2004) and used in routine by the CRIPI (Nolard et al. 2004). Briefly, air contaminants were directly sampled on Sabouraud chloramphenicol agar (Biorad, Temse, Belgium) slipped on HYCON® Agar Strips (Merck, Darmstadt, Germany), using the RCS plus (Biotest, Rupperswil, Switzerland) air sampler following the manufacturer's instructions and with a flow rate of 80L/min during 1 minute.

After an incubation of 5 days at 25 °C for mesophilic and 2 days at 45°C for thermophilic fungi, the species determination was performed by microscopic visualization (Nolard et al. 2004). The level of contamination was evaluated by counting of the colony forming units (CFU) on plate and expressed as CFU/m³ according to the guidelines defined by Nolard and co-workers (2004).

The second set of air samples (1.5 m³ for each sample) were taken in duplicate with the Coriolis® μ cyclone collector (Bertin Technology, Montigny-le-Bretonneux, France) and collected in 15 ml of ultra-pure water containing Tween® 20 (0.01 %) (Sigma-Aldrich, St Louis, USA) in an appropriate sterile cone (Bertin Technology, Montigny-le-Bretonneux, France). A flow rate of 300 L/min during 5 minutes was applied in each room. In each room, two cones were collected; the first one was analyzed by culturing and microscopic visualization, the second one by qPCR for the detection of *A. versicolor*. The first cone was put at – 80°C until analysis.

To analyse the Coriolis® μ air samples with the culturing approach, the samples were centrifuged at 5000 *g* during 15 min and the supernatant was discarded. The pellet was suspended in 1.5 ml of Gibco® DNase, RNase, protease free water (Life Technologies, Gent, Belgium) and plated on Sabouraud chloramphenicol agar (Biorad, Temse, Belgium), using an inclusion method. Hereto, under laminar flow, the 1.5 ml samples were put into empty petridishes. Afterwards, Sabouraud chloramphenicol medium was poured into the plates and kept at room temperature until the medium was solidified. The plates were incubated 5 days at 25 °C for mesophilic and 2 days at 45°C for thermophilic fungi as it was done for the routine protocol described above. The species

determination was performed by microscopic visualization (Nolard et al. 2004).

To analyse the Coriolis® μ air samples by qPCR for the presence of *A. versicolor*, samples were first centrifuged at 5000 *g* during 15 min and the supernatant was discarded. Next, the pellet was suspended in 1.5 ml of Gibco® DNase, RNase, protease free water (Life Technologies, Gent, Belgium) and the extraction was performed with the DNA extraction protocol used for pure cultures (see section Culture conditions and DNA extraction). The DNA amount and purity was evaluated with a Nanodrop® 2000 (Thermo Scientific, Wilmington, USA). The qPCR reactions were performed on 10 µl of eluted DNA i.e. 10 % of the total DNA eluted from in 1.5 m^3 of air sampled, corresponding to the amount of DNA mentioned in Table 2.4.

2.3 Results

2.3.1 Design and selection of A. versicolor qPCR primer pair

To select the *A. versicolor* primers, all the at the time of the primer design publicly available *A. versicolor* 18S rDNA sequences were searched. This collection of sequences was extended with those available for *A. creber* and *A. sydowii*. These species are both members of the *Versicolores* group as is also *A. versicolor*. These 3 species are difficult to be morphologically distinguished. Based on an in silico analysis of these sequences, 7 forward primers and 2 reverse primers targeting a conserved region of the full ITS-1 region of *A. versicolor* were designed (Table 2.2, Fig. 2.1).

Unfortunately, in the conserved ITS-1 region for *A. versicolor*, it was not possible to design specific primers which are not conserved in *A. creber* and *A. sydowii* (Fig. 2.1). The amplicons obtained with the *Aversi_ITS* f/r primers present 100 % of identity when using the sequences for these 3 species available in NCBI (Fig. 2.1). Preliminary experimental specificity tests were performed on DNA of *A. versicolor*, *A. fumigatus* and *P. chrysogenum*. The *Aversi_ITS*_1f/r primer pair (Fig. 2.1, Table 2.2) was selected for the detection of *A. versicolor* as it was the unique combination of forward and reverse primers allowing the amplification of *A. versicolor* DNA and not that of the closely genetically related *A. fumigatus* and *P. chrysogenum* (data not shown).

2.3.2 *Aversi_ITS* assay: Performance assessment

The performance of this *Aversi_ITS* assay was assessed according to the guidelines defined for the validation of qPCR detection and identification methods in other fields (Barbau-Piednoir et al. 2013b; Broeders et al. 2014; ENGL 2015). The performance assessment includes the evaluation of the following criteria: the selectivity of the primers, the dynamic range, the PCR efficiency, the LOD, and the *Aversi_ITS* assay repeatability.

Selectivity of the qPCR SYBR®green *Aversi_ITS* assay

The assessment of the selectivity was performed in two steps, i.e. an inclusivity and an exclusivity test.

To perform the inclusivity test, DNA extracted from 16 BCCM/IHEM strains of *A. versicolor* was amplified. DNA of all the strains was amplified (16/16) with a C_q range of 24.26 ± 0.44 to 28.67 ± 0.23 for 1,000 copies of gDNA. The T_m values ranged between 76.25± 0.35 and 76.75 ± 0.29 °C (Table 2.1, Fig. 2.2). The sequence of these BCCM/IHEM strains matches perfectly the ones published on NCBI for the corresponding region. All of the amplicons showed 100 % identity (Fig. 2.1). The obtained T_m for each amplicon corresponds to the theoretical T_m (76.50 °C) (Fig. 2.2)

Subsequently, the exclusivity test was performed using DNA of non-target species (*A. creber*; *A. sydowii*; *A. fumigatus*; *A. alternata*; *C. cladosporoïdes*; *C. herbarum*; *C. sphaerospermum*; *P. chrysogenum*; *S. charatum*; *U. botrytis*), closely related to *A. versicolor* and/or occurring in the same environment i.e. mold species from indoor air. As expected based on the in silico analysis, no DNA from non-targeted species considered as a background set of indoor air fungal species (i.e. *A. fumigatus*; *A. alternata*; *C. cladosporoïdes*; *C. herbarum*; *C. sphaerospermum*; *P. chrysogenum*; *S. charatum*; *U. botrytis*) was amplified during the test (Table 2.1), except for the *A. sydowii* (3 strains tested IHEM 895, 1360, 20347) and *A. creber* (IHEM 2646) strain. Although the in silico analysis of the targeted ITS region of *A. versicolor*, *A. creber* and *A. sydowii* with the available sequences in the NCBI database did not show any sequence dissimilarities (Fig. 2.1), for a same genome copy number (i.e. 1,000 theoretical genomic copies), the C_q values ranged between 30.18 ± 0.13 and 37.34 ± 1.84 for *A. sydowii* and 30.70 ± 0.70 for *A. creber* (Table 2.1). To clarify this issue, the obtained amplicons were sequenced. The sequence of the ITS-1 amplicon obtained for the experimentally tested *A. creber* (IHEM 2646) differs from that of *A. versicolor* with 17 nucleotides, of which 7 in the annealing site of the primers; Amplicons of *A. sydowii* IHEM 895, IHEM 1360, IHEM 20347 differ with 8 nucleotides of which 3 in the annealing site of the forward primer (Fig. 2.1). But despite of the differences in nucleotide composition, the obtained T_m values for the amplicon of *A. creber* and *A. sydowii* were in the same range as that of the target T_m (76.25 - 76.75) with respectively 76.50°C and 76.25°C (Table 2.1). The BLAST analysis of the ITS-1 and ITS-2 regions obtained by sequencing confirmed the IHEM 895, IHEM 1360 and IHEM 20347 as *A. sydowii* with 93 % of identity and the IHEM 2646 as *A. creber* with 81 % of identity. Despite this latter low identity, the strain was confirmed as a *A. creber* based on morphological analysis and sequencing of the regions 5.8 S and ITS-2 by BCCM/IHEM (data not shown).

In each assay, an NTC was included to verify that no contamination occurred during the preparation of the qPCR mixes and filling of the 96-well plates. In none of the assays a signal was

observed for the NTC. This absence of signal also demonstrates that no dimerization of primers occurred during the analysis, as predicted during the in silico test (Fig. 2.2).

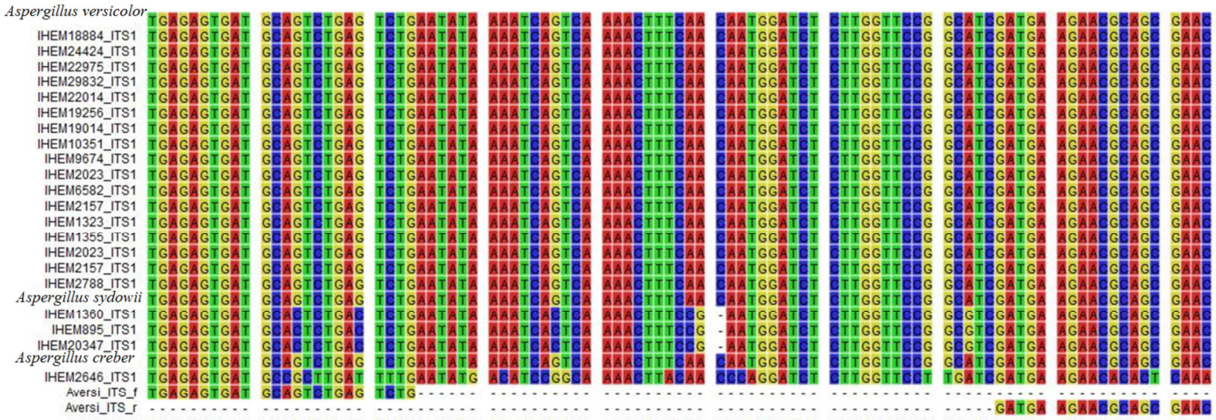

Figure 2.1: Alignment of selected forward and reverse *Aversi_ITS* primers on ITS-1 region sequences of *Aspergillus versicolor*, *A. creber* and *A. sydowii*

This alignment was made using publicly available ITS sequences of *Aspergilus versicolor, A. creber* and *A. sydowii*, extended with ITS sequences from the strains from the BCCM/IHEM collection used during the validation of the qPCR assay (indicated with IHEM prefix) and with the primers designed in this study (*Aversi_ITS*_f and *Aversi_ITS*_r). Because no nucleotide variation was detected for each of the public sequences used, only one sequence was introduced for this alignment, as a representative for that species. The accession numbers of all the NCBI sequences used in this study are listed hereunder *i.e.* for *Aspergillus versicolor* AJ937751.1/ AJ937753.1/ AJ937754.1/ AJ937755.1/ AM883155.1/ AM883156.1/ AY728196.1/ EF125026.1/ EU042148.1/ FJ878627.1/ FJ878625.1/ FJ461692.1/ FJ904814.1/ KJ466864.1/ JN205048.1, for *A. creber* KJ775474.1, for *A. sydowii* DQ114468.1/ FJ807779.1/ HQ625522.1/ JN94914.1/ KJ775568.1/ KJ775569.1/ KJ775570.1/ KJ775571.1/ KJ775574.1. The ITS-1 region of the BCCM/IHEM strains of *A. versicolor* (8), *A. creber* (1) and *A. sydowii* (3) used during the validation of the qPCR assay was sequenced and aligned to those publicly available. Consensus (last line of the alignment) corresponds to a consensus sequence defined by the software. The conservation level among each sequence (0 to 100 % of conservation) is represented by the pink rectangles at the bottom of the figure. The alignment was made with MEGA 6.06. software and visualized with CLC sequence viewer 7.

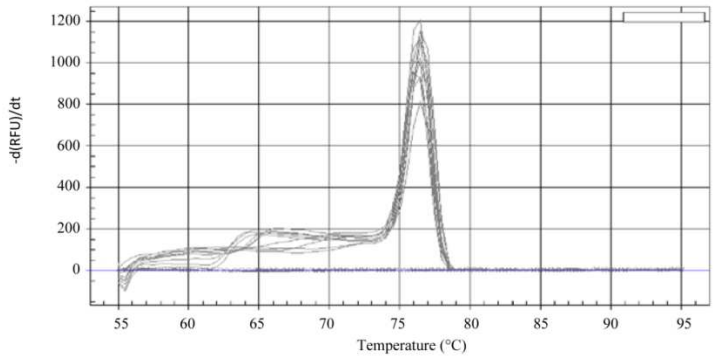

Figure 2.2: Melting curves obtained with the *Avesi_ITS* qPCR assay for the *A. versicolor* pure strains listed in Table 1. The melt curves were obtained with the Biorad IQ 5 software V. 2 (Biorad, Temse, Belgium). The X axis shows the temperature (°C). The Y-axis presents the inverse of the first derivative of the best-fitted curve of the measured fluorescence decrease. The grey curves correspond to the *A. versicolor* listed in Table 1. The blue flat curves represent the non-template controls.

Limit of detection and PCR repeatability

Based on 6 independent runs with a total of 18 repetitions (Table 2.3), the LOD for this SYBR®green assay was determined as being 1 or 2 copies of *A. versicolor* genomes (C_q 35.32 ± 0.78 and 34.65 ± 0.91) (Table 2.4). The *r* and *RSDr* were respectively 2.4 and 6.889 % for the C_q value at LOD.

Table 2.3: C_q values obtained during the 6 runs of the limit of detection estimation for qPCR SYBR®Green assay *Aversi_ITS*

	Run 1						Run 2						Run 3					
Theoretical copy number of gDNA	*Rep. 1*		*Rep. 2*		*Rep. 3*		*Rep. 4*		*Rep. 5*		*Rep. 6*		*Rep. 7*		*Rep. 8*		*Rep. 9*	
10	32.36	31.89	31.76	31.63	31.59	31.81	31.58	31.96	31.56	30.98	31.53	31.47	32.32	32.14	31.87	32.83	32.04	31.78
5	32.87	33.22	32.7	32.92	32.88	33.42	32.42	32.86	32.33	32.93	31.9	32.82	34.07	33.23	33.27	33.66	32.23	33.36
2	34.38	33.78	33.55	34.12	33.85	33.83	33.36	34.46	34.12	34.34	33.33	34.37	34.96	34.71	35.04	35.86	34.74	32.49
1	35.04	34.77	34.37	35.12	35.68	34.96	34.35	35.71	35.59	34.33	33.87	33.95	37.06	36.53	35.79	36.02	35.15	34.7
0.5	35.03	35.68	37.45	35.31	37.1	35	34.79	34.89	36.32	35.14	35.09	35.8	37.8	35.58	37.98	35.64	36.07	36.61
0.2	36.8	35.9	37.68	36.6	N/A	35.65	37.97	35.95	N/A	36.58	36.77	36.85	37.38	37.72	36.82	N/A	N/A	N/A
0.1	36.96	35.65	N/A	N/A	37.3	36.25	38.21	N/A	N/A	N/A	36.66	N/A	N/A	N/A	37.63	N/A	38.01	N/A
	Run 4						Run 5						Run 6					
Theoretical copy number of gDNA	*Rep. 10*		*Rep. 11*		*Rep. 12*		*Rep. 13*		*Rep. 14*		*Rep. 15*		*Rep. 16*		*Rep. 17*		*Rep. 18*	
10	33.17	32.97	32.77	33.8	33.28	33.84	33.17	32.97	33.38	33.18	33.33	33.12	33.89	34.51	33.17	32.97	33.38	33.18
5	34.54	32.88	33.76	33.89	33.52	33.04	34.54	32.88	33.82	34.16	33.64	34.02	33.34	33.41	34.54	32.88	33.82	34.16
2	34.79	35.4	34.97	34.8	35.19	36.37	34.79	35.4	34.21	34.62	34.59	34.73	37.58	35.76	34.79	35.4	34.21	34.62
1	36.18	34.51	36.7	35.83	N/A	35.74	36.18	34.51	35.28	35.46	35.36	34.94	35.73	N/A	36.18	34.51	35.28	35.46
0.5	N/A	N/A	38.45	34.9	36.29	N/A	N/A	N/A	36.45	36.63	36.04	36.73	37.27	36.79	N/A	N/A	36.45	36.63
0.2	N/A	N/A	36.9	N/A	N/A	37.34	N/A	N/A	36.59	36.79	36.03	37.84	N/A	38.16	N/A	N/A	36.59	36.79
0.1	N/A	37.87	36.94	36.61	37.02	N/A	N/A	37.87	N/A	N/A	N/A	N/A	N/A	N/A	N/A	37.87	N/A	N/A

Mean of C_q value obtained for 6 repetitions (Repetition 1 to 18) of 6 independent runs (Run 1 to 6) of a serial dilution of genomic DNA of *A. versicolor* (concentration expressed in copy number of genomes of the IHEM 18884 strain). The LOD is defined by the dashed line.

Table 2.4: Limit of detection results (C_q mean, SD and % positive) for *Aversi_ITS* SYBR®Green qPCR assay

Theoretical copy number	C_q mean ± SD	% positive
10	32,29 ± 0.86	100.00 (36/36)
5	33.33 ± 0.66	100.00 (36/36)
2	34.65.± 0.91	100.00 (36/36)
1	35.32± .078	94.44 (34/36)
0.5	36.2 ± 1.00	80.56 (29/36)
0.2	36.90 ± 0.09	61.11 (22/36)
0.1	37.21 ± 0.75	38.89 (14/36)

Mean of C_q value obtained for 6 repetitions of 6 independent runs of a serial dilution of genomic DNA of *A. versicolor* (concentration expressed in theoretical copy numbers of genomes of the IHEM 18884 strain), the standard deviation (± SD) and the percentage of positive response observed at each dilution point. The number of positive signal per assay is given between brackets. The LOD is defined by the dashed line.

Dynamic range and PCR efficiency

Based on a dilution series of 8 levels, corresponding to 1000 to 1 theoretical genomic copies of *A. versicolor*, the dynamic range and efficiency of the *Aversi_ITS* assay was determined. Between the tested range, a linear model with a R^2 of 0.9919 and an efficiency of 88.34 % were obtained for this qPCR method (Fig. 2.3).

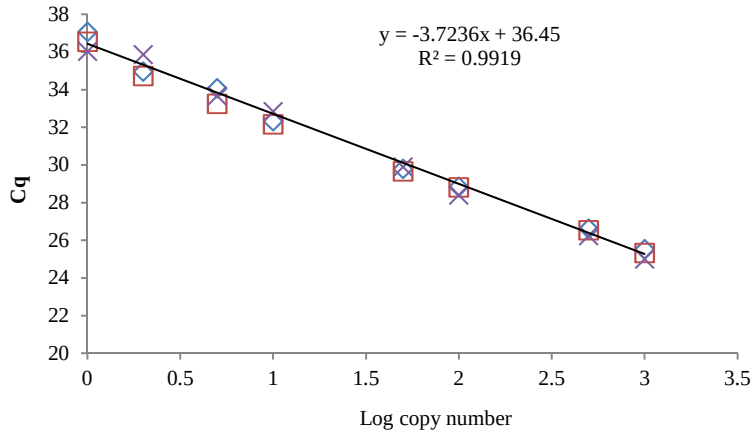

Figure 2.3: R^2 and PCR efficiency of the *Aversi_ITS* qPCR assay.
The PCR efficiency (E) was evaluated in duplicate on a serial dilution of gDNA (1000 to 1 theoretical copy number of gDNA) obtained by 2 independent extractions of *A. versicolor* IHEM 18884. R^2: the coefficient of determination (R^2) regarding a linear correlation curve. Log copy number: logarithm of the theoretical copy number of gDNA. C_q: C_q values obtained by qPCR for each repetition of each gDNA dilution.

2.3.3 Proof of concept: Environmental testing

At first, an inhibition test was performed to verify that no inhibition occurred during the amplification of the DNA extracted from the environmental samples. Theoretically, with a 100 % efficient amplification, a 10-fold dilution corresponds to a C_q difference of 3.3. The obtained C_q value was 25.06 ± 0.34 for the undiluted DNA extract of the collection fluid spiked with pure *A. versicolor* and 28.28 ± 0.64 for the 10-fold dilution. This test showed that no inhibition of the amplification occurs.

The *Aversi_ITS* assay was subsequently tested on environmental samples collected with the Coriolis® *μ* air sampler and compared to the results obtained with classical detection and identification method used by CRIPI in routine. Additionally, environmental samples collected with the Coriolis® *μ* were also analyzed using culturing and microscopic visualization, in order to be able to evaluate whether possible observed differences are due to the differences in the sampling method or in the detection method used (Table 2.5).

With the classical routine method (culturing, counting and microscopic visualization), *A. versicolor* was detected in the 2 sampled houses with a quantity ranging between 13 and 50 CFU/m³. *P. chrysogenum* was found as the most important contaminant in each of the sampled houses with a range of 75 to 300 CFU/m³. Other taxa, i.e. *A. glaucus*, *Cladosporium* spp. and an undetermined species were also observed, though in the same range of quantities as *A. versicolor*. These taxa occur in the list of most frequently found fungal contaminants of indoor environments.

Infertile mycelia were also present in the sampled houses. The range of counts for *A. versicolor* correlated with the results obtained with the *Aversi_ITS* qPCR assay. For each sample where *A. versicolor* was identified by microscopic analysis, a positive qPCR signal ranging between C_q 30.26 ± 0.49 to C_q 35.85 ± 0.07 was obtained (Table 2.5). The samples where *A. versicolor* was not detected on plate, gave a negative signal in qPCR.

Similar observations were made for the results obtained with culturing of the Coriolis® *μ* air samples. For the different sampling places, the same species as identified for the samples collected with the RCS plus sampler were found, with in two rooms (room of house 1 and the bathroom of house 2) some additional undetermined species detected for the Coriolis® *μ* sampler. The number of colonies on plate for these species was for both air samplers in the same range, including for *A. versicolor*. As for the RCS plus based sampling, also for the air samples collected with the Coriolis® *μ*, for each sample where *A. versicolor* was identified by microscopic analysis, a positive qPCR signal was obtained (Table 2.5). A negative signal in qPCR was obtained for the samples where *A. versicolor* was not detected on plate.

Table 2.5: Environmental sampling, comparison of classical analysis methods with the SYBR®green qPCR *Aversi_ITS* assay

Sampling place	Species	Classical method			Molecular method		
		RCS plus sampler and culturing		Coriolis® μ sampler and culturing	Coriolis® μ sampler and qPCR		
		Number of colonies per plate	CFU/m³ [a]	Number of colonies per plate	Amount of total DNA/PCR reaction (ng) [b]	C_q mean ± SD [c]	Theoretical copy number of gDNA for 1 m³ [d]
House 1							
Room	*A. versicolor*	3	38	9	19.8	32.15 ± 0.49	67
	P. chrysogenum	7	88	17			
	Infertile mycelium	0	0	1			
Kitchen	*A. versicolor*	1	13	2	21.7	35.25 ± 0.21	7
	A. glaucus	1	13	1			
	P. chrysogenum	8	100	7			
	yeast (undetermined)	1	13	1			
Living room	*P. chrysogenum*	24	300	25	53.3	N/A	/
Bathroom	*A. versicolor*	4	50	6	50	30.26 ± 0.35	93
	infertile mycelium	4	50	4			
	P. chrysogenum	15	188	17			

Table 2.5: *Continued*

Sampling place	Species	Classical method			Molecular method		
		RCS plus sampler and culturing		Coriolis® μ sampler and culturing	Coriolis® μ sampler and qPCR		
		Number of colonies per plate	CFU/m³ [a]	Number of colonies per plate	Amount of total DNA/PCR reaction (ng) [b]	C_q mean ± SD [c]	Theoretical copy number of gDNA for 1 m³ [d]
House 2							
Room 1	infertile mycelium	4	50	4	51.5	N/A	/
	P. chrysogenum	17	213	15			
Room 2	infertile mycelium	2	25	1	10.4	N/A	/
	P. chrysogenum	3	38	4			
Kitchen	*P. chrysogenum*	6	75	5	12.5	N/A	/
	infertile mycelium	3	38	4			
Living room	*A. versicolor*	1	13	2	49.5	35.85 ± 0.07	7
	P. chrysogenum	18	225	20			
Bathroom	*A. versicolor*	1	13	2	45	33.9 ± 0.28	33
	Cladosporium spp.	1	13	1			
	P. chrysogenum	15	188	13			
	yeast (undetermined)	0	0	2			

aThe value for CFU/m³ is an estimation of fungal contamination based on the number of colonies per plate. The Coriolis® μ samples that were put into culture to serve as a qualitative control for the species identified with the RCS plus sampler and for A. versicolor detected in the Coriolis® μ samples with the qPCR method. b 10 µl of extracted DNA from 1.5 m3 sampled air (and eluted in 100 µl of water) were use in a 25 µl -PCR reaction.
c Cq values are Cq means (≤ 40) ± standard deviation (SD) obtained with the validated Aversi_ITS primers. N/A defined as no amplification, i.e. A. versicolor was considered as not detected in the sample. d Theoretical copy number of gDNA based on the Aspergillus versicolor IHEM 18884 strain defined as the strain of reference for the validation of the Aversi_ITS assay (Table 2.1).

2.4 Discussion

Currently, indoor fungal contamination is considered as an important public health problem (World Health Organization 2009). Among the different species described, *A. versicolor* is considered as one of the most important (Beguin and Nolard 1994; Benndorf et al. 2008; de Ana et al. 2006; Melkin et al. 2004) and is suspected to have a link with asthma (Mendell et al. 2011; Sharpe et al. 2014; Verhoeff and Burge 1997). However, to provide more scientific-based, causal evidence on this, efficient screening and monitoring of indoor airborne fungal communities is crucial and necessary.

In this study we developed a SYBR®green qPCR method for the detection of *A. versicolor*. This *Aversi_ITS* assay targets the ITS-1 region, which is the same sublocus than the one targeted by the TaqMan®-based EPA assays (including the one for *A. versicolor*) (Haugland et al. 2004). This region from the 18S rDNA is considered as the most variable locus of this complex and therefore it is the most efficient species marker for fungi (Nilsson et al. 2008), making it the most appropriate region of the fungal DNA for the development of a molecular screening tool. This some intra-species variability of ITS sequence might impact TaqMan®-based methods and the probe's hybridization when different strains of a specific species need to be detected with the same probe. An alternative to the hydrolysis probe can be found in the SYBR®green chemistry. Less expensive than TaqMan based assays, SYBR®green assays theoretically allow for species specificity and species discrimination based on a melting curve and the T_m value of the amplicon.

Most published PCR and qPCR assays to detect molds are not uniformly assessed for their performance with clear guidelines or norms (Costa et al. 2001; Haugland et al. 2004; Johnson et al. 2012; Melkin et al. 2004; Roussel et al. 2013). In the present study, we propose a strategy to evaluate the performance of qPCR assays applied to mold detection. Therefore, using the same strategy than the one published for foodborne pathogens (Barbau-Piednoir et al. 2013b), the guidance existing for GMO was used to select a set of performance criteria for the qPCR assay (Broeders et al. 2014; ENGL 2015); namely, the selectivity, PCR efficiency, dynamic range, sensitivity and repeatability parameters were assessed to evaluate the performance of our developed *Aversi_ITS* assay.

First, the results of the specificity test showed that all of the tested *A. versicolor* strains were detected with the *Aversi_ITS* assay. However, for an identical number of genomic copies (i.e. 1000 gDNA copy numbers), a variation of approximately 4 C_q between these strains was observed. Because no differences in the ITS sequences were observed between the tested BCCM/IHEM stains (Fig. 2.1), including the sites of primer annealing, this difference in C_q might be explained

by variation in the copy number of the 18S rDNA operon to which the targeted ITS region belongs. This ITS region is known to vary not only between species but also within the species (Black et al. 2013; Corradi et al. 2007; Iwen et al. 2002; Schoch et al. 2012). For example, for *A. fumigatus*, the intra-species variation factor was estimated at 2.5, implying that the 18S rDNA gene complex could vary between 38 and 91 copies per genome (Herrera et al. 2009). A similar variation rate for *A. versicolor* might explain the observed difference in C_q values between different strains of this species. However, this hypothesis needs more investigation of the copy number of 18S rDNA in *A. versicolor* strains, similar to what was described by Herrera et al. for *A. fumigatus* (Herrera et al. 2009). Consequently, qPCR assays developed on the ITS region, such as this *Aversi_ITS* assay, are qualitative detection methods. To develop a quantitative method based on ITS, the copy number of the 18S rDNA should be determined for each target strain of the species.

The *Aversi_ITS* assay did not amplify DNA extracted from 10 non-target strains selected among the most common airborne fungal species. This indicates a good selectivity of the *Aversi_ITS* assay (Beguin et al. 1994). However, the assay did amplify DNA of 2 other members of the *Aspergillus* section *Versicolores* i.e. *A. creber* and *A. sydowii*. This was expected, based on the primer sequences and the alignments of already publicly available ITS sequences, which were identical to the one of *A. versicolor* in the selected region. These species are very close and their ITS sequences in general differ only in a few nucleotides (Hinrikson et al. 2005; Jurjevic et al. 2012). However, the C_q obtained for the amplicons of *A. sydowii* and *A. creber* were not in the expected range based on the identity of the publicly available sequences, although they resulted in the expected T_m. Therefore additional tests for strain identification were performed. These additional tests confirmed the identity of the strains used for the exclusivity test. They also confirmed the obtained experimental T_m, coinciding with the one calculated based on the obtained sequence which however shows mismatches as compared to the ITS-1 target region of *A. versicolor* or of the publicly available sequences of *A. creber* and *A. sydowii* used for the primer design. Although normally the T_m allows for species discrimination, in the selected amplicon region, this is not the case for *A. versicolor*, *A. creber* and *A. sydowii*. The obtained C_q is however higher for *A. creber* and *A. sydowii* for the same amount of template DNA, which might be due to the mismatches in the primer annealing sites, in addition to the difference in ITS copy number. These observations demonstrate the need for more publicly available sequences for these closely related species to be used for qPCR assay development.

These non-target amplifications of *A. sydowii* were also previously reported for the TaqMan® assays of EPA with probes *Avers2* and *Asydo3* developed respectively for the detection of *A. versicolor* and *A. sydowii* (Haugland et al. 2004; United States Environmental Protection Agency

2014). These probes amplified each time both species. *A. creber* is a recently described species, isolated from indoor air samples and identified through multilocus DNA sequencing (Jurjevic et al. 2012), and has not yet been commonly used for the testing of TaqMan® *A. versicolor* specific assays. However, these aspecific detections should have a limited impact on a possible use of the *Aversi_ITS* assay in routine analysis in Belgium. Indeed, *A. creber* and *A. sydowii* are not frequently detected in indoor environment, as compared to *A. versicolor*. Further investigations are needed to evaluate the difference between these 3 species concerning the impact of their presence in indoor air on public health.

To improve the discrimination between these 3 closely related species based on merely molecular methods, an alternative approach should be developed. One possibility could be the use of a combination of different markers similar to what has already been done for the detection of GMO (Van den Bulcke et al. 2010) and food-borne pathogens (Barbau-Piednoir et al. 2013a). The presence or the absence of an amplification signal for each of the markers and their combination defines a decision-taking tree. For fungi, this approach to improve the species discrimination could be possible if we would combine our primers of the *Aversi_ITS* assay with primers targeting another gene marker or a discriminatory SNP. In case that the sample is composed of a mixture of DNA from different species (e.g. from an air sample), the results will hint to a "candidate mold" which should be further confirmed by downstream analysis, such as sequencing. This idea is supported by Schoch and co-workers in 2012 who suggested that the use of a combination of different DNA regions, like ITS-1 and β-tubulin or another region, i.e. the DNA barcode principle, should improve the phylogenetic analysis of fungi (Schoch et al. 2012). Hereto, more genomic sequences of both species (*A. creber* and *A. sydowii*) should become publicly available in order to design primers for such discriminatory regions.

Subsequently, the PCR linearity and efficiency were evaluated as quality criteria for the developed assay. The BCCM/IHEM 18884 strain was collected and purified from a contaminated house by CRIPI and is used as a reference strain for allergy studies by the CRIPI. Therefore, this strain was selected as a reference to determine the parameters of our qPCR assay during the performance assessment.

The efficiency of this assay (E) was estimated to be 88.34 %, which complies with the guidelines for qualitative qPCR methods (Broeders et al. 2014). Our method is also characterized by a high R^2 value (0.9919) which demonstrated the linearity of our assay and of the accuracy of our experimental setup.

Moreover, the *Aversi_ITS* assay is sensitive with a LOD defined as1 or 2 genomic copies per reaction, and therefore it complies with the acceptance criteria, i.e. LOD below 25 copies (ENGL

2015). This LOD is similar to the results reported by Johnson et al. (2012) for their qPCR detection method of *A. fumigatus* which has an LOD ranging between 6 and 0.6 genomes (Johnson et al. 2012). Our *Aversi_ITS* showed also to be repeatable, with a $RSD_{r,}$ for all dilutions above the LOD, lower than 25 %, thereby fulfilling the requirements of the validation guidelines used in this study.

As discussed above, we mainly used the guidelines for GMO detection for the evaluation of our qPCR assay. As it was recently shown (Barbau-Piednoir et al. 2013a), these guidelines were successfully used to evaluate with high standard the performance of the SYBR®green qPCR methods for the detection of bacterial pathogens. However, requirements defined for food and feed analysis are not necessarily adapted for indoor airborne fungi monitoring, as the laws and the control measures are more numerous for food than for microbiological air pollution and the impact of food contamination on public health is more documented and better understood than for fungal air contamination. The increasing development and use of molecular tools for the identification and the monitoring of indoor airborne microbiological contamination imposes the establishment of guidelines for harmonization of performance requirements and threshold values for the parameters of the qPCR assays. Ultimately, this may contribute to the establishment of standardized and reproducible microbiological methods, highly needed to determine a causal link between indoor fungal contamination and health effects.

To deliver a proof-of-concept of our developed qPCR assay aimed to be used for indoor fungal contamination monitoring, the *Aversi_ITS* assay was tested on environmental samples and results were compared to those obtained with traditional methods of screening (i.e. culture, CFU counting and microscopic identification). This last part of our study confirmed that the *Aversi_ITS* assay is a valuable alternative for the currently used classical methods. The results of the qPCR assay were comparable to the ones obtained with the classical routine protocol as to indicate whether the targeted species *A. versicolor* was present or not, and this using a shorter time period to obtain these results with the qPCR method, i.e. 2 days maximum for the qPCR analysis (including DNA extraction with an overnight lyophilisation step and qPCR analysis) compared to 5 to 10 days for the classical methods. Based on the obtained C_q and an extrapolation from the *A. versicolor* data obtained for the LOD estimation, a theoretical copy number of *A. versicolor* gDNA per m³ of air could be estimated in order to attempt a more detailed comparison of the results from molecular and the classical methods (Table 2.5). The theoretical number of genomic copies for *A. versicolor* ranged between 7 and 67 for 1 m³ of sampled air for a number of CFU/m³ ranging between 13 and 50 for those collected with the RCS plus air sampler in the routine classical method. A similar range of colonies of *A. versicolor* was obtained for the samples collected with the Coriolis® μ. The culturing of Coriolis® μ samples was included as a control to verify that different samplers do not

lead to different species detected on plate, especially for *A. versicolor.*

The *A. versicolor* amounts obtained are in a similar range when comparing the culturing results to the qPCR results, despite the following considerations. The small difference in amounts could be attributed to the sampling method, the performance of the sampler used and the conversion factor used to express the results in m^3, which are different for the RCS plus and Coriolis® μ sampling methods. Also, the possibility that one colony would grow from an aggregate of fungal cells should be taken into account, explaining why the results are not exactly coinciding. Additionally, qPCR will also include the non-culturable *A. versicolor* fraction, while this fraction remains undetected using the classical methods. It is also important to note that the estimation of gDNA copies was based on the strain IHEM 18884, which is not necessarily the strain that was present in the contaminated houses. As observed during the evaluation of the assay, the real copy number of the ITS regions of the strains detected here could differ from that of the strain IHEM 18884. This would lead to another amount of gDNA copies. Nevertheless, the trend for the contamination by *A. versicolor* was similar as detected by the different methods i.e. the house 1 was the most contaminated and bathrooms presented more contamination than other rooms.

Our environmental test also showed, as expected, that other species than *A. versicolor* are present inside buildings. Therefore it will be interesting to develop and assess a more exhaustive multiplex tool targeting different indoor airborne fungal species. Different types of multiplex technologies can be envisaged, including the SYBR®green technology, where the discrimination between species could be based on the T_m values of the amplicons obtained with universal primers for the ITS region. However, this might imply the use of more accurate technologies such as high resolution melting (HRM) qPCR to perform the discrimination of some closely related taxa. Indeed, some species differ by a few nucleotides and are hardly discriminated with traditional qPCR methods. Based on a very fine temperature gradient, HRM could be an interesting tool to perform the discrimination and the identification of the airborne fungal community (McCarthy et al. 2013). It would also be interesting to investigate whether HRM in combination with the primers of the *Aversi_ITS* assay might offer a solution to discriminate the 3 species of the *Aspergillus* section *Versicolores.*

In conclusion, the developed *Aversi_ITS* method based on the SYBR®green chemistry is a convenient qualitative assay for the detection of *A. versicolor.* With the increased risk for public health linked to the augmentation of indoor fungal contamination, such a molecular assay is a first step in offering a valuable alternative to the currently used classical methods in the framework of routine monitoring of indoor air fungal contamination by a public health laboratory. A reduced time of detection of both the culturable and non-culturable fungal fraction, allows to reduce the time of reaction and taking of measurements aiming at reducing the impact on human health. With

an extension towards other important indoor airborne fungal contaminants, tools as the one developed in this study, based on harmonized guidelines to be urgently established, will contribute to an improved indoor air quality monitoring and ultimately to an improved insight into the causal effect between indoor airborne fungal contaminants and health effects. Once this causal link is established, meaningful regulatory individual exposure standards for well-defined airborne mold allergens can be established (AIHA 2011; Verhoeff and Burge 1997).

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Conflict of interest

The authors declare that they have no conflict of interest.

Chapter 3

A molecular approach for the rapid, selective and sensitive detection of *Exophiala jeanselmei* in environmental samples: Development and performance assessment of a real-time PCR assay

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A molecular approach for the rapid, selective and sensitive detection of *Exophiala jeanselmei* in environmental samples: Development and performance assessment of a real-time PCR assay

Abstract

Exophiala jeanselmei is an opportunistic pathogenic black yeast growing in humid environments such as water reservoirs of air-conditioning systems. Because this fungal contaminant could be vaporised into the air and subsequently cause health problems, its monitoring is recommended. Currently, this monitoring is based on culture and microscopic identification which are complex, sometimes ambiguous and time-demanding, i.e., up to 21 days. Therefore, molecular, culture-independent methods could be more advantageous for the monitoring of *E. jeanselmei*. In this study, we developed a SYBR®green real-time PCR assay based on the internal transcribed spacer 2 from the 18S ribosomal DNA complex for the specific detection of *E. jeanselmei.* The selectivity (100 %), PCR efficiency (95.5 %), dynamic range and repeatability of this qPCR assay were subsequently evaluated. The limit of detection for this qPCR assay was determined to be 1 copy of genomic DNA of *E. jeanselmei*. Finally, water samples collected from cooling reservoirs were analyzed using this qPCR assay to deliver a proof of concept for the molecular detection of *E. jeanselmei* in environmental samples. The results obtained by molecular analysis were compared with those of classical methods (i.e., culture and microscopic identification) used in routine analysis and were 100 % matching. This comparison demonstrated that this SYBR®green qPCR assay can be used as a molecular alternative for monitoring and routine investigation of samples contaminated by *E. jeanselmei*, while eliminating the need for culturing and thereby considerably decreasing the required analysis time to 2 days.

Key words

Black yeast, detection, environment, *Exophiala jeanselmei*, real-time PCR, molecular method.

3.1 Introduction

Exophiala is a fungal genus containing numerous species recognized to be pathogenic (Nucci et al. 2001, 2002; Packeu et al. 2012a,b; Woo et al. 2013), and it is a member of the black yeasts. This genus is described as ubiquitous and has been isolated from diverse substrates (*e.g.* wood, soil, sludge, water and feed) (Dixon et al. 1980; Nishimura et al. 1987; Nucci et al. 2002). Within this genus, *Exophiala jeanselmei* is usually causing cutaneous or subcutaneous infections. However, *E. jeanselmei* infections are also frequently observed as a systemic infection or as a causal agent of cystic fibrosis in immunosuppressed patients (Nucci et al. 2001, 2002). Nevertheless, although limited data on the number of cases occurring are available and the epidemiology of airways infections caused by *E. jeanselmei* is poorly documented. Inside buildings the way of contamination by this species is presumably linked to water reservoirs of air-conditioning units or pipings (Badali et al. 2012; Wang et al. 2001). As this species can cause health problems, its monitoring is needed.

Currently, monitoring of *E. jeanselmei* contamination of buildings is complicated and requires specific expertise. Indeed, in routine analysis, this monitoring is often based on culture, microscopic visualization and visual counts (Anaissie et al. 2003). This approach depends on the growth of the culture, which for *E. jeanselmei* can take up to 21 days (Anaissie et al. 2003). In addition, this culturing is also influenced by the growth media chosen, the culture conditions such as temperature and humidity, or by competition between species (Pitkaranta et al. 2011; Vesper 2011). Moreover, *E. jeanselmei* is a complex species belonging to a group of morphologically difficult (or impossible) to differentiate species (Kawasaki et al. 2005; Zeng and De Hoog 2008; Zeng et al. 2007), i.e., the *Exophiala spinifera* clade (De Hoog et al. 2003), which groups among others *E. jeanselmei*, *Exophiala exophialae*, *Exophiala lecanii-corni* and *Exophiala xenobiotica* (Kawasaki et al. 1999; Wang et al. 2001; Woo et al. 2013; Zeng and De Hoog 2008). Also *Exophiala dermatitidis*, considered as an important and much studied pathogen inside the *Exophiala* genus, is morphologically similar to *E. jeanselmei* (Kawasaki et al. 1990, 2005; Masuda et al. 1989). A molecular approach could bring a solution to these bottlenecks in classification and identification of *E. jeanselmei* (Haase et al. 1995; Hee and Yoon 2002; Kawasaki et al. 1990).

Actually, the use of molecular tools (such as polymerase chain reaction (PCR), real-time polymerase chain reaction (qPCR) and sequencing) is being increasingly used for the detection, monitoring and the identification of fungi in general (Chemidlin Prevost-Boure et al. 2011; Hospodsky et al. 2010; Melkin et al. 2004; United States Environmental Protection Agency 2015). For example for the genus *Exophiala*, some PCR (Nagano et al. 2008; Najafzadeh et al. 2013; Sudhadham et al. 2010), qPCR and other molecular tools have been developed for the detection

and identification of *E. dermatitidis* (Wang et al. 2013). Most often these molecular tools target the 18S rDNA regions and especially the internal transcribed spacer 1 and 2 (ITS-1 and ITS-2). This ITS region is fully documented and a vast amount of data is available for in silico analysis. In 2012, Schoch and co-workers proposed to define these internal regions as a part of the barcode marker for fungi (Schoch et al. 2012). For *E. jeanselmei*, these ITS regions offer the possibility to develop molecular tools to discriminate the species, even inside the *E. spinifera* clade (Wang et al. 2001; Woo et al. 2013; Zeng and De Hoog 2008). Despite these previous studies investigating the *E. jeanselmei* genotype (Badali et al. 2012; Kawasaki et al. 2005, 1999; Woo et al. 2013), molecular tools for the specific detection of this fungus are however still poorly documented.

Based on ITS-1 and ITS-2, diagnostic molecular tools for *E. jeanselmei* infections were developed using PCR and classical Sanger sequencing (Nagano et al. 2008; Najafzadeh et al. 2013; Packeu et al. 2012b; Wang et al. 2001; Woo et al. 2013). But although these approaches have proven their effectiveness, they are not really adapted to routine analysis of many environmental samples due to the low specificity of the universal primers used in the PCR assay the time needed for the analysis and the cost. Indeed, environmental samples could contain more than one species. Then, in these samples the use of universal primers would produce a mix of amplicons which would be poorly discriminated with Sanger sequencing. This problem could be solved by next generation sequencing (NGS) tools but the associated costs and expertise needed (especially in data analysis) might be too high for routine analysis.

qPCR is an alternative approach offering specificity and a reduction in analysis time and cost, especially when many samples need to be analysed. Also, a qPCR instrument might be more in the reach of a routine laboratory, as compared to a DNA sequencing instrument. Today, despite the development of qPCR tools for the detection of several fungal contaminants such as the monitoring of airborne molds from outdoor and indoor environment (Hospodsky et al. 2010; Libert et al. 2015; Nagano et al. 2008; Vesper 2011), no qPCR assay specific to *E. jeanselmei* yet exists.

In this context, this paper describes a SYBR®green qPCR assay for the specific detection of *E. jeanselmei*. To assess the performance of the developed qPCR assay, the selectivity, sensitivity and efficiency were evaluated. As previously discussed (Libert et al. 2015), no harmonized guidelines exist for this performance assessment of qPCR assays for fungal detection. Therefore, we followed a similar approach as the one previously reported for *Aspergillus versicolor* (Libert et al. 2015) which is based on the guidelines developed for the qPCR detection of genetically modified organisms (GMO) (Broeders et al. 2014) and foodborne pathogens (Barbau-Piednoir et al. 2013b) and the minimum information for publication of quantitative real-time PCR experiment (MIQE) proposed by Bustin et al. in 2009 (Bustin et al. 2009). Finally, a proof of concept for the

detection of *E. jeanselmei* in environmental samples was provided, comparing the routine protocol based on classical techniques involving culturing steps, and the molecular qPCR method developed and evaluated in this study. Therefore, this study offers a new molecular tool based on SYBR®green chemistry which could be used as a routine protocol for the detection and/or the monitoring of *E. jeanselmei* in environmental samples, by all actors concerned.

3.2 Materials and methods

3.2.1 Fungal strains

Table 3.1 lists all the fungal species (*Acrenonium strictum, Alternaria alternata, Aspergillus fumigatus, Cladosporium cladosporioides, Cladosporium herbarum, Cladosporium sphaerospermum, E. dermatitidis, E. exophialae, E. jeanselmei, E. lecanii-corni, E. spinifera, E. xenobiotica, Penicillium chrysogenum, Stachybotrys chartarum* and *Ulocladium botrytis*) and strains (19 strains in total) used in this study. They were all purchased from the BCCM/IHEM collection (Scientific Institute of Public Health in Brussels, Belgium).

3.2.2 Culture conditions

The fungal strains were grown as described previously (Libert et al. 2015). Briefly, the fungal strains were spiked into a S10 Sabouraud liquid medium (Biorad, Temse, Belgium) and were grown at 25 °C with constant agitation between 3 to 21 days according to the species' growth conditions.

3.2.3 DNA extraction

DNA of the fungal cultures was prepared as previously reported (Libert et al. 2015). Briefly, after the incubation time, 300 mg of wet sample were transferred to cryotubes containing 0.25 ml of acid washed glass beads (Sigma Aldrich, Diegem, Belgium) and put at -80 °C during 40 minutes and freeze-dried overnight with a freeze-dryer Epsilon 1-6D (Martin Christ, Osterode am Harz, Germany). Freeze-dried fungi were then beat-beaten with a Mini bead beater (Biospec Products, OK, USA) during 1 minute at maximal speed.

Subsequently, the total DNA was extracted with an adapted phenol chloroform (24:1) protocol (Ashktorab and Cohen, 1992) and purified with the Qiagen CTAB genomic Tip-20 kit (Qiagen Benelux – B.V., KJ Venlo, the Netherlands) according to the manufacturer's protocol. 100 µl Gibco® DNase, RNase, protease free water (Life Technologies, Gent, Belgium) was used to elute the DNA. The DNA integrity was verified on an 2% agarose gel. The DNA concentration and purity were evaluated with a Nanodrop® 2000 (Thermo Scientific, Wilmington, USA).

Table 3.1: Selectivity evaluation of SYBR® green qPCR *Ejeanselmei_ITS* assay

Genus	Species	Reference BCCM/IHEM*	Positive signal	C_q mean ± SD	T_m mean ± SD (°C)
Exophiala	*Jeanselmei*	IHEM 4740	yes	23.57 ± 0.38	79.75 ± 0.29
Exophiala	*Jeanselmei*	IHEM 4741	yes	20.86 ± 0.22	79.25 ± 0.50
Exophiala	*Jeanselmei*	IHEM 22665	Yes	21.81 ± 0.43	79.63 ± 0.25
Exophiala	*Dermatitidis*	IHEM 9780	No	/	/
Exophiala	*Exophialae*	IHEM 5976	No	/	/
Exophiala	*Exophialae*	IHEM 20759	No	/	/
Exophiala	*lecanii-corni*	IHEM 3662	No	/	/
Exophiala	*Spinifera*	IHEM 20752	yes**	26.04 ± 0.05	78.50 ± 0.00
Exophiala	*Xenobiotica*	IHEM 6582	No	/	/
Acremonium	*Strictum*	IHEM 993	No	/	/
Alternaria	*Alternata*	IHEM 4969	No	/	/
Aspergillus	*Fumigatus*	IHEM 3562	No	/	/
Cladosporium	*Cladosporioides*	IHEM 0859	No	/	/
Cladosporium	*Herbarum*	IHEM 2268	No	/	/
Cladosporium	*Sphaerospermum*	IHEM 1011	No	/	/
Penicillium	*Chrysogenum*	IHEM 4151	No	/	/
Penicillium	*Chrysogenum*	IHEM 20859	No	/	/
Stachybotrys	*Chartarum*	IHEM 0359	No	/	/
Ulocladium	*Botrytis*	IHEM 0328	No	/	/

The strain in bold is the reference used for the performance assessment and is fully characterised as *Exophiala jeanselmei* (IHEM 4740). Positive signal (*yes*) is defined as an amplification with a $C_q \leq 40$, and T_m value (°C) as expected; ** *Tm* is different*; no* defined as no amplification. C_q mean ± SD and T_m mean ± SD are based on two runs per extract from two independent DNA extracts for each strain which has given a positive signal in qPCR using 1,000 theoretical genomic copies.

* IHEM/BCCM collection, Mycology and Aerobiology, Scientific Institute for Public Health, rue Juliette Wytsman 14, 1050 Brussels, Belgium.

3.2.4. Primer design

First, a collection of publicly available 18S rDNA sequences from *E. jeanslemei* strains and other closely related species (namely *E. lecanii-corni* and *E. spinifera*) and from the common water contaminant *A. fumigatus* (Heinemann et al. 1994; Parat et al. 1996), was made (NCBI, GenBank). The following sequences were included: for *E. jeanselmei*: AB531492.1/AF549447.1/AF050271.1/ AJ866273.1/AY857530.1/ AY163553.1/ AY163549.1/ AY163550.1 AY163552.1/ AY163556.1/ DQ836791.1/ DQ836793.1/ DQ836795.1/ JN625228.1/ JX192603.1/ JX473278.1/ EF025410.1/ EF025411.1/ EF025412.1/ EU910261.1/ JX473276.1; for *A. fumigatus*: KC411924.1/ KC237295.1/ KC237291.1/ KC237292.1/ KC142152.1/ HE864321.1/ KC119199.1/ KC119200.1/ JX944178.1/ JX944118.1; for *E. lecanii-corni*: GQ426959.1/ GQ426975.1/ GQ426980.1/ JN675374.1/ JN675375.1/ JX473283.1/ JX473285.1/ JX681038.1/ JX681039.1/ JX681040; for *E. spinifera*: AB025891.1/ AB025892.1/ AB025857.1/ AB025876.1/ AY156966.2/ AY156970.1/ EF551539.1/ EF551459.1/ KC952672.1/ NR_111131.1. These sequences were aligned with the "MegAlign" software V10.0.1 (Lasergene, Madison, USA) to identify the ITS-1 and ITS-2 sequence regions of interest wherein different primer pairs were designed using the "Primer 3 V.0.4" software (http://bioinfo.ut.ee/primer3-0.4.0/) (Untergasser et al. 2007). Primer dimers and secondary structure formation was evaluated and predicted during the design with Primer3. The "wprimersearch" software (https://wemboss.uio.no/wEMBOSS/) (Sarachu and Colet 2005) was used to perform an *in silico* specificity test, allowing to select the primer pair that only amplifies the target sequences. Also BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to evaluate the specificity of the primers.

3.2.5 Qualitative SYBR®green qPCR assay

The qPCR assay for the detection of *E. jeanselmei* (*Ejeanselmei_ITS* qPCR method) was performed as previously described for *A. versicolor* (Libert et al. 2015), using the SYBR®green chemistry and a real-time PCR IQ5 ™ system (Biorad, Temse, Belgium). All the primers used in this study were synthetized by Eurogentec (Liège, Belgium).

Briefly, the reaction mix (25 µl final volume) contained 12.5 µl of 2x SYBR®green PCR Mastermix (Diagenode, Liège, Belgium), 0.25 µl of *Ej_ITS* forward and reverse primers (0.2 µM) (Table 3.2) and 7 µl of Gibco® DNase, RNase, protease free water (Life Technologies, Gent, Belgium). To this mix, 5 µl of genomic DNA (gDNA) at 200 theoretical genomic copy numbers per µl was added. The number of genomic DNA copies was calculated according to the formula presented below i.e.,

$$C_n = \frac{m \times A_c}{M_w \times G_s}$$

with C_n = genomic copy number; m = the amount of gDNA (grams) and determined by Nanodrop®2000 (Thermo Scientific, Wilmington, USA); *Ac* the Avogadro's constant (Mohr et al. 2008). M_w = base pair mean molecular weight (649 Da) and G_s = Genome size (expressed in bp) of *E. jeanselmei* = 30,000,000. Because no information on the genome size of *E. jeanselmei* is currently publicly available, we used an estimation based on the average of the genome size of *E. dermatitidis, E. xenobiotica, E. spinifera* (Broad Institute 2015). We are also aware that some strain-dependent deviations may exist.

The optimization of the qPCR conditions was performed with the *E. jeanselmei* strain BCCM/IHEM 4740. Therefore, this species was considered as a reference and used as a positive control added in each run performed in this study.

In each run, a "no template control" (NTC) was included for the analysis whereby the DNA template was replaced by ultrapure water in the reaction mix. This NTC aimed at verifying that no contamination occurred and that no primer dimers were formed.

Following thermal cycling conditions were used for all runs: 1 cycle of 95 °C for 10 minutes (Taq activation), followed by 40 amplification cycles of 15 s at 95 °C (denaturing step) and 60 °c for 1 min (annealing and extension step). Subsequently, a melting curve was made with a gradual increase of temperature of 0.5 °C/6s from 55 to 95 °C during 15 min. The Biorad IQ 5 software V. 2 (Biorad, Temse, Belgium) automatically determined the threshold level for the reaction.

3.2.6 Inhibition test (pure cultures)

Because PCR inhibitors (e.g. co-extracted substances or RNA contaminations) could affect the amplification, the validation results and also the detection of low amount of the targeted DNA, it is important to verify that all of these substances were removed during the DNA extraction step. This was done using an inhibition test. The workflow of this inhibition test was based on that proposed in 2012 by Broeders et al. for the assessment of absence of inhibitors in DNA extracts. Briefly, gDNA was extracted independently from 2 pure cultures of *E. jeanslemei* strain BCCM/IHEM 4740. Afterwards, a calibration curve was made based on the analysis of each set of gDNA, diluted 10, 100 and 1000, 5000 fold, with the *Ejeanselmei_ITS* SYBR®green assay. Two criteria exist to assess the absence of inhibitors in the DNA extracts; i.e., the slope of the calibration curve which should be between -3.6 and -3.1; and the difference (ΔC_q) observed between the experimentally obtained C_q values and an extrapolated C_q obtained by the regression of the C_q from the undiluted sample (Broeders et al. 2012). In the absence of inhibition, the ΔC_q

should be ≤ 0.50 for each dilution (Broeders et al. 2012, ENGL 2011).

3.2.7 Sequencing

The identity of the strains of *E. jeanselmei* IHEM 4740, IHEM 4741, IHEM 22665 and of *E. spinifera* IHEM 20752 that resulted in a specific amplicon in the qPCR assay was confirmed based on the sequence of their ITS-1 and ITS-2 regions. Hereto a dideoxy sequence analysis with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Life Technologies, Gent, Belgium) and an ABI3130xl Genetic Analyzer apparatus (Applied Biosystems, Life Technologies, Gent, Belgium) was used according to the manufacturer's recommendations. Primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) were first used to amplify the ITS-1 and ITS-2 regions. Primers ITS1 and ITS2 (White et al. 1990) were used for the subsequent sequencing reaction. The consensus sequences of each of the targeted regions (based on the forward and reverse sequence of each target region) were aligned with the MEGA v6.06 (http://www.megasoftware.net/) (Tamura et al. 2013) software and visualized with the CLC sequence viewer v7.0.2 (Qiagen Benelux – B.V., KJ Venlo, the Netherlands). Using BLASTn (http://blast.ncbi.nlm.nih.gov/), the amplification of the targeted DNA regions and their identity was confirmed by comparing them to the sequences available in the NCBI database.

3.2.8 Theoretical T_m calculation

The online tool from IDT (http://eu.idtdna.com/calc/analyzer) (IDT, Leuven, Belgium) with the consensus sequences obtained through sequencing as input and under the PCR conditions described above, was used to *in silico* calculate the theoretical T_m of the amplicon obtained in the *Ejeanselmei _ITS* assay.

3.2.9 *Ejeanselmei_ITS* assay performance assessment

For the performance assessment of the qPCR assay, the approach as previously outlined by Libert et al. (2015), which on its own is based on the study of Barbau-Piednoir et al. (2013), was followed. Different parameters of the qPCR method were evaluated i.e., the selectivity (based on inclusivity and exclusivity), the PCR efficiency, the limit of detection (sensitivity test) and the repeatability.

Selectivity test

The selectivity test was composed of a preliminary and a larger selectivity test.

For the preliminary selectivity test, the target species (*E. jeanselmei* IHEM 4740) and 1 non-target species (*E. dermatitidis* IHEM 9780) were used, both at 15,000 theoretical copies of gDNA. The amplicon obtained for the *E. jeanselmei* strain IHEM 4740, which was taken as a reference in the

performance assessment study, was confirmed by sequencing analysis.

The larger selectivity test aimed at evaluating the inclusivity (i.e., the selected primers should amplify the DNA of each tested strain from the target species) and the exclusivity (i.e., DNA of non-target species close to the target or described to frequently occur in the same environment as the target species should not be amplified by the selected primers, with a specific T_m) of the *Ejeanselmei_ITS* qPCR method, using the selected primers and the qPCR conditions as described above. So, a result has been considered as positive when the sample is amplified and the observed T_m corresponds to the T_m defined in silico for the target. If these two conditions are not observed, the result has been considered as negative.

For the inclusivity 3 target strains (i.e., *E. jeanselmei*) from the BCCM/IHEM collection were selected. The experimental design of the exclusivity test included 14 non-target strains i.e., 5 species closely related to *E. jeanselmei* (i.e., *E. dermatitidis, E. exophialae, E. lecanii-corni, E. spinifera* and *E. xenobiotica*,) (Zeng and De Hoog 2008) and 9 other common species in wet environment as described by Heinemann et al. (1994) (i.e., *A. strictum, A. alternata, A. fumigatus, C. cladosporioides, C. herbarum, C. sphaerospermum, P. chrysogenum, S. chartarum* and *U. botrytis).*

The conditions described above were used for each qPCR run using a total of 1000 theoretical copies of gDNA per reaction (evaluated for each target with its own corresponding genome size).

Based on the selectivity test, the false positives ratio (FPR) and false negatives ratio (FNR), the sensitivity and the selectivity values (%) were calculated according to the formulas presented by Blakely and Salmond (2002), i.e.,

FPR = False positives / (False positives + True negatives)

FNR = False negative / (False negatives + True positives)

Sensitivity = True positives / (True positives + False positives)*100

Selectivity = True negatives / (True negatives + False negatives)*100

PCR efficiency estimation

The qPCR analysis of a serial dilution, in duplicate, of gDNA (1000, 500, 100, 50, 10, 5, 2, and 1 theoretical copy number of gDNA obtained by 2 independent extractions) of *E. jeanselmei* IHEM 4740 was used to assess the linearity of the SYBR®green qPCR assay. Based on this analysis, 2 parameters can be evaluated, i.e., the coefficient of determination (R^2) and the PCR efficiency. R^2 is an indicator of the correlation of the data regarding the linear regression curve. The PCR efficiency (*E*) calculation was previously described by Rutledge and Cote (Rutledge and Cote

2003). As previously described for *A. versicolor* (Libert et al. 2015), according to the most recent guidelines developed for GMO detection with qPCR SYBR®green (ENGL 2015), the R^2 and amplification efficiency are not applicable to qualitative methods. However, a $R^2 \geq 0.98$ and a PCR efficiency ranging between 80 % and 120 % have previously been indicated as performance criteria for the validation of qualitative qPCR methods (Broeders et al. 2014).

Limit of detection (Sensitivity test)

The limit of detection (LOD) is defined as the lowest concentration of an analyte which is detected with a probability of 95 % (Barbau-Piednoir et al. 2013; Broeders et al. 2014). Six dilutions (i.e., 100, 50, 10, 5, 2, 1, 0.5, 0.2 and 0.1 theoretical copies of gDNA) of genomic DNA of *E. jeanselmei* IHEM 4740 were tested in 6 independent runs (using the qPCR conditions described above), each with 6 repetitions, to estimate the LOD of the *Ejeanselmei_ITS* assay. The LOD should be below 25 copies according to definition of minimum performance requirements for analytical methods of GMO testing (ENGL 2015; Libert et al. 2015).

PCR repeatability

This repeatability limit (*r*) is defined as the maximal difference of 2 results obtained under identical experimental conditions with a probability of 95 % (Barbau-Piednoir et al. 2013b). The experimental design used for the LOD evaluation (see above) was also applied for the evaluation of the *r* value of the *Ejeanselmei_ITS* qPCR method.

As described previously (Barbau-Piednoir et al. 2013b; Libert et al. 2015), the relative standard deviation of the repeatability (*RSDr*) was calculated as the absolute value of the coefficient variation (%). For these criteria, there is no limit fixed for qualitative qPCR methods (ENGL 2015). The *RSDr*, evaluated for the C_q values, should be below 25 % for all dilutions above the LOD for quantitative methods (Barbau-Piednoir et al. 2013b; Broeders et al. 2014).

3.2.10 Proof-of-concept: Environmental testing

Sampling

The sampling method for air-conditioning systems was previously described by Nolard and co-workers (Nolard et al. 2004). Briefly, 1 L of water was collected in a sterile Duran bottle with a vacuum pump at a distance between 1 and 5 cm of the bottom of the tank of the air-conditioning system. In total, 8 tanks from different air-conditioning system were sampled. The water samples were stored at 4 °C until their analysis.

Classical analysis: culture, microscopic analysis and cell counting

Under laminar flow, a first part of the water sample was diluted 10 and 100 fold and each dilution, as also an undiluted aliquot of 1 ml, was poured into an empty petridish. Afterwards, Malt Extract

Agar (MEA) chloramphenicol liquid medium (Biorad, Temse, Belgium) was put in the petridish and the plate was kept at room temperature till the medium had solidified. Then, the plate was incubated at 25 °C during 21 days. A first microscopic analysis was made after 5 days of incubation in order to determine the fastest growing species and a second after 21 days of incubation to determine other species such as the *Exophiala* species.

Molecular analysis: DNA extraction and qPCR analysis

Additionally, aliquots of 15 ml were taken of mixed water samples used for the classical analysis described above. After 15 min of centrifugation at 5000 g, DNA from aliquots was extracted with the DNA extraction protocol used for pure cultures (see above). The DNA concentration and purity was evaluated with a Nanodrop® 2000 (Thermo Scientific, Wilmington, USA). The qPCR reactions were performed on 5 µl of eluted DNA i.e., 10 % of the total DNA eluted from water samples corresponding to the amount of DNA mentioned in the Table 3.4.

PCR inhibition test (spike test)

To verify that no inhibition from the anti-fungal chemistry used to clean the air-conditioning occurred on the PCR amplification, as part of these chemicals might have been retained in the DNA extract, 1000 theoretical copy numbers of *E. jeanselmei* gDNA (IHEM 4740) were spiked into 5 µl of DNA extract from sample 3 (considered as an "environmental" no template control, as by classical analysis, no *E. jeanselmei* was detected) and analysed with the *Ejeanselmei_ITS* qPCR assay. To determine whether inhibition occurred during the qPCR reaction, the obtained C_q value with the spiked sample was compared to the C_q obtained with 1000 copy numbers of gDNA of *E. jeanselmei* IHEM 4740 in pure water (Table 3.1).

3.3 Results

3.3.1 Design and selection of qPCR primer pair

For the design of the *E. jeanselmei* specific qPCR primers, all the *E. jeanselmei* 18S rDNA sequences that were publicly available at that time, were used. This selection of sequences was extended with those available for the closely related species *E. lecanii-corni* and *E. spinifera*, which are belonging together with *E. jeanselmei* to the *E. spinifera* clade (Wang et al. 2001; Zeng and De Hoog 2008), and those available for *A. fumigatus*, a common species observed in water (Heinemann et al. 1994; Parat et al. 1996). Based on an alignment of these sequences, two couples of *E. jeanselmei* primers targeting a conserved region within the ITS-2 region of the 18S rDNA of *E. jeanselmei* were designed (Table 3.2, Fig. 3.1). It was however not possible to design primers in this region that were exclusively specific to *E. jeanselmei*, as part of the primers were also conserved in *E. spinifera*, with some nucleotide variations especially in the forward primer

annealing site. The impact of this sequence conservation was addressed during the specificity test as part of the performance assessment (see below).

Preliminary specificity tests with these 2 primer couples were performed on the ITS-2 regions of *E. jeanselmei* (IHEM 4740) and *E. dermatitidis* (IHEM 9780) as a negative control, showing that only the ITS-2 region of *E. jeanselmei* was amplified (data not shown). Finally, based on the best combination of primers in terms of amplification efficiency (data not shown), the *Ej_ITS*_f and *Ej_ITS* r primers (Table 3.2) were selected for the detection of *E. jeanselmei* in this qPCR assay (*Ejeanselmei_ITS* assay).

In order to verify that no inhibition occurred during the PCR amplification, an inhibition test was performed with the selected primers. The slope of the calibration curve obtained with the two sets of gDNA dilutions was determined to be -3.35 (Fig. 3.2). This slope is in the range of -3.1 to -3.6 as recommended by the ENGL (2011). ΔC_q values were calculated for each dilution i.e. 0.27 for the 5000-fold dilution, 0.43 for the 1000-fold dilution, 0.02 for the 100-fold dilution and 0.33 for the 10-fold dilution. According to the acceptance criteria from the ENGL for GMO for the assessment of the absence of inhibitors in the DNA extracts (ENGL 2011), no inhibition occurred.

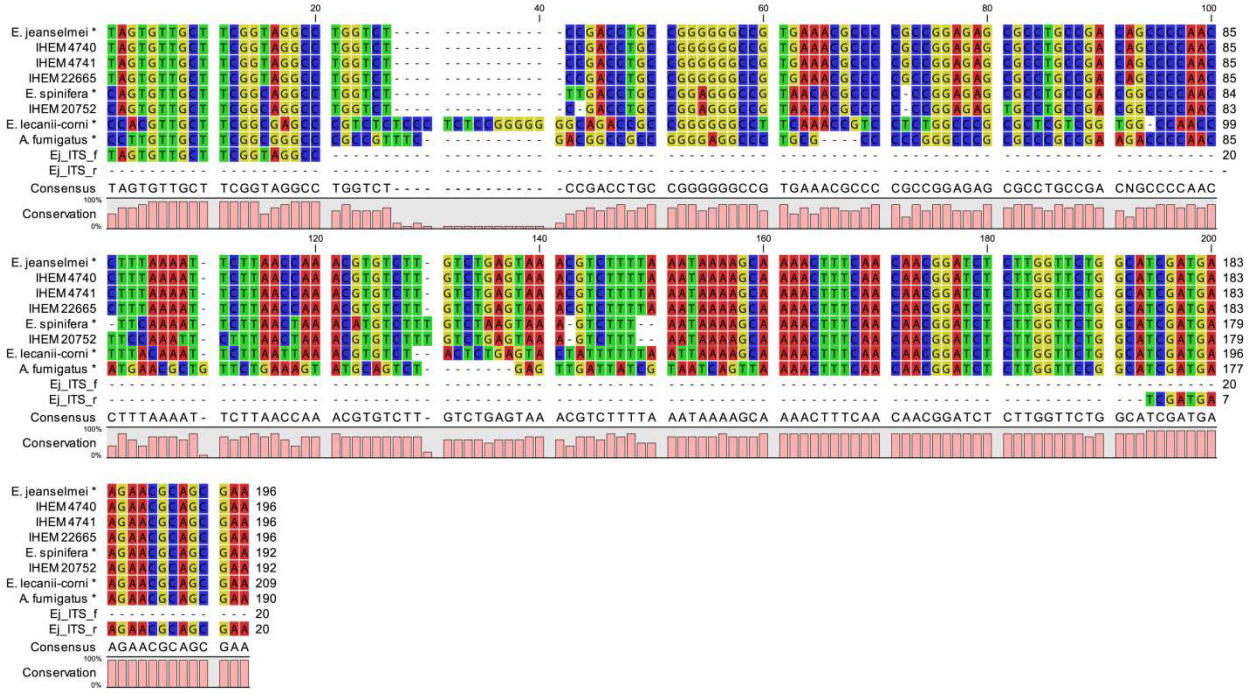

Figure 3.1: Alignment of region of ITS sequences of *E. jeanselmei*, *E. lecanii-corni*, *E. spinifera* and *A. fumigatus* strains, and of selected primers

This alignment was made using publicly available ITS sequences of *E. jeanselmei*, *E. lecanii-corni*, *E. spinifera* and *A. fumigatus* extended with ITS sequences from the strains from the BCCM/IHEM collection used during the performance assessment of the qPCR assay (indicated with IHEM prefix) and with the primers designed in this study (*Ej_ITS*_f and *Ej_ITS*_r). Because no nucleotide variation was detected for each of the public sequences used, only one sequence (*) was introduced for this alignment, as a representative for that species. Consensus (last line of the alignment) corresponds to a consensus sequence defined by the software. The conservation level among each sequence (0 to 100 % of conservation) is represented by the pink rectangles at the bottom of the figure. The alignment was made with MEGA 6.06 software (http://www.megasoftware.net/) and visualized with CLC sequence viewer 7 (Qiagen Benelux – B.V., KJ Venlo, the Netherlands).

Table 3.2: Primer sequences developed *in silico*

Name	Purpose	Sequence 5' to 3'	Amplicon size (bp)
Ej_ITS_f	Forward primer	Ccgagttagggtcctcaca	
Ej_ITS_r	Reverse primer	ggcctaccgaagcaacata	70
Ej_ITS_2f	Forward primer	Cccggtacactgagcatctt	107
Ej_ITS_2r	Reverse primer	Cctacctgatccgaggtcaa	

The amplicon size is expressed in base pairs (bp). In bold, the primers selected as efficient primer couple in the preliminary specificity test for the detection of E. jeanselmei (IHEM 4740).

3.3.2 *Ejeanselmei_ITS* assay performance assessment

To evaluate the quality of this *Ejeanselmei_ITS* assay, a performance assessment was done as previously described for *A. versicolor* (Libert et al. 2015), according to the guidelines defined for the validation of qPCR detection and identification methods in other fields (Barbau-Piednoir et al. 2013b; Broeders et al. 2014). The following criteria were evaluated: the selectivity of the primers, the LOD, the PCR efficiency, the dynamic range, and the *Ejeanselmei_ITS* assay repeatability.

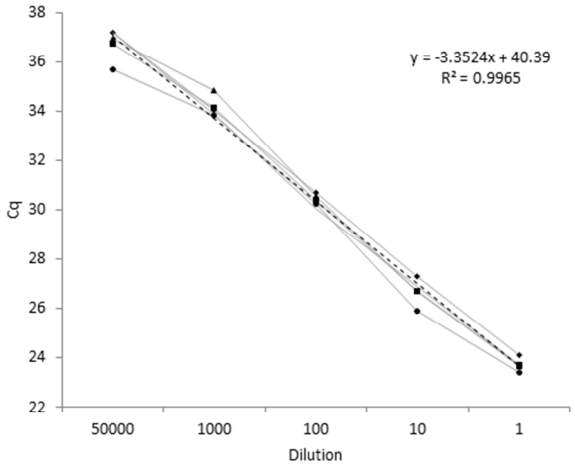

Figure 3.2: Calibration curve (inhibition test)
Data were obtained with 4 replicates of 4 dilutions (5000, 1000, 100, 10 fold) of gDNA of *E. jeanselmei* (IHEM 4740). The dotted line corresponds to the trend line.

Selectivity of the *Ejeanselmei_ITS* qPCR assay

First, an inclusivity test was performed with DNA of 3 *E. jeanselmei* strains from the BCCM/IHEM collection. We have included all in this collection available *E. jeanselmei* strains originating from water. DNA of each of the selected strains (*E. jeanselmei* BCCM/IHEM 4740, BCCM/IHEM 4741, BCCM/IHEM 22665) was amplified (3/3) with a C_q of, respectively, 20.86 ± 0.22, 21.81 ± 0.43 and 23.57 ± 0.38 for 1,000 copies of gDNA (Table 3.1). Themelting curve analyses showed T_m values between 79.25 ± 0.50 and 79.75 ± 0.25 (Table 3.1, Fig. 3.3).

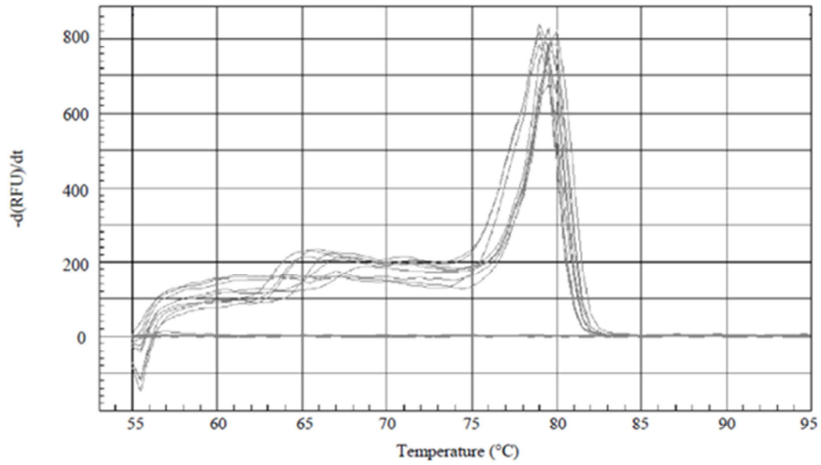

Figure 3.3: Melting curves obtained with the *Ejeanselmei_ITS* qPCR assay for the *E. jeanselmei* pure strains listed in Table 3.1

The meltcurves were obtained with the Biorad IQ 5 software V. 2 (Biorad, Temse, Belgium). The X-axis shows the temperature (°C). The Y-axis presents the inverse of the first derivate of the best-fitted curve of the measured fluorescence decrease.The gray curves correspond to *the E. jeanselmei* listed in Table 1. The *blue flat curves* represent the NTC.

The sequence of these BCCM/IHEM strains matches perfectly with the ones publicly available for the corresponding region and all amplicons showed 100 % identity (Fig. 3.1). The obtained T_m for each amplicon corresponds to the theoretical T_m, which was calculated to be 79.50 °C. No false negatives were obtained.

Then, an exclusivity test was performed on DNA of non-target species (i.e., *A. strictum, A. alternata, A. fumigatus, C. cladosporioides, C. herbarum, C. sphaerospermum, E. dermatitidis, E. exophialae, E. lecanii-corni, E. spinifera, E. xenobiotica, P. chrysogenum, S. chartarum* and *U. botrytis*). These species are closely related to *E. jeanselmei* and/or are occurring in the same environment (i.e., water reservoir) and/or in indoor environment (Al-gabr et al. 2014; Anaissie et al. 2003; De Hoog et al. 2003; Heinemann et al. 1994; Kawasaki et al. 1999). No false positives rewere observed (Table 3.1). Indeed, with this *Ejeanselmei_ITS* qPCR assay, except for DNA extracted from the *E. spinifera* IHEM 20752 strain, no DNA from non-targeted species was amplified. The amplification of *E. spinifera* was not unexpected based on the in silico analysis including the alignment (Fig. 3.1), which included all the publicly available ITS sequences of *E.*

jeanselmei and *E. spinifera*. Because the sequence between the two primers was found to be identical for all the sequences available for one species, only one sequence for each species has been represented in the figure.

However, the *E. spinifera* amplification should be considered as a true negative results based on the SYBR® green characteristic. Indeed, for a same copy number estimation (i.e., 1,000 theoretical genomic copy number), the obtained C_q value for *E. spinifera* was 26.04 ± 0.05 (Table 3.1) and the T_m was 78.50 °C (Table 3.1, Fig. 3.4), which are different from the ones obtained for *E. jeanselmei*. Based on these amplification results and Tm values, no false negative values were observed, i.e., FPR and FNR values of 0 % were obtained.

To explain these differences, amplicons of *E. jeanselmei* and *E. spinifera* were sequenced and aligned (Fig. 3.1). The amplicon of *E. spinifera* differs from that obtained for *E. jeanselmei* with 11 nucleotides, of which 2 in the annealing site of the forward primer (Fig. 3.1). These results were expected and met those obtained during the primer design (Fig. 3.1). These nucleotide differences explained the difference in the observed T_m for the two species (Table 3.1). The BLAST analysis of the ITS-1 and ITS-2 regions confirmed the IHEM 20752 as *E. spinifera* with 97 % of identity.

Based on these results, a sensitivity of 100 % and a specificity of 100 % were observed. An NTC was included in each assay to verify that no contamination occurred during the qPCR assay preparation (preparation of the mixes, filling of the qPCR plates). In none of the assays, the NTC resulted in a signal. This also showed that no dimerization of primers occurred during the analysis, as predicted during the in silico test (Fig. 3.3).

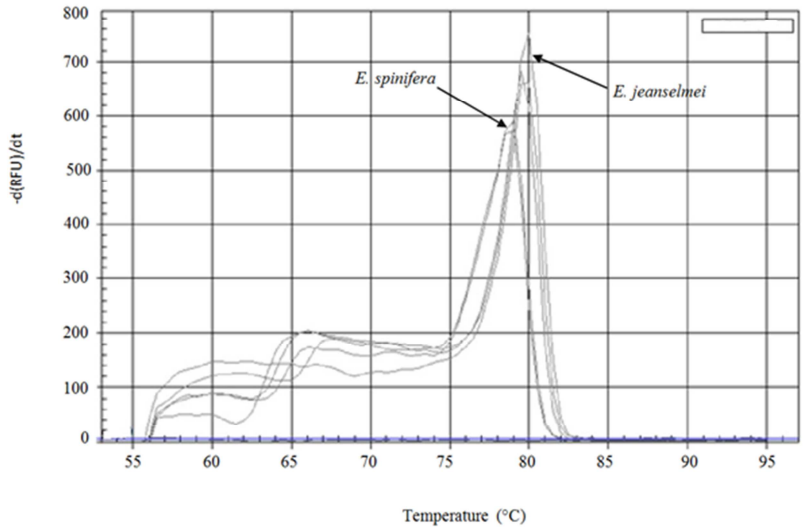

Figure 3.4: Melting curves obtained with the *Ejeanselmei_ITS* qPCR assay for the *E. jeanselmei* and *E. spinifera* pure strains listed in Table 1.
The melt curves were obtained with the Biorad IQ 5 software V. 2 (Biorad, Temse, Belgium). The X-axis shows the temperature (°C). The Y-axis presents the inverse of the first derivative of the best-fitted curve of the measured fluorescence decrease. The *blue flat curves* represent the NTC.

Limit of detection (sensitivity test) and PCR repeatability

The limit of detection (LOD) of the *Ejeanselmei_ITS* assay, based on 6 independent runs with a total of 18 repetitions, was determined to be 1 theoretical copy number (Table 3.3 and Table 3.4) (C_q = 34.86 ± 0.90). The *r* and *RSDr* were 3.45 and 9.73 % respectively for this assay.

Dynamic range and PCR efficiency

A serial dilution of 1,000 to 0.1 theoretical genomic copy numbers of *E. jeanselmei* permitted to define the dynamic range and PCR efficiency of the *Ejeanselmei_ITS* assay. A linear model with a R^2 of 0.9977 and an efficiency of 95.5 % were obtained with this SYBR®green assay (Fig. 3.5).

Table 3.3: Cq values obtained during the 6 runs of the limit of detection estimation for qPCR SYBR®Green assay *Ejeanslemei_ITS*

	Run 1						Run 2						Run 3					
Theoretical (estimated) copy number of gDNA	Rep. 1		Rep. 2		Rep. 3		Rep. 4		Rep. 5		Rep. 6		Rep. 7		Rep. 8		Rep. 9	
10	33.47	32.63	32.55	32.55	32.42	32.93	32.03	31.65	31.81	31.38	31.58	31.78	30.73	30.41	29.98	30.43	30.14	30.42
5	33.91	33.88	33.91	34.02	34.16	34.07	32.78	32.74	32.07	32.06	32.43	32.82	31.74	31.55	31.58	30.82	31.07	31.12
2	36.50	35.16	35.03	35.05	35.35	35.18	33.20	33.37	33.38	33.78	33.20	33.65	32.84	32.75	32.70	32.33	33.15	33.78
1	36.89	36.27	35.54	35.87	36.38	36.18	35.67	33.84	33.77	34.43	36.13	34.59	33.92	34.12	34.17	33.81	34.07	34.07
0.5	37.98	36.61	37.92	37.46	37.79	36.55	35.64	35.94	35.81	36.08	36.66	36.09	34.35	35.09	34.61	35.14	34.40	36.31
0.2	N/A	37.59	38.21	37.91	38.50	38.72	37.01	N/A	N/A	36.69	37.97	37.31	35.95	N/A	35.94	36.22	36.51	N/A
0.1	N/A	39.59	N/A	39.44	N/A	39.28	38.17	N/A	38.66	N/A	N/A	36.81	N/A	N/A	N/A	N/A	N/A	N/A
	Run 4						Run 5						Run 6					
Theoretical copy number of gDNA	*Rep. 10*		*Rep. 11*		*Rep. 12*		*Rep. 13*		*Rep. 14*		*Rep. 15*		*Rep. 16*		*Rep. 17*		*Rep. 18*	
10	31.76	31.68	31.79	32.05	31.55	31.82	30.87	31.07	31.03	30.96	31.00	31.34	31.61	31.59	31.30	31.28	31.09	31.70
5	32.71	32.07	32.33	32.70	32.83	32.77	31.76	31.72	32.06	31.97	31.63	31.98	33.26	32.31	32.16	32.63	32.06	32.43
2	34.15	33.17	33.35	33.51	33.85	34.50	32.57	32.89	33.52	32.48	32.64	32.78	34.75	34.72	33.99	34.53	34.09	34.18
1	34.33	34.33	36.08	34.96	34.46	34.03	35.45	33.86	33.66	34.76	35.29	34.20	35.12	N/A	34.62	35.44	34.48	35.19
0.5	37.83	36.17	36.67	35.50	35.91	36.24	36.56	35.33	37.09	35.43	34.69	35.88	38.39	37.13	N/A	N/A	37.13	36.71
0.2	37.64	38.06	39.55	N/A	38.91	38.02	37.30	36.07	35.88	36.91	38.14	37.75	38.31	38.32	38.91	39.55	37.08	N/A
0.1	N/A	36.61	N/A	N/A	N/A	N/A	N/A	39.61	37.34	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Mean of C_q value obtained for 6 repetitions (Repetition [*Rep.*] 1 to 18) of 6 independent runs (Run 1 to 6) of a serial dilution of genomic DNA of *E. jeanselmei* (concentration expressed in copy number of genomes of the IHEM 4740 strain). The LOD is defined by the dashed line.

Table 3.4: Limit of detection results for *Ejeanselmei_ITS* qPCR SYBR®green assay

Theoretical (estimated) copy number	C_q mean ± SD	% positive
10	31.51 ± 0.78	100.00 (36/36)
5	32.45 ± 0.88	100.00 (36/36)
2	33.78 ± 0.98	100.00 (36/36)
1	34.86 ± 0.90	97.22 (35/36)
0.5	36.27 ± 1.07	94.44 (34/36)
0.2	37.62 ± 1.06	80.56 (29/36)
0.1	38.39 ± 1.21	25.00 (9/36)

The table shows the mean C_q value obtained for 6 repetitions of 6 runs of a serial dilution of genomic DNA of *E. jeanselmei* (concentration expressed in copy number), the standard deviation (± SD) and the percentage of positive response observed at each dilution point. The LOD is defined by the discontinuous line.

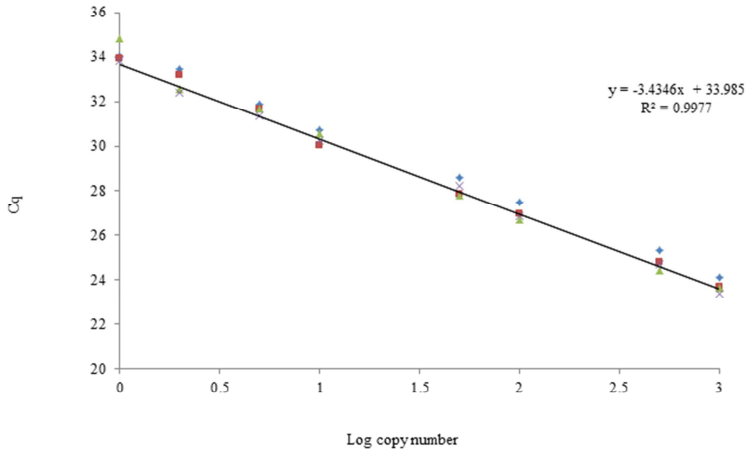

Fig. 3.5: Coefficient of determination and PCR efficiency of *Ejeanselmei_ITS* qPCR assay
Data are obtained with four replicates for each concentration i.e., 1,000 to 0.1 theoretical copy number of genome of *E. jeanselmei* (IHEM 4740).

3.3.3 Environmental testing

To evaluate the performance of this *E. jeanselmei_ITS* method on real-life samples, a test was performed on different water samples collected from air-cooled systems in office buildings. This proof of concept allows to test the *Ejeanselmei_ITS* assay on environmental samples and to compare this method of detection with a classical routine analysis method (Table 3.5).

The detection method used in routine (culture, counting and microscopic identification) allowed to detect *E. jeanselmei* in 3 water samples with an amount ranging between 2 CFU/ml and 4 CFU/ml (Table 3.4). All of these samples contained one or two other contaminants frequently recovered from water reservoirs (Table 3.5) i.e., *Acremonium* sp., *A. fumigatus* and a sterile mycelium (undetermined).

The results from the *Ejeanselmei_ITS* assay were in accordance with those of the classical method for the detection of *E. jeanselmei*. Indeed, this qPCR assay gave positive signals for the same water samples where *E. jeanselmei* was detected using the classical methods, i.e., sample n° 1, 2 and 3 (Table 3.5). The obtained C_q values were all ranging around 35 and the T_m values around 79.5 °C, as expected. No signal was observed for the samples where no *E. jeanselmei* was detected on plate. The spike test confirmed that no inhibition from potentially remaining anti-fungal chemistry products occurred during the qPCR analysis as a C_q of 22.50 ±0.68 was obtained which corresponds to the value shown in Table 3.1 for *E. jeanselmei* IHEM 4740.

Table 3.5: Environmental testing on water from air-conditionning reservoirs

	Classical method		Molecular method		
Sample number	Species	CFU/ml [a]	Amount of total DNA/PCR reaction (ng) [b]	C_q mean ± SD [c]	Theoretical copy number of gDNA for 1 ml [d]
1	*E. jeanselmei*	2	2.93	35.20 ± 0.72	1
	Sterile mycelium	5			
2	*Acremonium* sp.	5	2.53	N/A	
	Penicillium sp.	1			
3	N/D	0	0.36	N/A	
4	*E. jeanselmei*	4	3.27	34.63 ± 0.76	2
	Acremonium sp.	2			
5	N/D	0	0.94	N/A	
6	*A. fumigatus*	1	1.73	N/A	
7	*E. jeanselmei*	3		35.24 ± 0.34	1
	Acremonium sp.	1	3.07		
	Sterile mycelium	1			
8	*Acremonium* sp.	17	9.80	N/A	

[a] The value for CFU/ml is an estimation of the fungal contamination based on the number of colonies per plate. [b] 5 µl of the extracted DNA from 15 ml of sampled water (and eluted in 100 µl of extra pure DNAase, RNAase, protease free water) were used in a 25 µl-PCR reaction. [c] C_q values are C_q means ($\leq$ 40) ± standard deviation (SD) obtained with the validated *Ejeanselmei_ITS* assay with 4 technical replicates. [d] Theoretical copy number of gDNA based on the *E. jeanselmei* IHEM 4740 strain defined as the strain of reference of the performance assessment of this *Ejeanselmei_ITS* qPCR SYBR®green assay.

3.4 Discussion

E. jeanselmei is frequently found inside buildings in canalisations, reservoirs of drinking water, non-drinking water reservoirs for air-conditioning. As this species is an opportunistic pathogen causing health problems (Al-Gabr et al. 2014; Nucci et al. 2001; Nucci et al. 2002; Zeng et al. 2007), its monitoring is important. Classical approaches used for the detection of this pathogenic fungus are difficult because of morphologically closely related species and are moreover time-consuming. Indeed *E. jeanselmei* requires up to 21 days of incubation at 35 °C before microscopic identification (Najafzadeh et al. 2013; Nishimura et al. 1987; Nolard et al. 2004). This is why molecular analysis, such as the in this study developed *Ejeanselmei_ITS* assay, could be attractive for a more efficient monitoring and diagnosis of a contamination by *E. jeanselmei*. The real-time PCR analysis, and especially the SYBR®green qPCR chemistry, offers the advantage to be fast, sensitive, instrumental-wise more accessible and more cost-effective than other molecular techniques like direct sequencing, especially when many samples need to be analysed. Indeed, the SYBR®green chemistry is cheaper than the chemistry used for sequencing, with a cost of less than 1 euro per reaction. In comparison with the classical tools, this technique is more expensive. However, the time saving (2 days

against 21 for the classical approach) is a key advantage in terms of monitoring and diagnosis.

Another advantage is that the specificity is based on a primer couple and on the associated T_m value of the generated amplicon, thereby avoiding a sequencing step which is needed when using classical PCR approaches (Klein, 2002). In this assay, the primers were designed in the ITS-2 region from the 18S rDNA complex.

Because no guidelines nor norms exist for the development of qPCR methods for the detection of fungi, the performance assessment flow was based on guidelines and recommendations given for GMO detection and foodborne pathogen (Barbau-Piednoir et al. 2013; Broeders et al. 2014), as was previously done for a qPCR assay for the specific detection of *A. versicolor* (Libert et al. 2015). To evaluate the performance of this *Ejeanselmei_ITS* SYBR®green assay, the selectivity, PCR efficiency, dynamic range, sensitivity and repeatability parameters were investigated.

First, the inclusivity tests revealed that the *Ejeanselmei_ITS* assay detects all the tested *E. jeanselmei* strains. Nevertheless, between these two amplified species, a variation of approximately 3 C_q was observed. As shown by sequencing analysis (Fig. 3.1), no variation occurred between the amplicon obtained for each of these strains, and the sequence between the primers matched 100% with the corresponding one retrieved for all publicly available *E. jeanselemi* ITS-2 sequences. This variation in the C_q could however be explained by a dissimilarity of the 18S rDNA copy number which is known to have an interspecies and an intraspecies variability (Black et al. 2013; Corradi et al. 2007; Iwen et al. 2002; Schoch et al. 2012), and which was also suggested for *A. versicolor* (Libert et al. 2015). Because no variation rates for the 18S rDNA copy number are known for *E. jeanselmei* or closely related species, this *Ejeanselmei_ITS* assay is a qualitative qPCR. To develop a quantitative tool based on the ITS sequence, the range of variation of the 18S rDNA copy number should be determined for each targeted strain in order to extrapolate the quantity expressed in genome copy numbers.

The exclusivity test, the second step of the specificity test, showed that no non-targeted strains from indoor environment, including the air-conditioning system, were detected with the *Ejeanselmei_ITS* assay. This demonstrates the good specificity of the *Ejeanselmei_ITS* assay. For none of the closely related and morphologically difficult to discriminate species the DNA was amplified with the *Ejeanselmei_ITS* assay, except for *E. spinifera*. However, despite this amplification, the melting curve analysis allowed the discrimination between *E. spinifera* and *E. jeanselmei*, as the T_m differs with 1 °C between the two species (T_m *E. spinifera* = 78.50 °C, T_m *E. jeanselmei* = 79.50 °C) (Fig. 3.4). This difference in T_m can be explained by the interspecies diversity observed in the ITS region of species from the *E. spinifera* clade which includes *E. jeanselmei* (Wang et al. 2001; Woo et al. 2013; Zeng and De Hoog 2008) (Fig.

3.1). The post-analysis based on the melting temperature to discriminate a species is currently used with the SYBR®green chemistry also in other fields to discriminate two different strains amplified by the same couple of primers (Barbau-Piednoir et al. 2013b), without the need for sequencing confirmation.

In the future, high resolution melting (applied to the *Ejeanselmei_ITS* assay) might be used to obtain an even more pronounced discrimination of these two *Exophiala* species based on the T_m of the amplicon, if needed. Nevertheless, the *Ejeanselmei_ITS* assay allows to discriminate between species that are frequently confused with *E. jeanselmei* with culture dependent analysis and some molecular methods like classical PCR.

Then, efficiency and PCR linearity were evaluated for this SYBR®green assay. To evaluate these parameters, the strain *E. jeanselmei* IHEM 4740 was selected as the reference strain. Because the C_q values obtained for this strains were the highest amongst those for the strains tested, the results obtained for these parameters correspond to the worst case scenario for this assay. With an efficiency of 95.5 %, this SYBR®green assay is efficient according to the criteria defined for qualitative analysis of GMO (Broeders et al. 2014). Moreover, the obtained R^2 value (0.9977) shows the linearity and the accuracy of this qPCR SYBR®green assay.

Furthermore, with an LOD defined at 1 theoretical genomic copies per reaction, this *Ejeanselmei_ITS* is sensitive and well within the criteria put forward by the GMO community (ENGL 2015). This *Ejeanselmei_ITS* assay is also repeatable with *r* (3.45) and *RSDr* (9.73 %) values below the limits defined by the guidelines used in this study (Barbau-Piednoir et al. 2013b; Broeders et al. 2014). Because no information was available on the genome size of *E. jeanselmei*, the copy number estimation was done with an average of the genome size based on the size of 3 *Exophiala* species (i.e., *E. dermatitidis*, *E. spinifera* and *E. xenobiotica*). Therefore, this estimation should be reviewed when the real genome size of *E. jeanselmei* will be available. Nevertheless, if the genome size of *E. dermatitidis* (i.e., 26.35 Mb) is used as "worst case" scenario to define the copy number of gDNA of *E. jeanselmei*, the underestimation of the copy number at LOD is evaluated to be 1 % only, which should not influence drastically our results.

These performance assessment tests were done using DNA extracted from pure strains from the BCCM/IHEM collection, which are well characterised. In order to test this *Ejeanselmei_ITS* assay under environmental conditions, water samples from air-conditioning were submitted to our qPCR method. The obtained results were compared with those obtained with the routine protocol based on the classical analysis method (i.e., culture, microscopic visualisation and counting). As similar results were obtained for both classical and qPCR method, this proof of concept demonstrated that the *Ejeanselmei_ITS* assay could be useful for

the monitoring of *E. jeanslemei*. Concerning the time period to obtain these results, this *Ejeanselmei_ITS* assay seems to be a better alternative to the classical analysis. Indeed, using the classical methods, the incubation period was 21 days, while our SYBR®green analysis (lyophilisation step and DNA extraction included) required 2 days. A low diversity, maximum two species per sample, was observed with the classical method as it was previously observed in other study (Hamada and Fujita 2002; Kelkar et al. 2005; Parat et al. 1996). In total, 5 species were observed (*Acremonium* sp., *A. fumigatus, E. jeanselmei, P. chrysogenum*) (Table 3.4). This implies that the water reservoirs were regularly cleaned with anti-fungal chemistry to avoid health problem as allergies, asthma or sick building syndrome. Therefore, it was verified that this anti-fungal chemicals did not inhibit the qPCR reaction, which was found not to be the case. Based on the C_q values obtained for the LOD estimation, an extrapolation of the DNA theoretical copy number was performed in order to compare the results from the classical and the molecular methods. According to this estimation, the theoretical (estimated) copy number of genomic DNA for *E. jeanselmei* was evaluated to 1 and 2 copy number of DNA per ml of analysed water. These estimations were in the range of those obtained with classical methods (between 2 and 4 CFU/ml). However, some considerations should be made. Firstly, this extrapolation was done based on the values obtained for the reference strain (with the estimated genome size of 30 Mb), and it is not so unlikely that another strain of *E. jeanselmei* was present in the real life samples. Therefore the effect of the variation in 18S rDNA copy number should be taken into account. Secondly, this comparison supposed that one CFU observed on plate corresponds to one copy of gDNA. However, a colony can originate from more than one copy of gDNA if aggregates were present. Therefore, although the range of detected *E. jeanselmei* contamination was comparable for both methods, and therefore the qPCR assay could be regarded as a semi-quantitative method, the absolute quantities of *E. jeanselmei* are difficult to be compared between the two analysis methods. However, this was expected, because as elaborated above, the ITS-based qPCR method is a qualitative method. If an absolute quantification is needed, the classical methods should still be used.

The results of this proof of concept demonstrate that molecular methods such as qPCR could also be used for the detection of other contaminants present in the water reservoir of air-conditioners. In order to reduce even more the analysis time, it should also be interesting to develop a multiplex tool for the detection of several contaminants simultaneously. This will allow achieving the same advantage of the classical method, which is not limited to the detection of *E. jeanselmei* only.

In addition, the use of this SYBR®green qPCR assay should not be reduced to water environments. It would also be useful for the detection of *E. jeanselmei* in other environmental or in clinical samples. As qPCR methods, being based on DNA amplification, offer the advantage of detecting also non-culturable/dead organisms, it would be interesting to

apply this method to the monitoring of *E. jeanselmei* in environments where its occurrence has not yet been reported. Indeed, because *E. jeanselmei* requires a humid environment to be kept alive and the currently used monitoring methods for this pathogen are based on culturing (which implies that the to be detected organism should be alive and culturable), its presence in for instance indoor air, where the organism could suffer from desiccation impacting its growth, could not yet be demonstrated.

In conclusion, this paper reports on a novel SYBR®green qPCR assay for the specific detection of *E. jeanselmei*, called *Ejeanselmei_ITS*. Because classical methods are time consuming, and *E. jeanselmei* has demanding growth conditions, this qualitative assay, and molecular tools like qPCR in general, offers the possibility to reduce the analysis time period and to extend the monitoring to other environments. This will contribute to an improved response against fungal contamination and a better insight into the causal link between *E. jeanselmei* and health problems.

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 4

Discrimination of three genetically close *Aspergillus* species by using high resolution melting analysis applied to indoor air as case study

This chapter was previously submitted for publication as Libert X., Packeu A., Bureau F., Roosens N.H., De Keersmaecker S.J.C. "Discrimination of Three genetically close *Aspergillus* Species by Using High Resolution Melting Analysis Applied to Indoor Air as Case Study".

Abstract

Background: Indoor air pollution caused by fungal contamination is suspected to have a public health impact. Monitoring of the composition of the indoor airborne fungal contaminants is therefore important. To avoid problems linked to culture-dependent protocols, molecular methods are increasingly being proposed as an alternative. Among these molecular methods, the polymerase chain reaction (PCR) and the real-time PCR are the most frequently used tools for indoor fungal detection. However, even if these tools have demonstrated their appropriate performance, some of them are not able to discriminate between species which are genetically close. A solution to this could be the use of a post-qPCR high resolution melting (HRM) analysis, which would allow the discrimination of these species based on the highly accurate determination of the difference in melting temperature of the obtained amplicon. In this study, we provide a proof-of-concept for this approach, using our previously developed qPCR SYBR®Green method to detect *Aspergillus versicolor* in indoor air, an important airborne fungus in terms of occurrence and cause of health problems. Despite the good performance observed for that qPCR method, no discrimination could previously be made between *A. versicolor*, *Aspergillus creber* and *Aspergillus sydowii.*

Results: Using HRM analysis, the discrimination of the 3 *Aspergillus* species could be made. No false positive, nor false negatives were observed during the performance assessment including 15 strains of *Aspergillus*. The limit of detection was determined for each species i.e., 0.5 pg/µl of gDNA for *A. creber* and *A. sydowii*, and 0.1 pg/µl of gDNA for *A. versicolor*. The HRM analysis was also successfully tested on environmental samples.

Conclusion: We reported the development of HRM tools for the discrimination of *A. versicolor*, *A. creber* and *A. sydowii*. However, this study could be considered as a study case demonstrating that HRM based on existing qPCR assays, allows a more accurate identification of indoor air contaminants. This contributes to an improved insight in the diversity of indoor airborne fungi and hence, eventually in the causal link with health problems.

Keywords

Aspergillus, High resolution melting analysis, Indoor air, Public health, molecular method

4.1 Background

Today, the contamination of indoor air of buildings by fungi is suggested to be associated with public health problems (World Health Organization 2009). However, the causal link between fungal air contamination and respiratory problems is still not well understood. This is partly due to the issues related to the detection and identification of fungal species in indoor air. Indeed classically, the detection and monitoring workflow of indoor fungal contamination are based on the microscopic identification of fungi obtained after a cultivation step (Beguin and Norlard 1994; Nolard et al. 2004). This culture-dependent workflow leads to some bias in the diversity observed due to e.g., species competition on plate, uncultivable species or dead fungi(Beguin and Norlard 1994; Nolard et al. 2004; HUD 2006; Pitkaranta et al. 2011) However, these by classical workflow undetected species could affect human health (HUD 2006). To avoid this bias, the use of culture-independent, molecular techniques seems to be more advantageous than the classical workflow (Pitkaranta et al. 2011; Vesper 2011). That is why PCR (Martin et al. 2005; Zhou et al. 2000) and real-time PCR (qPCR) are currently increasingly used for the monitoring of indoor airborne fungi (Bellanger et al. 2009; de Ana et al. 2006; Haugland et al. 2004; Martin et al. 2005; Morrisson et al. 2004).

Although qPCR methods are specific and allow the identification up to species level, genetically close species are sometimes difficult to be discriminated using these molecular tools. For example, we previously proposed a qualitative qPCR SYBR®green method targeting the ITS2 region (*Aversi_ITS* assay) for the detection of *Aspergillus versicolor* (Libert et al. 2015), an important indoor fungal contaminant (Andersen et al. 2011; Beguin and Norlard 1994; Packeu et al. 2012a). Among the 10 species from the indoor air background that had been included in the specificity test, this tool developed for the specific detection of *A. versicolor* resulted in false positives only for the DNA of 2 genetically close species, i.e., *Aspergillus creber* and *Aspergillus sydowii*, belonging to the same group of *Versicolores.*

As previously elaborated (Libert et al. 2015), these 3 species are difficult to be discriminated, both morphologically as genetically. The available Taqman assays of the United States Environmental Protection Agency (EPA) for the specific detection of *A. versicolor* and *A. sydowii* respectively, also amplify both species each time (Haugland et al. 2004; United States Environmental Protection Agency 2015). For *A. creber*, no Taqman assays have been developed and/or tested yet. Nevertheless, it has been observed that closely related species could show different antifungal patterns, which is important information to choose the appropriate therapeutic regime (Jurjevic et al. 2012). Additionally, Jurjeciv et al. (2013) reported that, in a same environmental context, the different species belonging to this *Aspergillus* group (including *A. creber* and *A. versicolor*) produce different concentrations of sterigmatocystin, a precursor of the aflatoxin B1 which is a well-known carcinogenic mycotoxin. *A. sydowii* is known as a non-sterigmatocystin producer (Rank et al. 2011). These

observations indicate that these 3 genetically closely related species belonging to the *Versicolores* group could have a different effect on health. However, it has not yet been investigated what the difference is concerning the impact of their presence in indoor air on public health. Hereto, a rapid, culture-independent discriminative method is currently lacking. Therefore, this is an interesting case study for the development of a molecular method that can discriminate genetically close species of indoor airborne fungi.

In the present study, we developed a molecular method based on our previously proposed SYBR®green qPCR method for the detection of *A. versicolor* (Libert et al. 2015), for the discrimination of *A. versicolor*, *A. sydowii* and *A. creber*. Indeed, the advantage of SYBR®green includes the possibility to discriminate different amplicons based on their melting temperature (T_m). However, despite nucleotide variations between the 3 amplicons obtained for respectively *A. versicolor*, *A. sydowii* and *A. creber*, poor discrimination could be made with a classical melting curve analysis (Libert et al. 2015). In this context, the technology of high resolution melting (HRM) could offer a good alternative method for the discrimination of species closely related at the genetic level. Unlike the SYBR®green chemistry, the dye used for HRM analysis is a saturation dye. Consequently, all amplicons obtained after the DNA template amplification are saturated by the dye improving the detection of nucleotide variation, in combination with a high resolution qPCR instrument (Reed et al. 2007). Based on this particularity, genetically closely related species can be distinguished with HRM analysis as it was shown for *Candida* species and some other invasive fungal species (Lengerova et al. 2014; Nemcova et al. 2015; Somogyvari et al. 2012) including some *Aspergillus* species (Alonso et al. 2012). The HRM analysis groups together (i.e., clusters) samples with similarities in the shape of the melting curves. By including positive controls for each of the expected species, the discrimination in species-specific clusters can be done.

By taking 3 *Aspergillus* species closely related at the genetic level as a case study, we deliver the proof-of-concept that existing SYBR®Green qPCR methods can be further developed using HRM into more discriminating molecular methods. These offer the possibility to improve the identification of indoor airborne fungi, thereby eventually contributing to establishing the causal link between these contaminants and adverse health effects.

4.2 Results

4.2.1 HRM assay development

The HRM analysis reported in this study is based on a previously published qPCR SYBR®Green assay (Libert et al. 2015), called *Aversi_ITS*. In that study (Libert et al. 2015), during the performance assessment, the specificity was tested for 10 species frequently found

in indoor air i.e., *Alternaria alternata, A. creber, Aspergillus fumigatus, A. sydowii, A. versicolor, Cladopsorium cladosporioides, Cladosporium herbarum, Penicillium chrysogenum, Stachybotrys chartarum* and *Ulocladium botrytis*. This test showed to be specific for *A. versicolor*, i.e. no amplification for the non-targeted species, with the exception of *A. creber* and *A. sydowii* which yielded an amplicon with the same T_m than the one observed for *A. versicolor* (i.e., 76.5 °C).

Therefore, the species discrimination using the *Aversi_ITS* assay was optimized using the HRM approach as elaborated in Materials and Methods for the species for which amplification was obtained with the *Aversi_ITS* assay. Hereto, firstly, the HRM assays were performed in duplicate using gDNA extracted from pure strains acting as reference strains in Libert et al. (2015) i.e., the BCCM/IHEM strain 18884 for *A. versicolor*, the BCCM/IHEM strain 20347 for *A. sydowii* and the BCCM/IHEM strain 2646 for *A. creber*. The 3 reference strains showed an expected melting temperature (T_m) ranging between 76.40 ±0.28 and 76.50 ±0.14 and could be classified each in a different cluster (Fig. 4.1a,b; Table 4.1). Cluster 1 grouped together all the replicates of the *A. versicolor* BCCM/IHEM 18884 with a confidence of 98.6 ±0.9. Cluster 2 was defined by all the replicates from the *A. sydowii* BCCM/IHEM 20347 strain with a confidence of 99.8 ±0.1. Finally, all the replicates from the *A. creber* BCCM/IHEM strain 2646 were classified in cluster 3 with a confidence of 97.9 ±1.2.

Subsequently, all other strains available in the BCCM/IHEM collection and belonging to the species *A. creber, A. versicolor* or *A. sydowii* were tested. The T_m of the obtained amplicon was determined to verify the PCR amplification step. As previously reported (Libert et al. 2015), gDNA of all species tested resulted in an amplicon with a T_m around 76.5 ±0.18 °C (Table 4.1). No amplification was observed in any of the NTCs added to the assays. All the strains of *A. versicolor* were grouped in cluster 1 defined by the reference strain BCCM/IHEM 18884 with a confidence defined between 97.6 ± 2.2% and 99.0 ± 0.5%. Those of *A. sydowii* were grouped in cluster 2 defined by the reference strain BCCM/IHEM 20347 with a confidence ranging between 99.1 ± 0.4% and 99.2 ± 0.3% and those of *A. creber* in cluster 3 defined by the reference strain BCCM/IHEM 2646 with 98.9 ± 1.3 % of confidence.

P. chrysogenum was tested as negative control because of its phylogenetic proximity with the *Aspergillus* genus (van den Berg et al. 2008) and its importance in indoor air contamination (de Ana et al. 2006; Hyvarinen et al. 2001). Two different strains were included, but they did not yield an amplicon as expected based on the selectivity previously determined for the *Aversi_ITS* assay (Libert et al. 2015).

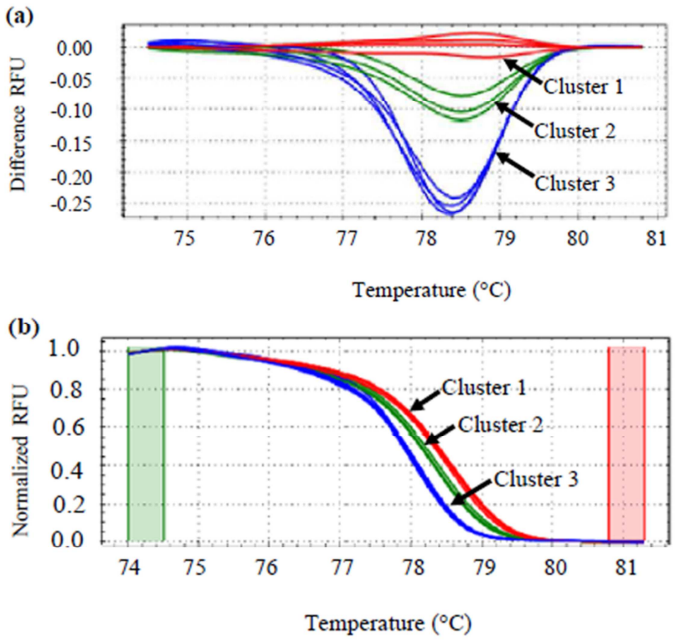

Figure 4.1: High resolution melting analysis plots. HRM analysis results are illustrated with (a) the normalized melt curves. Each cluster was defined with the Biorad Precision Melt Analysis software 1.2 (Temse, Belgium) (b) the difference temperature plot. The red curves correspond to *A. versicolor* strains, the green curves to *A. creber* strains and the blue curves to *A. sydowii*. gDNA of each strain, i.e., *A. creber* IHEM 2646 (5 ng of gDNA), *A. versicolor* IHEM 18884 (5 ng of gDNA) and *A. sydowii* IHEM 20347, was tested in duplicate in 2 independent runs.

Sensitivity test: Limit of detection

To define the lowest amount of gDNA that can be discriminated with the HRM analysis, a serial dilution, from 1000 to 0.01 pg in 10 steps repeated in duplicate in 6 independent runs was tested (Table 4.2). Because the HRM supermix cannot be used with an amount of input DNA lower than 0.1 ng, all the dilutions were made in salmon sperm DNA. No amplification was observed in the negative controls i.e., NTC_{water} and $NTC_{salmon\ sperm}$ (data not shown). For *A. versicolor*, the LOD of the *Aversi_ITS* qPCR assay was previously defined at 1 copy of gDNA (Libert et al. 2015) which corresponds to 0.05 pg of gDNA. In the HRM assay, *A. versicolor* was amplified until 0.1 pg of gDNA with 12/12 positive detection events and until 0.05 pg of gDNA with 10/12 positive detections, i.e. correct amplicon and cluster and confidence >95%. Below this limit of 0.05 pg, no amplification was observed for *A. versicolor* in the tested concentrations. *A. creber* and *A. sydowii* were detected with minimum 11/12 positive detection events until 0.5 pg of gDNA (Table 4.2). As shown in Table 4.2, the discrimination in the 3 different clusters could be made with HRM until 0.5 pg of gDNA, under this limit no discrimination could be made between *A. creber* and *A. sydowii*. Based on these observations, the LOD for the HRM assay was defined at 0.5 pg of gDNA for *A. creber* and *A. sydowii* and at 0.1 pg for *A. versicolor* (Table 4.2).

Table 4.1: Species discrimination by HRM analysis

Genus	Species	Reference BCCM/IHEM [a]	T_m mean ± SD (°C) [b]	Cluster [c]	Confidence mean ± SD [d] (%)
Aspergillus	*versicolor*	IHEM 1323	76.45 ±0.07	1	98.4 ± 0.4
Aspergillus	*versicolor*	IHEM 1355	76.30 ±0.14	1	97.6 ± 2.2
Aspergillus	*versicolor*	IHEM 2023	76.40 ±0.28	1	98.9 ± 0.9
Aspergillus	*versicolor*	IHEM 2157	76.35 ±0.21	1	98.6 ± 0.4
Aspergillus	*versicolor*	IHEM 2983	76.30 ±0.14	1	98.6 ± 1.0
Aspergillus	*versicolor*	IHEM 6598	76.40 ±0.28	1	98.2 ± 0.4
Aspergillus	*versicolor*	IHEM 9674	76.55 ±0.07	1	98.9 ± 0.4
Aspergillus	*versicolor*	IHEM 10351	76.30 ±0.14	1	99.0 ± 0.5
Aspergillus	*versicolor*	IHEM 18884	76.40 ±0.28	1	98.6 ± 0.9
Aspergillus	*versicolor*	IHEM 19014	76.35 ±0.21	1	98.8 ± 0.6
Aspergillus	*versicolor*	IHEM 19210	76.40 ±0.01	1	98.5 ± 0.9

Table 4.1: *Continued*

Genus	Species	Reference BCCM/IHEM [a]	T_m mean ± SD (°C) [b]	Cluster [c]	Confidence mean ± SD [d] (%)
Aspergillus	*versicolor*	IHEM 19256	76.40 ±0.28	1	98.8 ± 0.2
Aspergillus	*versicolor*	IHEM 22014	76.35 ±0.21	1	98.3 ± 1.1
Aspergillus	*versicolor*	IHEM 22975	76.30 ±0.14	1	98.6 ± 1.0
Aspergillus	*versicolor*	IHEM 24424	76.50 ±0.42	1	99.0 ± 0.5
Aspergillus	*sydowii*	IHEM 895	76.35 ±0.21	2	99.1 ± 0.4
Aspergillus	*sydowii*	IHEM 1360	76.40 ±0.00	2	99.2 ± 0.3
Aspergillus	*sydowii*	IHEM 20347	76.50 ±0.14	2	99.8 ± 0.1
Aspergillus	*creber*	IHEM 2646	76.40 ±0.28	3	97.9 ± 1.2
Aspergillus	*creber*	IHEM 2916	76.38 ±0.08	3	98.9 ± 1.3
Penicillium	*chrysogenum*	IHEM 20859	/	ND	/
Penicillium	*chrysogenum*	IHEM 4151	/	ND	/

The strains in bold are considered as a reference used for the assay development and are fully characterised as respectively *A. creber*, *A. sydowii* and *A. versicolor*. [a] Pure strains from the BCCM/IHEM collection. [b] Average of the T_m ± standard deviation (SD) obtained for each strain analyzed in duplicate during 2 independent runs. [c] Cluster 1, 2 and 3 were defined with the Biorad Precision Melt Analysis software 1.2 (Temse, Belgium). ND: d not detected. [d] Average of the percentage of confidence (± standard deviation SD) from the mean of the cluster, defined with the Biorad Precision Melt Analysis software 1.2 (Temse, Belgium). The percentage of confidence threshold was defined as 95 %, below this threshold the result is considered as not acceptable as a true positive.

Table 4.2: Limit of detection of HRM assay

Parameter	Genus	Species [a]	Cluster [b]	gDNA amount (pg)									
				1000	500	50	10	5	1	0.5	0.1	0.05	0.01
Detection [c]	*Aspergillus*	*creber*	3	12/12	12/12	12/12	12/12	12/12	12/12	11/12	6/12	0/12	0/12
	Aspergillus	*sydowii*	2	12/12	12/12	12/12	12/12	12/12	12/12	11/12	9/12	0/12	0/12
	Aspergillus	*versicolor*	1	12/12	12/12	12/12	12/12	12/12	12/12	12/12	12/12	10/12	0/12
Confidence (%) [d]	*Aspergillus*	*creber*	3	97.98 ±1.28	96.66±1.54	99.03±0.59	99.30±0.29	99.70±0.02	97.99±0.53	96.65±0.36	97.99 ±0.53.	/	/
	Aspergillus	*sydowii*	2	98.72 ±0.39	99.25 ±0.26	99.28±1.66	99.67±1.12	98.840±2.34	96.83±3.11	95.63±1.63	95.83 ±3.11	/	/
	Aspergillus	*versicolor*	1	98.99 ±0.26	99.23±0.35	99.49±2.01	98.93±0.60	99.76±096	98.52±2.32	97.49±0.11	97.60±1.06	96.37±1.540	/

[a] gDNA extracted from pure strains from the BCCM/IHEM collection. BCCM/IHEM strains used as reference: *A. creber* IHEM 2646, *A. sydowii* IHEM 26347, *A. versicolor* IHEM 18884

[b] Cluster defined with the Biorad Precision Melt Analysis software 1.2 (Temse, Belgium)

[c] Detection: Number of positive signals i.e., correct cluster and confidence > 95%, observed for each dilution, the LOD for each species is indicated in bold.

[d] Confidence (%) is the average and standard deviation (±SD)

4.2.2 Symmetric and asymmetric DNA concentration test

The capacity to detect and to discriminate the 3 *Aspergillus* species when they are mixed, was tested with two different types of mix composition i.e., a symmetric mix including an equal amount of gDNA from each targeted species (*A. creber*, *A. sydowii* and *A. versicolor*) and a asymmetric mix composed of a different amount of gDNA from the 3 *Aspergillus* species, including one species present at LOD, and mixes that contained the negative control *P. chrysogenum*.

The HRM analysis of the symmetric mix A (i.e., a mix of 5 ng of gDNA of each *Aspergillus* species) yielded 3 different clusters, corresponding to the 3 targeted *Aspergillus* species (Table 4.3).

In the 3 first asymmetric mixes (i.e., mixes B, C and D, Table 4.3), the amount of gDNA from one of the 3 *Aspergillus* species was taken at the LOD previously defined i.e., 0.1 pg for *A. versicolor* and 0.5 pg for the 2 others. The results obtained were similar (i.e., correct cluster, confidence % in the same range) to those observed with the symmetric mix, even when the amount of gDNA was at the LOD (Table 4.3). In the mixes E, F and G, one of the 3 species was added in a higher amount than that of the other 2. Once again, the 3 clusters were found with high confidence with all mix configurations (Table 4.3).

Table 4.3: Symmetric, asymmetric and specificity assessment of different mixes

Parameter	Species [a] Mix										
		A	B	C	D	E	F	G	H	I	J	K
DNA amount (ng)[b,c]	*A. creber*	5	LOD[d]	5	5	25	5	5	0	5	5	0
	A. sydowii	5	5	LOD	5	5	25	5	5	0	5	0
	A. versicolor	5	5	5	LOD	5	5	25	5	5	0	0
	P. chrysogenum	0	0	0	0	0	0	0	25	25	25	0
	NTC_{water}	/	/	/	/	/	/	/	/	/	/	5
Cluster [d]	*A. creber*	3	3	3	3	3	3	3	/	3	3	/
	A. sydowii	2	2	2	2	2	2	2	2	/	2	/
	A. versicolor	1	1	1	1	1	1	1	1	1	/	/
	P. chrysogenum	/	/	/	/	/	/	/	ND	ND	ND	/
	NTC_{water}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Confidence (%)[e]	*A. creber*	97.56 ±1.05	99.67 ±0.07	99.13 ±1.80	99.18 ±1.87	99.67±1.87	99.67±1.17	99.64±1.17	/	98.27 ±0.34	99.20 ±1.43	/
	A. sydowii	99.85 ±0.20	99.59 ±0.01	99.85 ±0.20	99.88±0.10	99.60±0.10	99.58±0.61	99.50±0.68	99.27 ±0.61	/	98.27 ±0.51	/
	A. versicolor	98.67 ±1.09	100.00 ±1.09	98.68 ±1.19	98.48 ±1.19	100.00±0.10	99.90 ±0.02	99.67±0.02	98.20 ±1.03	98.98 ±0.03	/	/
	P. chrysogenum	/	/	/	/	/	/	/	ND	ND	ND	/
	NTC_{water}	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] gDNA extracted from pure strains from the BCCM/IHEM collection

[b] Each assay contained 3 pure strains i.e., BCCM/IHEM 2646 for *A. creber*, BCCM/IHEM 895 for *A. sydowii*, BCCM/IHEM 10351 for *A. versicolor*, and BCCM/IHEM 4151 for *P. chrysogenum*. Each run was performed with 3 strains defined as positive control for each target i.e., *A. creber* BCCM/IHEM 2646, *A. sydowii* BCCM/IHEM 20347 and *A. versicolor* BCCM/IHEM 18884. *P. chrysogenum* BCCM/IHEM 20 859 was used as a negative control. Each HRM reaction was done with 5 µl of mix.

[c] LOD defined as the limit of detection defined for each species i.e., 0.1 pg for *A. versicolor* and 0.5 pg for *A. creber* and *A. sydowii*.

[d] Detection parameters i.e., clusters and percentage of detection defined with the Biorad Precision Melt Analysis software 1.2 (Temse, Belgium). /: not of application, as not present in the mix; ND: no amplicon obtained, no cluster detected

[e] Confidence (%) is the average of each percentage of confidence defined with the Biorad Precision Melt Analysis software 1.2 (Temse, Belgium) obtained for each repetition and standard deviation (±SD).

4.2.3 Specificity assessment

Although the *Aversi_ITS* assay was previously shown to be specific for *A. versicolor*, as evaluated for the 10 most frequent species from the indoor air background, the discrimination between *A. versicolor*, *A. creber* and *A. sydowii* was not observed in classical qPCR (Libert et al. 2015). To evaluate that the discrimination of these 3 *Aspergillus* species by the HRM method is not influenced by the presence of non-targeted species, even at a dominant concentration, 3 others mixes were made and analyzed with HRM.

In mixes H, I and J, one of the targeted species was removed from the mix (Table 4.3), and replaced by 5 ng of *P. chrysogenum* gDNA and analyzed with the *Aversi_ITS* HRM method. This analysis indicated that *P. chrysogenum* was not amplified in any of the mixes (Table 4.3), which was expected based on the results of Libert et al. (2015) and in agreement with the results obtained for mix K, where *P. chrysogenum* was the only species present. The targeted species were all classified in their corresponding clusters i.e., cluster 1 for *A. versicolor*, cluster 2 for *A. sydowii* and cluster 3 for *A. creber* (Table 4.3).

4.2.4. Proof-of-concept

In order to verify whether this HRM analysis could be used on real-life samples, 4 environmental air samples, previously analyzed by classical methods (i.e., plate counting and microscopic identification) and by qPCR for the detection of *A. versicolor* (Libert et al. 2015), were re-analyzed with the *Aversi_ITS* HRM assay.

As summarized Table 4.4, the *Aversi_ITS* HRM analysis defined 3 different clusters for the 3 positive controls with or without the addition of salmon sperm DNA. The 2 NTCs (NTC_{water} and $NTC_{salmon\ sperm}$) did not yield an amplicon.

Three environmental samples (i.e., samples 1, 2 and 4), with or without the addition of salmon sperm DNA, gave a positive signal for *A. versicolor* and were all classified in only one cluster corresponding to the one of the *A. versicolor* positive control. The number of positive detections (i.e., amplification and classification in the correct cluster and confidence above 95%) of the 8 repetitions was between 5/8 and 8/8 with a confidence ranging between 98.58 ±0.55 and 99.76 ±0.01 (Table 4.4). The lowest number of positive detections and the lowest confidence were obtained for a sample without the addition of salmon sperm DNA. These results were in accordance with those previously obtained by classical methods and qPCR where *A. versicolor* was found on plate and detected by qPCR (Libert et al. 2015). No amplification and consequently no cluster, was observed for the sample 3. This result was also obtained with the classical analysis (Libert et al. 2015), where no *A. versicolor* was detected on plate (Table 4.4).

Table 4.4: Environmental test

Sample	Sample type	Species [a]	Amount of DNA/HRM analysis (ng) [b]	Salmon sperm [c]	Cluster [d]	Percentage of confidence (%) [d]	Number of positive detections
Control							
NTC_{water}	Water only	Water only	0	No	N/A	/	
$NTC_{salmon\ sperm}$	Salmon sperm only	Salmon sperm only	10	Yes	N/A	/	
Positive control	Pure culture	*A. creber*	25	No	3	95.74 ±7.07	4/4
Positive control	Pure culture	*A. creber*	25	Yes	3	97.32 ± 3.90	4/4
Positive control	Pure culture	*A. sydowii*	25	No	2	97.91 ± 3.26	4/4
Positive control	Pure culture	*A. sydowii*	25	Yes	2	98.89 ± 0.97	4/4
Positive control	Pure culture	*A. versicolor*	25	No	1	99.16 ± 0.49	4/4
Positive control	Pure culture	*A. versicolor*	25	Yes	1	97.99 ± 2.38	4/4
Environmental sample							
Sample 1	Indoor air sample	*A. versicolor*	19.8	No	1	99.15 ± 0.08	6/8
				Yes	1	99.76 ± 0.01	7/8
		P. chrysogenum					
Sample 2	Indoor air sample	*A. versicolor*	21.7	No	1	98.10 ± 0.88	5/8 [$]
				Yes	1	98.93 ± 0.45	7/8
		A. glaucus					
		P. chrysogenum					
		Yeast (undetermined)					
Sample 3	Indoor air sample	*P. chrysogenum*	53.3	No	N/A		
				Yes	N/A		
Sample 4	Indoor air sample	*A. versicolor*	50	No	1	98.81 ± 1.90	5/8[$]
				Yes	1	98.58 ± 0.55	8/8
		Infertile mycelium *P. chrysogenum*					

[a] Determined with classical methods i.e. plate culture and microscopic analysis (determination and counting) in Libert et al. 2015

[b] Amount of DNA extracted from air samples of 1.5 m³; DNA amount determined with a Nanodrop® 2000; 5 µl of extracted DNA (5ng/µl) were used in a20 µl -HRM analysis.

[c] Salmon sperm (salmon sperm) DNA carrier added (10 ng).

[d] Cluster and % of confidence ± standard deviation (SD) defined with the Biorad Precision Melt Analysis software 1.2 (Temse, Belgium) .

[e] A sample is defined as positive for a specific species, if an amplicon is obtained, if the observed Tm corresponds to the Tm defined by Libert et al. (2015) for *A. versicolor* (i.e., 76.5 ±0.18 °C) and if the sample is classified in the same cluster as the cluster defined for its respective positive control with a confidence > 95%.

Based on the above described results, it could be concluded that the 3 environmental samples contained the *A. versicolor* species, and not one of the 2 species closely related at the genetic level belonging to the same *Versicolores* group. However, in order to verify that if one of the other 2 species would have been present, it would have been possible to be detected in the environmental sample, a spike test with each of the targeted species was performed as described in Materials and Methods. At the highest gDNA concentration, with or without salmon sperm DNA, all the species were detected and classified in the correct cluster defined by the corresponding positive control (Appendix A – Table A1). The same observations were made for all spikes at LOD with a confidence between 97.70 ±1.56% and 99.08 ±1.14% and a positive rate between 5 and 8 for the 8 repetitions depending on the addition of salmon sperm DNA (Appendix A – Table A1).

4.3 Discussion

Currently, an increasing amount of studies are focused on fungal indoor contamination and its impact on public health (Bellanger et al. 2009; Bennedorf et al. 2008; de Ana et al. 2006; Jones et al. 2011; Meheust et al. 2014; Packeu et al. 2012a; Rebroux et al. 2009; Vesper et al. 2013). To rapidly detect and identify the fungal contaminants, qPCR holds a great potential in comparison to the classical methods based on plate counting and microscopy. Indeed, this molecular technique is rapid, sensitive, easy to use and culture independent (Pitkaranta et al. 2011). However, some issues could occur especially for the discrimination of species closely related at the genetic level. This problem was highlighted by Libert et al. (2015) during the development of a SYBR®green qPCR tool for the detection of *A. versicolor* (*Aversi_ITS*), an important contaminant of indoor environment. Even if this *Aversi_ITS* assay is fast, efficient, sensitive and specific for *A. versicolor* as evaluated for the 10 most frequently occurring fungal species in indoor air, no discrimination between *A. versicolor*, *A. creber* and *A. sydowii* could be previously made. Indeed the insufficient variation of nucleotides inside their respective amplicons yielded a too similar T_m value impeding discrimination by qPCR (Libert et al. 2015). Nevertheless, the accurate identification of fungal contaminants is important to eventually determine the causal link between indoor airborne fungal pollution and respiratory health problems.

In this context, to improve the specific detection of *A. versicolor* in indoor air and to discriminate it from the other targets *A. creber* and *A. sydowii*, a post-qPCR HRM was optimized on the basis of the *Aversi_ITS* assay (Libert et al. 2015). The HRM analysis developed in this study showed the possibility to improve the *Aversi_ITS* qPCR assay by the discrimination of *A. versicolor* from *A.creber* and *A. sydowii*. All the strains for the 3 species that were available in the BCCM/IHEM

collection were not only detected but also classified with a high confidence in 3 different clusters (Table 4.1, Fig. 4.1 a,b), demonstrating the inclusivity and discriminative power of the assay.

The absence of amplification when using gDNA of the 2 *P. chrysogenum* negative controls (Table 4.1) confirms the exclusivity of the primers of the *Aversi_ITS* assay. These results confirmed those previously shown by Libert et al. (2015), during an exclusivity test on 10 species selected as the most detected fungal species in indoor air where no amplification was obtained for the non-targeted species.

During this study, a total of 20 strains of *Aspergillus* were tested, including all strains available for each species in the BCCM/IHEM collection i.e., 2 strains for *A. creber*, 3 for *A. sydowii*, and 15 for *A. versicolor*. In addition, based on a sequence alignment previously shown during the *Aversi_ITS* development (Libert et al. 2015) with all the sequences of *A. creber*, *A. sydowii* and *A. versicolor* available on NCBI at the date of the study.

Because these *Aspergillus* species are genetically grouped in the *Versicolores* group, primer exclusivity was also verified using an alignment of primers sequences and all the ITS sequences available in the NCBI database for all the species from this group, i.e. *Aspergillus amoenus, Aspergillus austroafricanus, Aspergillus cvjetkovicii, Aspergillus fructus, Aspergillus jensenii, Aspergillus protuberus, Aspergillus puulaauensis, Aspergillus subversicolor, Aspergillus tabacinus, Aspergillus tennesseensis* and *Aspergillus venenatus*. As these showed several nucleotide variations, i.e. more than 3 as is the case for *A. versicolor*, *A. creber* and *A. sydowii*, inside the amplicon (primers annealing site included) defined by the primers sequences, these species should be able to be discriminated from *A. creber*, *A. sydowii* and *A. versicolor* with qPCR (based on the T_m) (Appendix A – Figure A1 and Table A2).

The sensitivity for the HRM assay was also tested by defining the LOD for discrimination for each species. For *A. versicolor*, the LOD was previously determined for the *Aversi_ITS* qPCR assay at 1 or 2 copies of gDNA (Libert et al. 2015), corresponding to 0.05 pg of gDNA of *A. versicolor*. The LOD observed for *A. versicolor* in the HRM assay was determined at 0.1 pg of gDNA. This difference is due to the confidence threshold applied in this study (i.e., 95%). The dissimilarity observed between the LOD for the qPCR and HRM could be explained by the fact that an HRM analysis needs a higher amount of DNA templates to discriminate with high confidence. According to the user guide for the HRM analysis (Biorad 2016), the threshold for a HRM discrimination is observed around 30 C_q (corresponding to 0.5 pg of DNA of *A. versicolor*). Above this C_q limit, the results are too variable. As no LOD was defined in the previous study for *A. creber* and *A. sydowii*, a sensitivity test was performed for the HRM assay and the LOD for these two species was defined at 0.5 pg of gDNA (Table 4.2). Thus, as observed by Libert et al.

(2015) with the SYBR®Green chemistry, the *Aversi_ITS* primers are more efficient for the amplification of gDNA of *A. versicolor* than for the two other species. This difference of sensitivity could impact the level of detection in real-life samples where some species could be present in very low concentration. However, in addition to the sensitivity assessment, HRM assays were performed on gDNA mixes from pure cultures of *A. creber, A. sydowii* and *A. versicolor*, present at different amounts, including at the LOD. The results obtained during these tests demonstrated that the HRM technology can be used to discriminate a mix of species, even when the gDNA mix is not equilibrated (Table 4.3).

Sensitivity and discrimination were also observed for the environmental, i.e. indoor air samples containing a mix of gDNA from these 3 species. One of those samples, which was demonstrated to be negative for *A. versicolor* based on classical methods and qPCR (Libert et al. 2015), was used to spike the 3 targeted species for HRM analysis. No inhibition from the environmental sample matrix on the detection and discrimination of the 3 species was detected. The discrimination between the 3 species could be made, even if one of them was present at the LOD or if a non-targeted species was present (e.g., *P. chrysogenum)* (Tables 4.4, Appendix A – Table A1).

In the other non-spiked indoor air samples, *A. versicolor* was detected, in accordance with the previous results obtained with classical methods based on culture and microscopic determination (Table 4.4). In each of these positive samples, *A. versicolor* was present with other common indoor air species (i.e., *A. glaucus* and *P. chrysogenum*) or with undetermined strains. This however did not affect the HRM-based detection, with a detection and discrimination of *A. versicolor* in each of the samples where the SYBR®green *Aversi_ITS* qPCR method previously detected *A. versicolor* (Libert et al. 2015). However, based on the classical detection methods (Libert et al. 2015), it was observed that the level of contamination by *A. versicolor* in sample 2 was close to the LOD of the qPCR (i.e., 0.05 pg of gDNA). This could explain why the level of positive repetitions in the HRM analysis varied between 62.5% (5/8, without salmon sperm DNA added) and 100% (8/8, with salmon sperm DNA added) (Table 4.4). This might also indicate that adding salmon sperm DNA to the HRM reactions improves the performance of this assay, as a similar trend was observed for the other environmental samples. However, no statistical evidence could be obtained for this observation due to the low amount of total extracted DNA available per environmental sample for the analysis which limited the number of repetition which could be made.

4.4 Conclusions

Conclusively, our study, by taking 3 *Aspergillus* genetically closely related species as a case study, demonstrated that HRM analysis, based on existing qPCR methods, could be used to more accurately detect and identify indoor fungal contamination. HRM analysis offers the advantage to easily discriminate genetically close species which are difficult to be distinguished. This increase in accuracy will improve data on indoor air fungal contamination. This is especially important for currently difficult to be discriminated species, but that could however diverge in terms of toxicity, allergenicity or pathogenicity. In this study, the proof of concept was delivered for the 3 species from the *Versicolores* group based on a SYBR®green *Aversi_ITS* qPCR method previously developed for *A. versicolor* (Libert et al. 2015). In the future, other existing SYBR®green qPCR assays could be adapted for HRM for other species, to improve the discrimination of genetically close species. Eventually, the use of HRM in routine analysis performed in the framework of monitoring activities will contribute to the insight in the causal link between indoor fungal contamination and public health.

4.5 Material and methods

4.5.1 Strains, culturing and DNA isolation

All the species and strains used in this study were previously used to develop the qPCR SYBR®Green *Aversi_ITS* assay (Libert et al. 2015). All of these strains were purchased from the BCCM/IHEM collection (Brussels, Belgium) and are listed in Table 4.1, i.e., *A. creber*, *A. sydowii*, *A. versicolor* and *P. chrysogenum.* Culturing and the DNA extraction protocols were previously described in Libert et al. (2015).

4.5.2 QPCR and high resolution melting (HRM) conditions

The HRM assays were performed using a CFX96 Touch™ Real-Time PCR Detection System and the CFX manager 3.1 software (Biorad, Temse, Belgium).

The qPCR program was previously described and optimized (Libert et al. 2015). The following thermal cycling conditions were used i.e., 1 cycle at 95 °C for 2 min for the complete activation of the hot-start DNA polymerase, 40 cycles at 95 °C for 10 s for the denaturing step, followed by one step at 60 °C for 30 s (annealing and extension), and a final extension at72 °C for 30 s. The PCR amplification was followed by the HRM analysis which is performed in 2 stages, adapted from the instruction manual for the Precision Melt Supermix (Biorad, Temse, Belgium). The first

stage was the heteroduplex formation, i.e. 95 °C for 30 s and 60 °C for 1 min. The second step was the high resolution melting (HRM) itself between 63 and 95°C with an increment of 0.10 °C each 10 s.

As recommended by the manufacturer, the reaction mix (20 µl final volume) contained 10 µl of Precision Melt Supermix with Evagreen dye (Biorad, Temse, Belgium), 1.2 µl of *Aversi_ITS* f and *Aversi_ITS* r (Eurogentec, Liège, Belgium) at 300 nM final concentration each (Libert et al. 2015), and 2.6 µl of Gibco® DNase, RNase, Protease free pure water (Life Technologies, Gent, Belgium). In each well, an equal amount of 5 µl of each genomic DNA (gDNA) template (1 ng per µl, so 5 ng gDNA in total per well) was added to the reaction mix. During the optimization phase of the HRM assay, 5 ng of gDNA was analyzed in duplicate, each in two independent runs. In each assay a non DNA template control (NTC_{water}) composed of Gibco® DNase, RNase, Protease free pure water (Life Technology, Gent, Belgium) and 3 positive controls i.e., *A. creber* IHEM 2646 (5 ng of gDNA), *A. versicolor* IHEM 18884 (5 ng of gDNA) and *A. sydowii* IHEM 20347 (5 ng of gDNA) were added.

4.5.3 HRM data analysis

The melt-curve data were analysed with the Biorad Precision Melt Analysis software 1.2 (Biorad, Temse, Belgium).

A sample is defined as positive for a specific species, if an amplicon is obtained, if the observed T_m corresponds to the T_m defined by Libert et al. (2015) for *A. versicolor* (i.e., 76.5 ±0.18 °C) and if the sample is classified in the same cluster as the cluster defined for its respective positive control. The software also calculates a percent confidence. This value provides a percentage chance that a given well is correctly categorized within the assigned cluster. It is based on the number of standard deviations the sample is from the mean of the cluster. This assumes that the found "cluster means and standard deviations" are accurate descriptions of the real probability distributions of the data (Biorad 2016). The threshold of the percent confidence was fixed at 95%. Below this limit, the sample was considered as not acceptable as a true positive.

The clustering of each sample can be visualized by the software with different melt curve charts, i.e. the normalized melt curve chart and the difference curve chart. The first one (Fig. 4.1a) shows a normalized view of the melting curve of each sample (Pre-melt (initial) and post-melt (final) fluorescence signals of all samples are normalized to relative values of 100% and 0%, differences in background fluorescence between curves are eliminated) and plots their relative fluorescence unit (RFU) against the temperature. The second chart (Fig. 4.1b) magnifies curve differences by subtracting each curve from the most abundant type or from a user-defined reference. By setting a baseline, small differences between the RFU obtained for each cluster become visible.

4.5.3 Sensitivity test: Limit of detection

To evaluate the sensitivity of the use of the *Aversi_ITS* f/r primers for the HRM application, a serial dilution of gDNA of *A. creber* BCCM/IHEM 2646, *A. sydowii* BCCM/IHEM 20347 and *A. versicolor* IHEM 18884, defined as a reference by Libert et al. (2015) for the performance assessment of the *Aversi_ITS* assay, was made and the limit of detection (LOD) was determined. A serial of 10 dilutions from 1000 to 0.01 pg (i.e., 1000, 500, 50, 10, 5, 1, 0.5, 0.1, 0.05, 0.01 pg) was analyzed in duplicate with 6 independent runs. To comply with the Precision Melt Supermix requirements on the minimum amount of DNA to be present in the well, all the dilutions were made in 10 ng deoxyribonucleic acid sodium salt from salmon testes i.e., salmon sperm DNA (Sigma-Aldrich, Diegem, Belgium). In each assay, a negative control ($NTC_{salmon\ sperm}$), i.e., 10 ng of salmon sperm DNA (Sigma-Aldrich, Diegem, Belgium) and NTC (composed of Gibco® DNase, RNase, Protease free pure water) were added. The LOD was determined as the lowest amount of gDNA where for at least 11 out of the 12 repetitions the amplicon (with the correct T_m) and the correct cluster (with confidence > 95 %) were obtained.

4.5.4. Symmetric and asymmetric DNA concentration test

In order to evaluate the discriminative power of the HRM assay, 12 mixes of gDNA of *A. creber*, *A. sydowii* and *A. versicolor* were analyzed in duplicate. The mixes' composition is presented in the first part of Table 4.3. Briefly, first 5 ng of gDNA from a mix (A) containing an equal amount of gDNA from each targeted species (i.e., 5 ng of each species for 5 µl of total volume of mix) that were mixed together (Table 4.3) was analyzed with HRM. To assess the capacity of the HRM assay to detect and discriminate the 3 targets in an imbalanced mix of gDNA, as it could occur in environmental samples, a second (B to G) set of mixes was analyzed (Table 4.3). In mixes B to D, 2 species were introduced with an equal amount of gDNA (i.e., 5 ng both for 5 µl of total volume of mix) and a third one at the LOD; in mixes E to G, one species dominated the others (i.e., 25 ng for 5 µl of total volume of mix), while the 2 others were added at the same amount of gDNA (i.e., 5 ng both for 5 µl of total volume of mix). Each HRM analysis was performed in duplicate with 5 µl of gDNA mix. The strains used for the mixes were the BCCM/IHEM 2646 for *A. creber*, the BCCM/IHEM 895 for *A. sydowii* and the BCCM/IHEM 10351 for *A. versicolor*.

In each assay, an NTC (no DNA) and 3 positive controls were added to the assay i.e., *A. creber* IHEM 2646 (5 ng of gDNA), *A. versicolor* IHEM 18884 (5 ng of gDNA) and *A. sydowii* IHEM 20347 (5 ng of gDNA). At the time of these experiments, the strain BCCM/IHEM 2646 was the only confirmed strain of *A. creber* available in the collection. During the redaction of this manuscript, a new *A. creber* strain (BCCM/IHEM 2916) was added to the BCCM/IHEM collection. To verify whether a correct discrimination could be done for this new strain, a test was

performed following the conditions described in the sections qPCR and high resolution melt conditions and HRM data analysis (Table 4.1). The results matched with those previously obtained with the strain BCCM/IHEM 2646. Therefore it was decided that the strain *A. creber* BCCM/IHEM 2646 could be used as a representative for *A. creber* in the symmetric and asymmetric concentration tests.

4.5.5 Specificity assessment

In order to verify that the discrimination of the 3 targeted species (*A. creber, A. sydowii*, *A. versicolor*) can be done in the presence of a non-targeted species (*P. chrysogenum*), 3 additional mixes (H to J, Table 4.3) were tested, where *P. chrysogenum* replaced one of the 3 *Aspergillus* species. The pure strain *P. chrysogenum* BCCM/IHEM 20849 was selected as a negative control. The mixes H, I and J have equal composition containing 5 ng per *Aspergillus* species and 25 ng for *P. chrysogenum* (Table 4.3). The mix K was introduced as a negative control containing only 5 ng of *P. chrysogenum* gDNA extracted from the strain BCCM/IHEM 20 849 (Table 4.3). In each assay, an NTC_{water} and 3 positive controls were added i.e., *A. creber* IHEM 2646 (5 ng of gDNA), *A. versicolor* IHEM 18884 (5 ng of gDNA) and *A. sydowii* IHEM 20347 (5 ng of gDNA). The strains used for the mixes were the BCCM/IHEM 2646 for *A. creber*, the BCCM/IHEM 895 for *A. sydowii* and the BCCM/IHEM 10351 for *A. versicolor*.

4.5.6. Proof-of-concept using environmental air samples

To assess the performance of the *Aversi_ITS* assay to discriminate the 3 targeted *Aspergillus* spp. using the HRM technology, 4 environmental samples previously collected in a single contaminated house and previously analysed with the *Aversi_ITS* assay and classical identification methods (Libert et al. 2015), were used. The sampling protocol, the classical method of identification (i.e., counting and microscopic identification) and the gDNA extraction have been previously described (Libert et al. 2015). The HRM analysis was performed in duplicate in 4 independent repeats, as described above. In addition to using the DNA extracted from the environmental samples as such, in a second round of experiments, in order to avoid any issues with the HRM supermix, 10 ng of salmon sperm DNA was added to all DNA mixtures prior to HRM analysis. As mentioned above, each HRM analysis included a positive control for each of the species to be discriminated (25 ng of gDNA).

To be sure that no inhibition occurs during the HRM analysis and in order to verify that all the 3 species can be detected and discriminated with the HRM analysis, two sets of gDNA from pure cultures were spiked into one environmental sample where *A. versicolor* was not detected by classical nor qPCR methods (i.e., sample 3). A first set contained 25 ng gDNA extracted from pure culture (*A. creber* 2646 IHEM/BCCM, *A. sydowii* 895 and *A. versicolor* 10351) spiked into

the DNA extracted from the environmental sample 3. In addition, spikes were made with an amount of DNA for each of the targeted species at the LOD in order to verify whether a very small amount could be detected in this environmental sample. Similarly to what was done with the 'pure' environmental samples, a second set of sample was made with an addition of salmon sperm DNA (10 ng) into the spiked samples in order to increase the DNA amount available for the supermix (cfr. the sensitivity test). As mentioned above, in each assay, a NTC_{water}, a $NTC_{salmon\ sperm}$ and 3 positive controls were added i.e., *A. creber* IHEM 2646 (25 ng of gDNA), *A. versicolor* IHEM 18884 (25 ng of gDNA) and *A. sydowii* IHEM 20347 (25 ng of gDNA).

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Competing interests

The authors declare that they have no competing interests.

Chapter 5

Development and performance assessment of a Luminex xMAP® Direct hybridization assay for the detection and identification of indoor air fungal contamination

This chapter was previously submitted for publication as Libert X., Packeu A., Bureau F., Roosens N.H., De Keersmaecker S.J.C. "Development and performance assessment of a Luminex xMAP® Direct hybridization assay for the detection and identification of indoor air fungal contamination".

Abstract

Considered as a public health problem, indoor fungal contamination is generally monitored using classical protocols based on culturing. However, this culture dependency could influence the representativeness of the fungal population detected in an analyzed sample, including the dead and uncultivable one; moreover, they are often time-consuming. In this context, molecular tools are a powerful alternative, especially those that allow multiplexing. In this study a Luminex xMAP® assay was developed for the simultaneous detection of the 10 most frequently in indoor air found fungi that may cause health problems. This xMAP® assay is sensitive, i.e. the limit of detection is ranging between 0.05 and 0.01 ng of gDNA. The assay was subsequently tested with environmental air samples which were also analyzed with a classical protocol. All the species identified with the classical method were also detected with the xMAP® assay, however in a shorter time frame. These results demonstrate that the Luminex xMAP® fungal assay developed in this study could contribute to the improvement of public health and specifically to the indoor fungal contamination treatment.

Keywords

Luminex, indoor air monitoring, fungi, Public health, multiplex, molecular

Practical implications

Considered as a public health problem, indoor fungal contamination is generally monitored using classical protocols based on culturing. However, this culture dependency could influence the representativeness of the fungal population detected in an analyzed sample. Effectively, competition factors, time of incubation or the growth medium could impact the fungal diversity observed after culturing. This paper showed an original molecular tool based on the Luminex xMAP® technology for fungal detection which is simple to be performed, fast (maximum 3 days) and accurate which improved fungal detection and monitoring by an important reduction in turn-around-time, with a faster communication of the results to the involved medical team. This will improve the framework of fungal monitoring in indoor air.

5.1 Introduction

Currently, indoor airborne fungal contamination is suggested to be associated with public health problems World Health Organization 2009). Even if indoor air fungal contaminants could be allergenic or could have an implication in respiratory diseases, such as asthma, wheezing or rhinitis (Bellanger et al. 2009; Horner et al. 1995; Meheust et al. 2014; Mendell et al. 2011; Packeu et al. 2012a,b; Rebroux et al. 2010) the scientific evidence for the causal link between these molds and adverse health effects is still poorly documented. The use of classical methods involving culturing and microscopic visualization in routine monitoring analysis could be pointed out as one of the reasons explaining this lack of evidence in scientific literature. Even if culture-dependent tools are useful and well documented, these techniques are known to be affected by competition factors, selection of growth media or culture conditions (Pitakaranta et al. 2011; Vesper 2011) and they are only able to detect the living fungal fraction. Therefore, these technical problems could cause an underestimation of the diversity of the indoor air fungal population and reduce the evidence for the causal link between fungal contamination and health problems (Pitakaranta et al. 2011). Another drawback is that these classical tools are time-consuming, taking 5 up to even 21 days in some cases (Pitakaranta et al. 2011; Libert et al. 2015; Libert et al. 2016; Nolard et al. 2004).

In order to avoid these problems of culture dependency and being time-consuming, molecular tools are increasingly being used to detect indoor airborne molds. Amongst them are the real-time polymerase chain reaction (qPCR) tools which are more and more developed for their application in fungal monitoring (Black et al. 2013; Dean et al. 2004; Haugland et al. 2004). Besides being fast, accurate and specific, qPCR methods have also the advantage of being culture-independent. Furthermore, qPCR could be developed for multiplex detection, detecting simultaneously several different species, reducing once more the time and amount of sample needed for an analysis. Many qPCR assays are based on hydrolysis probes, such as the TaqMan® ones, which are highly specific and useful in multiplex analysis (Bellanger et al. 2009; Roussel et al. 2013; United States Environmental Protection Agency 2016). However, although multiplexing is possible and already successfully performed, the number of targets is still limited to 4 or 5, especially because the number of available fluorophores and quenchers (Gurvich et al. 2011), which can be detected at the same time, is limited.

The Luminex xMAP® technology has been demonstrated to be a valuable alternative to the qPCR multiplex. This technology is based on the detection of multiple sets of polystyrene microspheres (beads) characterized by a specific spectral emission. The number of sets that can be detected is

dependent on the type of Luminex instrument being used. For the benchtop model, the MagPix instrument, 50 sets of beads can be simultaneously detected. The coupling of each set of xMAP® beads with a specific oligonucleotide probe, specific to a certain target, permits to detect up to 50 different targets in a single assay (Dunbar et al. 2006). One set of coupled beads-probes is hybridizing to a specific PCR amplicon previously amplified with biotinylated primers. The addition of streptavidin-R-phycoerythrin as a reporter allows the detection of each hybridized PCR amplicon on a beads set coupled with the specific probe (Dunbar et al. 2006). So, if the target was present in the sample, it will be detected through a green fluorescent signal read out on the Luminex instrument. Today, fungal xMAP® assays have been mainly used for the diagnosis of relevant fungal pathogens in clinical samples as e.g., some *Aspergillus* sp., *Candida* sp., *Mucor* sp. or *Fusarium* sp. (Etienne et al. 2009; Landlinger et al. 2009). These assays included the testing of isolated colonies from biological samples such as biopsy's tissues, bronchoaveolar secretions or blood, and these were not yet tested on DNA extracted directly from environmental samples (Etienne et al. 2009; Landlinger et al. 2009). In fact, until now, no Luminex xMAP® tool is available for indoor air fungal monitoring.

In this study, a Luminex xMAP® assay was developed, for the first time, for the multiplex detection of the 10 airborne fungal species most frequently found in indoor air and that may cause health problems i.e., *Alternaria alternata*, *Aspergillus creber*, *Aspergillus fumigatus*, *Aspergillus sydowii*, *Aspergillus versicolor*, *Cladopsorium cladosporioides*, *Cladosporium herbarum*, *Penicillium chrysogenum*, *Stachybotrys chartarum* and *Ulocladium* botrytis (Beguin et al. 2004; Bellanger et al. 2009; Shelton et al. 2002) without prior cultivation. As it is required for the performance assessment of molecular tools, the specificity and sensitivity of the developed Luminex xMAP® assay was determined. Finally, the assay was tested on real-life environmental samples as a proof of concept demonstrating that the Luminex xMAP® technology can be used for the monitoring of indoor air fungal contamination. The development of a Luminex xMAP® assay aimed at the improvement of the framework of fungal monitoring in indoor air, which will eventually improve public health. Indeed, the simultaneous detection allowed by the multiplexing permits to reduce the time required for the analysis and for the communication of the results to the involved medical team. Moreover, the use of Luminex xMAP® technology has the advantage that the sample size requirements are reduced and that no skilled mycologist is needed to perform the microscopic-based identification analysis.

5.2 Materials and methods

5.2.1 Fungal strains and DNA isolation

All the fungal species and strains used in this study are listed in Table 5.1. All of them were purchased from the BCCM/IHEM collection located at the Scientific Institute of Public Health in Brussels (WIV-ISP, Belgium).

The culture and extraction protocol were both previously described in Libert et al. (2015). Briefly, all the strains were incubated with constant agitation in a S10 Sabouraud liquid medium (Biorad, Temse, Belgium) at 25 °C during 3 to 10 days depending on the species' culture conditions. Then, after a centrifugation of 1 min at 12 000g to eliminate all the Sabouraud liquid, 0.25 ml of acid washed glass beads (Sigma Aldrich, Diegem, Belgium) were added to the wet sample (300 mg) transferred into cryotubes and put at -80 °C during 40 minutes. After that, the samples were freeze-dried overnight with a freeze-dryer Epsilon 1-6D (Martin Christ, Osterode am Harz, Germany) and subsequently beat-beaten (1 minute, maximal speed) with a Mini bead beater (Biospec Products, OK, USA).

Finally, an adapted phenol chloroform (24:1) protocol (Ashktorab and Cohen 1992) was applied to extract DNA, which was then purified with the Qiagen CTAB genomic Tip-20 kit (Qiagen Benelux – B.V., KJ Venlo, the Netherlands) and eluted with 100 µl Gibco® DNase, RNase, protease free water (Life Technologies, Gent, Belgium). The purity and the amount of extracted DNA were evaluated with a Nanodrop® 2000 (Thermo Scientific, Wilmington, USA), and DNA integrity on a 2 % agarose gel.

Table 5.1: Fungal species and probes used in this study

Genus	Species	Reference BCCM/IHEM[a]	Probe name	Sequence 5' -> 3'	Length	Target	Modified from Reference
Alternaria	*alternata*	IHEM 4969	AaltP2.2	TGAATTATTCACCCTTGTCTTTTGCGTACT	30	ITS-1	17
Aspergillus	*creber*	IHEM 2646	VersP1	AGACTGCATCACTCTCAGGCATGAAGTTCA	30	ITS-1	17
Aspergillus	*sydowii*	IHEM 20347	VersP1	AGACTGCATCACTCTCAGGCATGAAGTTCA	30	ITS-1	17
Aspergillus	*versicolor*	IHEM 18884	VersP1	AGACTGCATCACTCTCAGGCATGAAGTTCA	30	ITS-1	17
Aspergillus	*fumigatus*	IHEM 3562	AfumP1	CCCGCCGAAGACCCCAACATGAACGCTGTT	30	ITS-1	20
Cladosporium	*cladosporioides*	IHEM 0859	CcladP1	CCGGGATGTTCATAACCCTTTGTTGTCC	28	ITS-2	17
Cladosporium	*herbarum*	IHEM 2268	CherbP1	CTGGTTATTCATAACCCTTTGTTGTCCGACT	31	ITS-1	17
Penicillium	*chrysogenum*	IHEM 20859	Pchris1	GCCTGTCCGAGCGTCATTTCTGCCCTCAAGC	31	ITS-2	17
Stachybotrys	*charatum*	IHEM 0359	StachP2	CTGCGCCCGGATCCAGGCGCCCGCCGGAGA	30	ITS-1	17
Ulocladium	*botrytis*	IHEM 0328	UloP1	TGAATTATTCACCCGTGTCTTTTGCGTACT	30	ITS-1	17

[a] Identification number as defined by the BCCM/IHEM collection, Mycology and Aerobiology, Scientific Institute for Public Health, Juliette Wytsman street 14, 1050 Brussels, Belgium

5.2.2 PCR amplification

The PCR amplifications were performed in duplex in order to amplify both the internal spacer 1 and 2 regions, using the following couples of universal primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')/ ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') and ITS3 (5'-GCATCGATGAAGAACGCAGC-3')/ ITS4 (5'- TCCTCCGCTTATTGATATGC-3') (White et al. 1990). All primers were manufactured by Eurogentec (Liège, Belgium) and purified with HPLC. The ITS1 and ITS3 forward primers were labeled at 5' with biotin.

Duplex PCR amplifications were performed on a Swift MaxPro and Aeris thermal cycler (Esco, Barnsley, the Netherlands). All the reactions contained 4 µl of 10 X Phusion High Fidelity PCR Buffer with 15 mM $MgCl_2$ (Thermo Fisher Scientific, Erembodegem, Belgium), 0.5 µl of each primer at 0.5µM, 0.4 µl of dNTPs each at 200 µM (Thermo Fisher Scientific, Erembodegem, Belgium) and 0.2 µl of High Fidelity TaqPolymerase enzyme at 0.02 U/µl (Thermo Fisher Scientific, Erembodegem, Belgium). Finally, 11.4 µl of Gibco® DNase, RNase, protease free water and 5 µl of pure gDNA template (1 ng/µl) were added to obtain a final volume of 20 µl per reaction.

The PCR amplifications were carried out with following thermal cycler programme: 30 s at 98 °C (initial denaturation); 35 cycles of 30 s at 98 °C (denaturation), of 1 min at 55 °C (annealing) and 1 min at 72 °C (extension); a final extension at 72 °C during 10 min and a final hold at 16°C.

5.2.3 Probe selection

All probes were selected from literature (qPCR Taqman probes transferred into Luminex probes, Table 1) and adapted if needed in order to have a similar range of length. Probe quality estimation (hairpins, ΔG values…) was performed with Visual OMP version 7.8.42.0 (DNA software, Washington, USA). An *in silico* analysis of each probe was done with the Thermoblast tool from Visual OMP version 7.8.42.0 (DNA software, Washington, USA).

All probes were manufactured by Eurogentec (Liège, Belgium) and tagged with a 5'-end amino modifier C12, followed by a RP-Cartridge-Gold purification.

5.2.4 Probe coupling to xMAP beads

According to the Luminex xMAP® technology, each specific probe is coupled to a specific set of beads. The coupling protocol used in this study is based on the Luminex recommendations for carbodiimide coupling of amine-modified oligonucleotides to MagPlex magnetic carboxylated microspheres (beads) (Angeloni et al. 2014). The final concentration of the coupled beads was 12500 beads/µl. These working stocks of coupled beads were stored in the dark at 4 °C until their use.

5.2.5 Coupled beads hybridization and MagPix analysis

For all Luminex analyses, every coupled bead set used was diluted with 1.5X tetramethylammonium chloride (TMAC) solution containing 5 M tetramethylammonium chloride (Sigma-Aldrich, Diegem, Belgium), 75 mM Tris (Sigma-Aldrich, Diegem, Belgium), 6 mM EDTA (Sigma-Aldrich, Diegem, Belgium), and 0.15% sarkosyl, pH 8.0 (Sigma-Aldrich, Diegem, Belgium) to arrive at a final concentration for each set of coupled beads of 76 beads /µl.

The hybridization mix contained per reaction 33 µl of a specific coupled bead set (76 beads/µl), 12 µl of Tris-EDTA buffer, pH 8 (Sigma-Aldrich, Diegem, Belgium) and 5 µl of fresh PCR amplicons. The hybridization reaction was performed on an Aeris thermal cycler (Esco, Barnsley, the Netherlands) according to the following protocol i.e., a first step at 96 °C during 1 min 30 s and a second one at 58 °C during 30 min. Before a third incubation step of 5 min at 58°C, 25 µl of reporter mix composed of 4 µg/ml of SAPE (Streptavidin-R-Phycoerythrin) (Life Technologies, Gent, Belgium) and 1X TMAC buffer (Sigma-Aldrich, Diegem, Belgium), were added to each sample.

Finally, all analyses were performed on a MAGPIX device (Luminex Corporation, Austin, USA) equipped with the xPONENT for MAGPIX v4.2 software (Luminex Corporation, Austin, USA). The protocol applied in all runs was performed at 58 °C with a minimum of 50 beads counted for each bead region. A wash of each sample was also carried out on the machine during the analysis.

Because each bead set has a unique spectral address (distinct red color code) and each PCR amplicon hybridized to the probe bound to the beads is labeled with SAPE, the fluorescence intensity (red and green) gives information on the amount of beads per region (bead set) and on the amount of beads bound to a PCR amplicon. This last information is given by the median fluorescence intensity value (MFI) and is defined for each region (bead set).

5.2.6 Data analysis and interpretation

The data analysis and the interpretation of the results were based on Wuyts et al. (2015). At the end of each run, the bead counts were checked to verify whether the bead count was homogenous for all of the coupled bead sets. If this was not the case, the run was repeated. Then, the median fluorescence intensity (MFI) of each target was used to calculate a signal-to-noise ratio (SN) with the following formula i.e.,

$SN_{\text{target } a} = MFI_{\text{sample target } a} / MFI_{\text{NTC target } a}$

$SN_{\text{target } a}$ = the signal-to-noise ratio observed for the set of coupled beads selected for the detection of the target *a*

$MFI_{\text{sample target } a}$ = MFI data (collected by the xPONENT software) observed for the target in a specific sample

$MFI_{NTC\ target\ a}$ = MFI obtained for the $NTC_{target\ a}$

According to Wuyts et al. (2015), a result was considered as positive if $SN_{target\ a} \geq 3.00$. For each SN ratio close to the limit (i.e. close to 3), a t-test (95 % confidence) was performed with the SN ratio obtained for the negative control. If the difference is significant (*), the results were considered as positive. If no difference was observed with the negative control, the data were considered as negative.

5.2.7 Specificity test

The specificity of each coupled beads-probes was tested with 3 different assays: a simplex analysis where each probe was tested only on its targeted species (i.e., one bead set, one species) and a multiplex analysis where each species was subjected to each coupled bead set at the same time. Finally, a mix of gDNA extracted from different species was tested.

Simplex analysis (DNA of 1 species, 1 set of beads)

The probe specificity was firstly tested during a simplex analysis. This test consisted of the one by one analysis of all the targeted species with their specific coupled beads –probe set. So, 10 specific hybridization mixes were made, i.e. one mix per set of beads and one mix per species, and analyzed in duplicate during 3 independent runs. All analyses were performed with 5 µl of PCR amplicon, obtained as described above. The repetitions were done with DNA template extracted from independent cultures. For each mix and run, one non-template-control PCR reaction (NTC_{PCR}) i.e., gDNA replaced by water, was introduced in order to evaluate the background linked to the analysis.

Multiplex analysis (DNA of 1 species, multiple sets of beads)

The second step of the specificity evaluation consisted of the multiplex analysis. In this test, the hybridization mix contained every of the coupled bead sets, and this mix was tested on each species in duplicate in 3 independent runs. Every analysis was performed with 5 µl of PCR amplicon, obtained for each species with 5 ng of gDNA from pure culture, as elaborated above. The repetitions were done with DNA template extracted from independent cultures. A NTC_{PCR} was added to all runs.

DNA mix analysis (DNA of multiple species, multiple sets of beads)

Subsequently, a Luminex analysis was performed on the product of a PCR reaction containing a mix of gDNA extracted from different species. Nine different PCR mixes were made (Table 5.2). Mix 1 contained 5 ng of gDNA from all targeted species as mentioned in the Table 5.2 and was considered as a positive control.

For the mixes 2 to 9, gDNA of all species targeted in the test was added in the PCR mix, except for one, whereby the missing species changed for each mix (Table 5.2). All analyses of mixes were done in duplicate in 3 independent runs. For every analysis, a NTC_{PCR} was added.

Sensitivity evaluation: Limit of detection

To evaluate the sensitivity of the Luminex assay developed in this study, a serial dilution of gDNA of each targeted species was made to determine the limit of detection (LOD). Because no guidelines exist on the development and the performance assessment of molecular methods for fungal detection, the LOD estimation performed in this study is based on the workflows elaborated for the validation of molecular methods for the detection of GMO and food-pathogens. In these fields, the LOD is defined as the lowest concentration of an analyte which is detected with a probability of 95% (Barbau-Piednoir et al. 2013b; ENGL 2015).

In order to estimate this LOD, 9 dilutions from 10 to 0.001 ng of gDNA of each species were amplified independently in a PCR reaction according to the above described PCR protocol. All of the PCR reactions were analyzed in duplicate in 3 independent runs.

Table 5.2: Composition of DNA Mixes analysis

Species	BCCM/IHEM strain [1]	Mix								
		1	2	3	4	5	6	7	8	9
A.alternata	3320	V	X	V	V	V	V	V	V	V
A. versicolor [2]	18884	V	V	X	V	V	V	V	V	V
A. fumigatus	3562	V	V	V	X	V	V	V	V	V
C. cladosporioides	859	V	V	V	V	X	V	V	V	V
C. herbarum	2268	V	V	V	V	V	X	V	V	V
P. chrysogenum	20859	V	V	V	V	V	V	X	V	V
S. chartarum	359	V	V	V	V	V	V	V	X	V
U. botrytis	328	V	V	V	V	V	V	V	V	X

[1] Identification number as defined by the BCCM/IHEM collection, Mycology and Aerobiology, Scientific Institute for Public Health, Juliette Wytsman street 14, 1050 Brussels, Belgium
V shows the presence of the species in the mix. X indicates the absence of the species in the mix.
[2] Only DNA from *A. versicolor* was added for the mixes analysis as the probe VersP1 is not specific and detects also the 2 closely related species, *A. creber and A. sydowii.*

5.2.8 Proof of concept with real-life environmental samples

The environmental testing was performed on real-life air samples previously collected from contaminated houses (Libert et al. 2015). The protocols for the sampling, the DNA extraction and microscopic determination were previously described in Libert et al. (2015).

The Luminex assay was applied on samples that were all collected in the same house in 4 different

rooms (i.e., bathroom, bedroom, kitchen and living-room). The analysis was performed in duplicate with 3 independent runs according to the protocols described above. The PCR amplifications were done with 5 ng of gDNA extracted from each sample. In each run a NTC_{PCR} and 8 positive controls (i.e., gDNA extracted from pure cultures of *A. alternata*, *A. versicolor*, *A. fumigatus*, *C. cladosporioides*, *C. herbarum*, *P. chrysogenum*, *S. chartarum* and *U. botrytis*) were added. It should be noted that neither *A. creber* nor *A. sydowii* positive controls were added, because their detection was done with the same probe than that for the detection of *A. versicolor*.

For each SN ratio close to the limit (i.e., close to 3), a t-test (confidence 95 %) was performed with the higher negative results considered as a negative control. If the difference is significant (*), the results were considered as positive. If the no difference was observed with the negative control, the data were considered as negative.

To verify that no inhibitors were present in the environmental samples extracts and to confirm that if the species would be present, it could be detected with the Luminex fungal assay, a final amount of 5 ng of gDNA from pure culture of each species not-detected during the Luminex assay and the classical monitoring were spiked into the DNA extracted from the 4 environmental samples. The spiked DNA extract comes from the same culture than that was used to prepare the DNA used as positive controls. The Luminex analysis was subsequently repeated.

5.3 Results

5.3.1 Probe selection

This Luminex assay aims at the detection of the 10 fungal species most frequently found in indoor air and that may cause health problems i.e., *A. alternata*, *A. creber*, *A. fumigatus*, *A. sydowii*, *A. versicolor*, *C. cladosporioides*, *C. herbarum*, *P. chrysogenum*, *S. chartarum* and *U.* botrytis (Beguin et al. 1994; Bellanger et al. 2009; Shelton et al. 2002). The Luminex xMAP technology used in this study is based on the direct DNA hybridization to a specific probe coupled with a unique set of beads. Specific probes to be coupled to the beads were selected in the internal transcribed spacer (ITS) regions of the ribosomal DNA. These ribosomal regions have the advantages to be conserved and to show, at the genus level, few polymorphisms (Chemidlin Prevost-Boure et al. 2011; Costa et al. 2001; Iwen et al. 2002), allowing the specific detection of particular species. Another advantage is the fact that the small subunit (SSU), 5.8 S and the large subunit (LSU) from the rRNA genes flanking the ITS-1 and ITS-2 regions, are sufficiently conserved among species to design some universal primers such as the primers ITS1 and ITS2 and ITS3 and ITS4 (White et al. 1990) to create the PCR amplicons to be hybridized to the probes. The probes were designed based on qPCR Taqman probes available in literature. Six probes i.e., AaltP2.2, UloP1, StachP2, AfumP1, CherbP1 and VersP1, detect amplicons in ITS-1, while Pchris1 and CcladP1 detect amplicons the ITS-2 region (Table 5.1). All of them are specific to

their target except the probe VersP1 from the EPA (Haugland et al. 2004; United States Environmental Protection Agency 2016) which can also hybridize to the ITS-1 region of *A. creber* and *A. sydowii*. The probes were evaluated *in silico* and adapted if needed in order to have a similar range of length (Table 5.1). Indeed, the stabilization of the formation of the hybridization complex between probe and PCR amplicon is assured by the addition of TMAC which reinforces AT base-pairs (Dunbar 2006). Consequently, the hybridization efficiency is more influenced by the length of the probe than by the nucleotide composition (Dunbar et al. 2006). According to the *in silico* testing, a consensus length for an optimal detection of each target was found to be between 29 and 31 nucleotides (Table 5.1).

5.3.2 Specificity test

The specificity of each probe was tested in three steps i.e., a simplex analysis, a multiplex analysis and the multiplex analysis of a mix of DNA. With the simplex analysis, it was verified whether the protocol and probe can detect the amplicon from the targeted species only (i.e., one species, one probe, one bead set for detection of one species); secondly, with the multiplex analysis, it was investigated if no aspecific annealing occurred when a specific amplicon was put into a mix of probes, the specific probe included (i.e., one species, multiple sets of beads mixed for detection of one species); thirdly, with the multiplex analysis of a mix of DNA, it was verified if no mis-detection happened when a mix of amplicons from different species was analyzed with a mix of different beads (i.e., multiple species mixed, multiple sets of beads mixed for multiple specific detection).

Simplex and multiplex analysis

During these tests, the PCR amplicon of each species was detected by its own specific coupled probe-bead set in every of the 6 repetitions and this both in the simplex analysis (Table 5.3) as well as in the multiplex analysis (Table 5.4). The simplex analysis yielded average SN ratios ranging between 3.50 ±0.25 (for *A. sydowii*) and 22.69 ±1.54 (for *U. botrytis*) (Table 5.3). The average SN ratios obtained in the multiplex analysis ranged between 3.52 ±0.05 and 27.15 ±0.18 for *C. cladosporioides* and *U. botrytis*, respectively (Table 5.4). During these analyses, no false positives as well as no false negatives were observed (Tables 5.3 and 5.4), according to the criteria defined for obtaining a positive result (i.e., MFI ratio ≥3.00).

Table 5.3: Simplex xMAP® analysis

	BCCM/IHEM [a]	Number of positives [b]							
		AaltP2.2	VersP1	AfumP1	CcladP1	CherbP1	Pchris1	StachP2	UloP1
A.alternata	3320	6/6							
A. creber	2646		6/6						
A. sydowii	20347		6/6						
A. versicolor	18884		6/6						
A. fumigatus	3562			6/6					
C. cladosporioides	859				6/6				
C. herbarum	2268					6/6			
P. chrysogenum	20859						6/6		
S. chartarum	359							6/6	
U. botritys	328								6/6
Species	BCCM/IHEM [a]	SN ratio [c,d]							
		AaltP2.2	VersP1	AfumP1	CcladP1	CherbP1	Pchris1	StachP2	UloP1
A. alternata	3320	4.15 ±1.10							
A. creber	2646		3.63 ±0.43						
A. sydowii	20347		3.50 ±0.25						
A. versicolor	18884		4.85 ±0.52						
A. fumigatus	3562			15.24 ±2.39					
C. cladosporioides	859				6.86 ±0.92				
C. herbarum	2268					7.6 ±0.66			
P. chrysogenum	20859						5.15 ±0.81		
S. chartarum	359							4.19 ±0.11	
U. botritys	328								22.69 ±1.54

[a] identification number as defined by the BCCM/IHEM collection, Mycology and Aerobiology, Scientific Institute for Public Health, Juliette Wytsman street 14, 1050 Brussels, Belgium

[b] Number of positive detections obtained during 3 independent runs done in duplicate.

[c] SN ratio defined as the average (±standard deviation) of the ratio between the MFI values for the sample and the NTC for a specific target, obtained with 3 independents runs of independent gDNA extracts of pure cultures (5 ng of gDNA)

[d] In bold are the values considered as positive (i.e., average SN ratio ≥3)

Table 5.4: Multiplex xMAP® analysis to test the bead- probe specificity

Species	BCCM/IHEM [a]	NTC [b]	Number of positives [c]							
			AaltP2.2	versP1	AfumP1	CcladP1	CherbP1	Pchris1	StachP2	UloP1
A.alternata	3320	0/6	6/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
A. creber	2646	0/6	0/6	6/6	0/6	0/6	0/6	0/6	0/6	0/6
A. sydowii	20347	0/6	0/6	6/6	0/6	0/6	0/6	0/6	0/6	0/6
A. versicolor	18884	0/6	0/6	6/6	0/6	0/6	0/6	0/6	0/6	0/6
A. fumigatus	3562	0/6	0/6	0/6	6/6	0/6	0/6	0/6	0/6	0/6
C. cladosporioides	859	0/6	0/6	0/6	0/6	6/6	0/6	0/6	0/6	0/6
C. herbarum	2268	0/6	0/6	0/6	0/6	0/6	6/6	0/6	0/6	0/6
P. chrysogenum	20859	0/6	0/6	0/6	0/6	0/6	0/6	6/6	0/6	0/6
S. chartarum	359	0/6	0/6	0/6	0/6	0/6	0/6	0/6	6/6	0/6
U. botrytis	328	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	6/6
Species	BCCM/IHEM [a]	NTC	SN ratio [c,d]							
			AaltP2.2	versP1	AfumP1	CcladP1	CherbP1	Pchris1	StachP2	UloP1
A.alternata	3320	/	14.06 ±1.05	1.12 ±0.55	0.42 ±0.11	0.19 ±0.09	0.21 ±0.12	0.25 ±0.46	1.78 ±0.23	1.72 ±0.17
A. creber	2646	/	1.46 ±0.12	3.98 ±0.37	1.17 ±0.08	1.24 ±0.02	1.28 ±0.03	1.26 ±0.01	1.3 ±0.04	1.21 ±0.11
A. sydowii	20347	/	1.95 ±0.25	3.87 ±0.05	1.63 ±0.11	1.20 ±0.18	1.31 ±0.18	1.15 ±0.15	2.43 ± 0.25	1.34 ±0.15
A. versicolor	18884	/	1.54 ±0.16	5.51 ±0.15	1.51 ±0.10	1.16 ±0.12	1.07 ±0.13	1.1 ±0.11	2.01 ±0.19	2.43 ±0.17
A. fumigatus	3562	/	1.15 ±0.18	1.42 ±.0.06	17.19 ±2.01	1.11 ±0.12	1.13 ±0.18	1.16 ±0.17	1.13 ±0.18	1.31 ±1.86
C. cladosporioides	859	/	1.02 ±0.16	2.01 ±0.34	1.45 ±0.06	3.52 ±0.05	1.50 ±0.07	1.32 ±0.15	1.69 ±0.69	1.45 ±0.20
C. herbarum	2268	/	2.19 ±0.70	2.94 ±0.06	1.29 ±0.89	1.98 ±0.89	4.02 ±1.35	1.63 ±0.35	1.67 ±0.75	2.36 ±0.32
P. chrysogenum	20859	/	1.04 ±0.23	2.95 ±0.87	0.87±0.03	1.03 ±0.18	0.99 ±0.13	10.69 ±4.14	1.16 ±0.23	0.88 ±0.07
S. chartarum	359	/	1.22 ±0.16	0.94 ±0.22	1.21 ±0.22	1.33 ±0.27	1.23 ±0.19	1.28 ±0.33	21.59 ±0.20	1.14 ±0.87
U. botrytis	328	/	2.03 ±0.99	1.05 ±0.07	1.26 ±0.14	1.44 ±0.02	2.00 ±0.13	1.38 ± 0.00	1.43 ±0.18	27.15 ±0.18

[a] Identification number as defined by the BCCM/IHEM collection, Mycology and Aerobiology, Scientific Institute for Public Health, Juliette Wytsman street 14, 1050 Brussels, Belgium

[b] NTC defined as no template control

[c] Positive results obtained with 3 independents runs of independent gDNA extracts of pure cultures run in duplicate. A result is considered positive when the average (±standard deviation) of the SN ratio is ≥3.00.

[d] In bold are the values considered as positive (i.e., average SN ratio ≥3.00)

DNA mix analysis

Subsequently, an analysis was performed on 9 different DNA mixes (Table 5.2), with a design allowing to verify that the detection of each species is still possible in the presence of other species and with a mix of set of beads. All the positive controls were correctly detected for each repetition (6/6) with a SN ratio ranging between 4.31 ±0.92 for *A. fumigatus* and 17.82 ±0.63 for *A. versicolor*. The first mix contained all of the targeted species. The Luminex analysis resulted in an SN ratio ≥3.00 (i.e. all positive) for all of the expected species and probes for each repetition (6/6 positive for all), with a lowest SN ratio observed at 4.69 ±0.68 (for *A. fumigatus*) and the highest SN ratio observed at 16.98 ±2.85 (for *A. versicolor*) (Table 5.5). In the other mixes (mixes 2 to 9) each time one species was omitted (Tables 5.2 and 5.5). The gDNA of each target species introduced in the mixes was each time detected (6/6) with SN ratios ranging from 3.37 ±0.25 for the detection of *S. chartarum* in the mix 7 to 26.10 ±1.37 for *P. chrysogenum* in the mix 4 (Table 5.5), indicating that no false negatives were obtained. No positive Luminex signal was obtained for any coupled bead sets for which no corresponding specific gDNA was added to the mix (SN ratios ranged between 0.92 ±0.20 and 2.62 ±0.25) (Table 5.5). This means that for the 6 repetitions, no false positives were observed during this test.

Sensitivity: Limit of detection

The sensitivity of this Luminex® assay was determined with 9 points of serial dilutions (from 10 to 0.001 ng of gDNA for each of the targeted species). The LOD was 0.05 ng for *A. alternata, A. creber, A. sydowii, A. fumigatus, C. herbarum, P. chrysogenum* and *S. chartarum* and 0.01 ng for *A. versicolor, C. cladosporioides* and *U. botritys* (Table 5.6).

Table 5.5: DNA Mixes analysis

Species	BCCM/IHEM strain [a]	NTC [b]	Number of positives [c]									
			Positive control [e]	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8	Mix 9
A.alternata	3320	0/6	6/6	6/6	0/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
A. versicolor	18884	0/6	6/6	6/6	6/6	0/6	6/6	6/6	6/6	6/6	6/6	6/6
A. fumigatus	3562	0/6	6/6	6/6	6/6	6/6	0/6	6/6	6/6	6/6	6/6	6/6
C. cladosporioides	859	0/6	6/6	6/6	6/6	6/6	6/6	0/6	6/6	6/6	6/6	6/6
C. herbarum	2268	0/6	6/6	6/6	6/6	6/6	6/6	6/6	0/6	6/6	6/6	6/6
P. chrysogenum	20859	0/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	0/6	6/6	6/6
S. chartarum	359	0/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	0/6	6/6
U. botrytis	328	0/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	0/6

Species	BCCM/IHEM strain [a]	NTC [b]	SN ratio ± SD [d,e] Positive control [e]	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8	Mix 9
A.alternata	3320	ND [f]	6.98 ±0.83	7.73 ±1.95	1.20 ±0.35	5.75 ±1.36	6.18 ±1.17	8.49 ±0.14	5.39 ±0.33	6.82 ±0.89	4.90 ±0.17	5.40 ±0.32
A. versicolor	18884	ND	17.82 ±0.63	16.98 ±2.85	13.88 ±0.04	1.23 ±0.03	18.83 ±0.37	18.29 ±0.06	17.34 ±0.29	10.93 ±0.54	17.35 ±1.31	15.52 ±0.57
A. fumigatus	3562	ND	4.31 ±0.92*	4.69 ±0.68	4.27 ±0.09	4.27 ±0.19	2.43 ±0.33	5.08 ±1.67*	4.06 ±0.63*	3.47 ±0.34*	4.58 ±0.17	5.85 ±1.02
C. cladosporioides	859	ND	10.04 ±0.74	5.40 ±0.15	5.52 ±1.65	10.08 ± 2.49	3.81 ±0.69*	1.81 ±0.63	5.39 ±0.46	8.72 ±1.03	5.85 ±1.69	7.33 ±0.96
C. herbarum	2268	ND	6.39 ±0.23	6.56 ±0.25	5.39 ±1.23	5.26 ±0.09	20.55 ±0.63	8.72 ±0.26	2.62 ±0.25	5.98 ±1.79	4.12 ±0.23	7.33 ±1.23
P. chrysogenum	20859	ND	6.66 ±1.26	7.69 ±1.48	4.39 ±0.02	4.07 ± 0.27*	26.10 ±1.37	9.01 ±1.97	4.85 ±0.26	0.92 ±0.20	9.68 ±1.25	8.41±2.01
S. chartarum	359	ND	7.04 ±0.75	6.54 ±1.53	5.87 ±0.01	5.87 ±1.21	4.41 ±0.82*	6.16 ±1.30	4.46 ±0.45	3.37 ±0.25*	1.77 ±0.41	4.42 ±0.10
U. botrytis	328	ND	8.90 ±1.02	8.16 ±1.90	8.31 ±0.21	7.32 ±1.62	6.02 ±0.54	9.18 ±0.19	5.31 ±0.64	7.53 ±1.38	5.06 ±0.29	1.81 ±0.05

[a] Identification number as defined by the BCCM/IHEM collection, Mycology and Aerobiology, Scientific Institute for Public Health, Juliette Wytsman street 14, 1050 Brussels, Belgium

[b] No Template control

[c] Number of positive detections obtained during 3 independent runs done in duplicate

[d] SN ratio defined as the average (± standard deviation) of the ratio between the MFI values for the sample and the NTC for a specific target, obtained with 3 independents runs of independent gDNA extracts of pure cultures (5 ng of gDNA). * indicates the significance of each result obtained during a t-test (confidence 95 %) performed between the SN ratios obtained for the mix and the negative control mix of the targeted species (in bold).

[e] In bold are the values considered as negative (i.e., average SN ratio <3.00)

[f] ND defined as not detected

Table 5.6: Limit of detection a of the fungal Luminex® assay

Species	DNA amount (ng)									LOD (ng)
	10	5	1	0.5	0.1	0.05	0.01	0.005	0.001	
A. alternata	100[b] (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	83 (5/6)	50 (3/6)	0 (0/6)	0.05
A. creber	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	83 (5/6)	50 (3/6)	0 (0/6)	0.05
A. sydowii	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	67 (4/6)	50 (3/6)	0 (0/6)	0.05
A. versicolor	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	83 (5/6)	33 (2/6)	0.01
A. fumigatus	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	50 (3/6)	33 (2/6)	0 (0/6)	0.05
C. cladosporioides	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	83 (5/6)	17 (1/6)	0.01
C. herbarum	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	50 (3/6)	50 (3/6)	17 (1/6)	0.05
P. chrysogenum	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	83 (5/6)	17 (1/6)	0 (0/6)	0.05
S. chartarum	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	67 (4/6)	67 (4/6)	50 (3/6)	0.05
U. botritys	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	50 (3/6)	17 (1//6)	0.01

[a] Limit of detection (LOD) obtained in duplicate with 3 independents runs of independent gDNA extracts of pure cultures. In bold: results defining the LOD. [b] The % of positive results. The number of positive results is indicated between brackets.

5.3.3 Proof of concept using environmental samples

Following the performance assessment, DNA extracted from environmental indoor air samples collected from different rooms inside a contaminated house was analyzed using the fungal Luminex assay. This gave the opportunity to test the molecular tool with real-life samples containing a mix of a priori unknown fungal species at unknown concentration. Therefore, in this study, the proof of concept using environmental samples allows not only to test the performance (detection of DNA at very low or variable concentrations, detection in a mix of species, etc.) of the developed tool but also to demonstrate that the developed tool can be used with uncharacterized strains (i.e., strains not coming from a culture collection) of targeted species which could be present in an environmental air sample.

The 4 samples used in this study were previously analyzed with classical methods (Libert et al. 2015) and only 3 species were determined i.e., *A. versicolor*, *Cladosporium* sp. and *P. chrysogenum*. *P. chrysogenum* was observed in each sample, while *A. versicolor* was retrieved in 2 of them (i.e., samples from bathroom and living room) and *C. cladosporioides* in sample 4 (i.e., sample collected in the bathroom) only. Infertile mycelia were also observed in the living room and the kitchen (Table 5.7a).

These 3 species were also detected in the same rooms by the fungal Luminex assay performed on the extracted DNA from these 4 real-life samples (Table 5.7b). SN ratios obtained for each species were 3.84 ± 0.18 for *C. cladosporioides*, 3.67 ±0.04 and 3.34 ±0.05 for *A. versicolor*, respectively in the bathroom and the living room and ranged between 3.77 ± 0.09 in the kitchen and 14.83 ±0.22 for *P. chrysogenum* in the bathroom (Table 5.7b). No other species were detected (Table 5.7b).

In order to verify that no inhibition occurred during the analysis of the environmental samples, and to verify that if a species would have been present, it could be detected in the 4 environmental samples, a spike of all species not detected by the Luminex® assay was performed into every environmental sample, according to the results obtained for the first part of the proof-of-concept analysis. So, according to the results obtained for the analysis of the 4 environmental samples, *A. versicolor* was spiked into the samples 1 and 2, but not into samples 3 and 4. *C. cladosporioides* was spiked into the samples 1 to 3 and not in sample 4. Finally, as it was detected in each sample, no spike of *P. chrysogenum* was performed. Every gDNA spiked into the 4 samples was properly detected (Table 5.7b). Indeed, in the 4 samples each species, spiked or not (as already present), was detected (SN ratio ≥3.00).

Table 5.7a: Proof of concept with environmental samples: Culture, microscopic determination and quantification [a]

Sampling place	Species	Number of colonies on plate	CFU/m³
Bedroom	infertile mycelium	4	50
	P. chrysogenum	17	213
Kitchen	*P. chrysogenum*	6	75
	infertile mycelium	3	38
Living room	*A. versicolor*	1	13
	P. chrysogenum	18	225
Bathroom	*A. versicolor*	1	13
	Cladosporium sp.	1	13
	P. chrysogenum	15	188

[a] The value for CFU/m³ is an estimation of fungal contamination based on the number of colonies per plate.

Table 5.7b: Proof of concept with environmental samples: Luminex xMAP® analysis

Species	Control [a,b,d]	Environmental sample [d]				Spike test [c, d]			
		1 Bedroom	2 Kitchen	3 Bathroom	4 Living room	Spike 1 (bedroom)	Spike 2 (kitchen)	Spike 3 (bathroom)	Spike 4 (living room)
A.alternata	4.16 ±0.68*	0.72 ±0.17	0.98 ±0.53	1.41 ±0.09	1.12 ±0.01	3.74 ±0.10*	4.29 ±0.09*	3.26 ±0.26*	4.03 ±0.57*
A. versicolor	4.22 ±0.22	0.94 ±0.16	1.91 ±0.53	3.67 ±0.04*	3.34 ±0.05*	4.91 ±0.55*	4.09 ±0.71*	*3.78 ±0.27**	*3.18 ±0.12**
A. fumigatus	3.41 ±0.14*	2.03 ±0.62	2.16 ±0.35	1.39 ±0.03	1.72 ±0.49	4.18 ±0.52*	3.69 ±0.21*	3.08 ±0.35 *	3.05 ±0.39*
C. cladosporioides	3.44 ±0.04*	0.72 ±0.20	0.76 ±0.25	3.84 ±0.18*	0.88 ±0.08	3.57 ±0.16*	3.58 ±0.17*	3.60 ±0.14*	*3.23 ±1.02**
C. herbarum	3.01 ±0.06*	0.62 ±0.17	0.71 ±0.22	1.59 ±0.62	1.11 ±0.01	4.12 ±0.76*	3.93 ±0.50*	4.10 ±0.28*	3.83 ±0.15*
P. chrysogenum	3.00 ±0.09*	7.77 ±0.22	3.77 ±0.09	14.83 ±0.22	12.27 ±0.23	*9.40 ±2.46*	*3.60 ±0.11*	*3.64 ±0.01*	*4.47 ±1.39*
S. chartarum	4.82 ±0.02	0.64 ±0.16	0.60 ±0.16	1.54 ±0.16	1.43 ±0.04	9.41 ±2.46*	7.09 ±0.07*	4.81 ±1.51*	3.99 ±0.66*
U. botrytis	8.68 ±0.52*	0.60 ±0.13	0.36 ±0.13	1.05 ±0.17	1.24 ±0.48	6.15 ±0.38*	4.92 ±1.13*	4.61 ±0.70*	3.70 ±0.99*

[a] BCCM/IHEM 3320 for *A. alternata*, BCCM/IHEM 18884 for *A. versicolor*, BCCM/IHEM 3562 for *A. fumigatus*, BCMM/IHEM 859 for *C. cladosporioides*, BCCM/IHEM 2268 for *C. herbarum*, BCCM/IHEM 20859 for *P. chrysogenum*, BCCM/IHEM 359 for *S. chartarum* and BCCM/IHEM 328 for *U. brotytis*.BCCM/IHEM collection, Mycology and Aerobiology, Scientific Institute for Public Health, Juliette Wytsman street 14, 1050 Brussels, Belgium

[b] SN ratio defined as the average (± standard deviation) of the ratio between the MFI values for the sample and the NTC for a specific target, obtained with 3 independents runs of independent gDNA extracts of pure cultures (5 ng of gDNA). In bold are the values considered as positive (i.e., average SN ratio ≥3.00). * indicates the significance of each SN ratio close to the limit (i.e., 3) was evaluated with t-test (confidence 95%) performed with the higher negative results considered as a worst negative control.

[c] In each air sample, 5 ng DNA extracted from each strain not detected in the indoor samples were spiked into the DNA extracted from the air samples. The SN ratios of each species detected in air samples (not spiked) were put in italic. DNA used for the spike comes from the same strains than those used as positive control.

[d] In bold are the values considered as positive (i.e., average SN ratio ≥3.00)

5.4 Discussion

For years, fungal indoor air contamination is considered as a public health problem, even if today no substantiated scientific evidence on the causal link exists. This lack of evidence is principally due to a scarcity of data on the full composition of indoor airborne fungal community. Indeed, most of the protocols used today for fungal contamination monitoring are culture-dependent and revealed some limitations, such as the non-representativeness of the full indoor fungal community or of being time-consuming. To improve the collection of data in terms of time and completeness, molecular methods, such as qPCR, have been developed for the detection of the indoor fungal community. However, even if the efficiency of most of these qPCR methods has been well established, their multiplex capacities are still too limited. With its ability to simultaneously analyze up to 50 different targets, the use of the Luminex xMAP® technology using a MagPix instrument could significantly upgrade the indoor fungal contamination monitoring as was previously demonstrated for the diagnosis of some relevant fungal and other pathogens from clinical samples (Christopher-Hennings et al. 2013; Dunbar et al. 2011; Etienne et al. 2009; Landlinger et al. 2009; Wuyst et al. 2015; Lin et al. 2011).

This study presents the first Luminex xMAP® assay developed for the monitoring of 10 fungal species most frequently found in indoor air and that may cause health problems, i.e., *A. alternata*, *A. creber*, *A. fumigatus*, *A. sydowii*, *A. versicolor*, *C. cladosporioides*, *C. herbarum*, *P. chrysogenum*, *S. chartarum* and *U.* botrytis (Beguin and Nolard 1994; Bellanger et al. 2009; Shelton et al. 2002). While *A. creber*, *A. versicolor*, *A. sydowii* and *P. chrysogenum* are typical indoor species, *A. alternata*, *A. fumigatus* and *C. herbarum* are known to be outdoor species. Despite their outdoor sources, these 3 species are commonly detected in indoor air samples, especially in the forms of spores, and they arrive in indoor environment through the draft and ventilation system (i.e., windows, ventilation or air-conditioning system).

Some of these species such as *A. alternata*, *A. versicolor*, *P. chrysogenum*, *U. botrytis* or *S. chartarum* are known to have implications in the worsening of respiratory diseases or allergies (Crameri et al. 2011; Denning et al. 2006; Knusten et al. 2011; Mendell et al. 2011; Piecková et al. 2004; Rosembaum et al. 2010). The detection of these multiple species is therefore important in the context of health issues. The Luminex xMAP® technology using different sets of beads, characterized by a specific spectral signature, gives the opportunity to perform in a single run a multiplex analysis able to detect multiple species with high specificity, defined itself by the specificity of the probes bound to the each set of beads.

To have a species-specific detection, the probes were designed to target the ITS-1 or ITS-2 regions from the ribosomal DNA, which are recognized as the most suitable region for the detection of fungi due to their low intra-species variablility (Nilsson et al. 2008; Schoch et al. 2012). There exist universal primer pairs (i.e., ITS1/2 (and ITS 3/4) (White et al. 1990) to amplify these regions in all fungi. Therefore, targeting of ITS-1 and ITS-2 also allowed to easily design a multiplex detection based on species-specific probes. Indeed, the targeting of the ITS regions and the use of one set of universal primers for each region allow to reduce the number of step and so, the time and amount of sample needed for the generation of a species-specific amplicon i.e., PCR amplifications could be done in a duplex PCR reaction with universal primers for fungal gDNA amplification (primers ITS1, 2, 3 and 4). Due to their universal character, the use of these primers also gives the possibility to extend the panel of species to be detected in the future, by adding specific probe-coupled bead sets, and so, to improve the powerful fungal monitoring based on this xMAP® assay. Indeed, the specific detection of the generated amplicons is based on the hybridization with specific probes coupled to Luminex bead-sets. Every probe used in this study was found in literature (Table 5.1) and developed at first for a TaqMan® qPCR method (Etienne et al. 2009; United States Environmental Protection Agency 2016). So, the specificity of each probe used in this study was previously validated and published. According to the Luminex' recommandations, the optimal probe length used for an xMAP® analysis is defined between 15 and 20 nucleotides (Dunbar et al. 2006). However, all probes used in this study, obtained from literature, are larger than 20 nucleotides. For most of them, according to the *in silico* analysis, their specificity was negatively impacted by a size reduction. Therefore, in this study the length defined for the best detection of each species targeted in our mulitplex assays was found to be around 30 nucleotides. To optimize the TMAC-based hybridization step, which is probe-size dependent, all the probes were adapted to have a length between 28 and 31 nuclotides.

The current assay showed a specific detection of each targeted species, as observed in the simplex and multiplex assays. The species-specific detection was further demonstrated with the DNA mixes test. The remark has to be made that the developed fungal Luminex® assay targets 10 different fungal species. Seven of them are detected uniquely with specific probes; the 3 remaining species (i.e., *A. creber*, *A. sydowii* and *A. versicolor*) are detected by the same probe. Therefore, no false negative nor false positives were observed, except for the probe VersP1 which detects the closely related species *A. versicolor*, *A. sydowii* and *A. creber*. The poor discrimination between these 3 *Versicolores* (*A. creber*, *A. sydowii* and *A. versicolor*) species was already observed in other studies (Haugland et al. 2004; Libert et al. 2015). To improve the specific identification of these 3 species, an additional marker could be added such as the gene coding for β-tubulin or mycotoxin genes (Etienne et al. 2009; Lezar et al. 2010; Nilsson et al. 2008; Schoch et al. 2012). However, the use of a new marker requires the optimization of the PCR workflow (multiplex

optimization or addition of an amplification step) and probably of the hybridization temperature in the xMAP® workflow, depending on the probe length. Indeed, with the use of TMAC, the specificity of the annealing depends only on the hybridization temperature, and not on the composition of the probe. So, to avoid any problem of specificity, adding markers (and thus probes) to a workflow will require the optimization of this parameter. When using multiple markers to target closely related species such as the 3 *Versicolores* in this study, a decision-tree-based workflow could be applied after the Luminex assay to perform the discrimination based on the combination of the signals obtained for each marker. With such a decision-tree-based workflow, the xMAP® assay developed in this study could already be used as a first screening without modifications. If a positive result is observed for the VesP1 probe, a second analysis could be performed with some new markers in order to identify the species. This kind of decision tree already exists in other fields e.g., for the detection of genetically modified plants (Van den Bulcke et al. 2010). Alternatively, another molecuar assay could be used, e.g. based on high resolution melting qPCR for the discrimination of closely related species (Libert et al. submitted). Being able to discriminate between these 3 species will contribute to our understanding of the impact of indoor fungal contamination and health problems, as currently, the presence of these 3 *Aspergillus* species in indoor air and their difference in impact on health could not yet be evaluated.

In addition to its specificity, the xMAP® assay developed in this study is also sensitive with a LOD ranging between 0.05 and 0.01 ng of gDNA. According to the limited information available on the genome size of each targeted species, these LOD correspond to a range from 2.51 theoretical genomic copy numbers for *C. cladosporioides* and *U. botritys* to 19.84 theoretical genomic copy numbers for *A. fumigatus* (Table 5.8). It should be noted that intraspecies gDNA copy number variations observed in some fungal species such as *A. alternata, A. fumigatus, A. verscicolor* or *C. cladopsorioides* could affect the LOD (Black et al. 2013; Herrera et al. 2009; Johnson et al. 2012; Libert et al. 2015), thereby impeding the theoretical genomic copy number variation estimation. Therefore, it has been decided to determine the LOD in mass, instead of in genomic copy numbers.

The proof of concept using environmental samples showed that the xMAP® technology can be used for the fungal monitoring in indoor environment. Indeed, the 3 species detected with the classical methods (i.e., *A. versicolor, C. cladosporioides* and *P. chrysogenum*) were also detected with the xMAP® technology. Also, the species not detected with the classical protocol were not detected with the xMAP assay. This test indicates that our Luminex assay can be used on mixed and unbalanced concentrations of fungal species without giving the problem of false detection. This observation is supported by the spike test results which showed that the species present in the

gDNA extracted from the environmental samples (not spiked) could still be detected even when a high amount of spiked DNA of other species was present. The spike test results also demonstrated that no inhibitors were present in the DNA mix, as the spiked DNA could be detected by the Luminex assay performed on the environmental samples. If the species would have been present in the environmental sample, it would have been detected by the Luminex® assay. So, if the species was not detected, it was not present in the environmental samples, or it was present at a concentration below the LOD. According to the observations based on culturing, besides *P. chrysogenum,* also some infertile mycelia were observed with classical analysis in samples from the kitchen and the bedroom while the xMAP® assay only detected *P. chrysogenum* (Tables 5.7a,b). According to these results and those from the spike test, these undetermined taxa do not belong to the targeted species, except for *P. chrysogenum*. As no DNA sequencing was performed on the infertile mycelia observed on plate, we cannot exclude that these infertile mycelia belong to an untargeted species of our xMAP® assay.

Table 5.8: Theoretical genomic copy number estimation of the LOD

Species	Genome size (Mb)	Reference	LOD	
			DNA amout (ng)	CN estimation [a]
A. alternata	32.99	47	0.05	14.06
A. creber	33.76 [b]	47	0.05	13.74
A. fumigatus	29.39	47	0.05	19.84
A. sydowii	34.38	47	0.05	13.49
A. versicolor	33.13	47	0.01	2.80
C. cladosporioides	36.91[c]	48	0.01	2.51
C. herbarum	36.91 [c]	48	0.05	12.57
P. chrysogenum	31.34	47	0.05	14.80
S. chartarum	40 [d]	49	0.05	11.60
U. botritys	36.91 [c]	48	0.01	2.51

[a] CN estimation defined as an estimation of the theoretical gDNA copy number (CN).
[b] No sequencing data available; genome size obtained as an average of the genome size from the other species from the Versicolores group i.e., *A. sydowii* and *A. versicolor.*
[c] No sequencing data available ; genome size considered as general estimation of the Ascomycota genome size.
[d] Genome size estimation based on the whole genome sequencing of the environmental strain *S. chartarum* 51-11.

However, if needed, probes for additional taxa, once determined, can be easily added to the assay, as elaborated above. Additionally, a 'general ITS' probe, detecting all fungal species, could be added to the assay. If none of the specific probes give a Luminex signal, but there is fungal DNA present in the sample (as detected by the general probe), other methodologies, including massive

parallel sequencing, could be applied to further characterize the sample, if needed. Nevertheless, this environmental test delivered the proof of concept for the use of the xMAP® technology, which is culture-independent and less time-consuming for the analysis of real-life samples i.e., 3 days for the xMAP® technology (sampling, DNA extraction included) compared to 5 to 21 days for the classical analysis (sampling and the culturing included).

To conclude, this study reported on a fast, specific and sensitive Luminex xMAP® assay targeting 10 important fungal contaminants frequently observed in indoor air and that could have health impacts. The use of the xMAP® technology allows a culture–independent analysis with a reduced turn-around-time compared to the classical protocols. The use of this molecular multiplex tool to investigate the indoor contamination could improve the monitoring of fungal diversity. The improvement of data on the fungal population in buildings will contribute to the knowledge concerning their impact on public health, especially on respiratory diseases.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 6

Exploiting the advantages of molecular tools for the monitoring of fungal indoor air contamination: First detection of *Exophiala jeanselmei* in indoor air

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Abstract

Today, indoor air pollution is considered a public health issue. Among the impacting pollutants, indoor airborne fungi are increasingly highlighted. Most of the monitoring protocols are culture-based, but these are unable to detect the uncultivable and/or dead fraction, even though this fraction could impact health. Among the contaminants suspected to be part of this fraction, *Exophiala jeanselmei* is an interesting case study. Known to be pathogenic, this black yeast grows in humid environments such as air-conditioning systems, where it has been previously detected using classical culture-based methods. However, until now, this fungus was never detected in indoor air.

This study shows the first detection of *E. jeanselmei* in indoor air collected from offices in contact with contaminated air-conditioning reservoirs. While its presence in indoor air could not be demonstrated with culture-based methods, it was found by qPCR and massive parallel sequencing. The latter also allowed obtaining a broader view on the fungal diversity in the tested samples. Similar approaches were applied on water samples collected from the conditioning reservoirs to trace the source of contamination. The comparison of results obtained with both methods confirmed that the molecular tools could improve indoor air monitoring, especially of dead and/or uncultivable contaminants.

Key words

Exophiala jeanselmei, air contamination, indoor, qPCR, NGS, detection, public health

Practical information

Indoor air fungal contamination monitoring is still frequently based on culture and microscopic identification. Consequently, based on these classical techniques, the dead and/or uncultivable fraction present in the indoor environment is insufficiently investigated. However, this dead and/or uncultivable fraction could have an impact on public health, independently of their viability. This study shows that the use of the biological air sampler Coriolis® μ combined with molecular tools such as the real-time polymerase chain reaction and/or massive parallel sequencing permits to detect uncultivable and/or dead fungal contaminants in indoor air such as Exophiala jeanselmei, which was for the first time detected in indoor air samples.

6.1 Introduction

Today, as most of their time is spent inside homes, offices and other workplaces, people are increasingly impacted by a low indoor air quality. Among the different indoor pollutants inventoried, fungi and molds appear to be important airborne contaminants in workplaces, houses and buildings and they are suspected to have an impact on health, especially on respiratory diseases such as allergy, asthma, asthma exacerbation or rhinitis. In this way, indoor fungal contamination is more and more considered as a public health issue by the scientific community (Bellanger et al. 2009; Dannemiller et al. 2016; Douwes et al. 2003; de Ana et al. 2006; Khan and Karuppayil et al. 2012; Mendell et al. 2011; Packeu et al. 2012a; Piecková et al. 2004; Meheust et al. 2014; Sharpe et al. 2014; Portnoy et al. 2014) and by preventive health care actors such as environmental agencies (European Environment Agency 2016; United States Environmental Protection Agency 2016) or the World Health Organization (World Health Organization 2009).

These fungal contaminants could originate from different sources, including from the outdoor via windows, aeration and ventilation such as is the case for *Alternaria alternata* and *Cladosporium* (Adams et al. 2013; Horner et al. 2004; Kelkar et al. 2004; Ponsoni and Gonçalves Raddi 2010; Shelton et al. 2002) species or could occur in water contained in reservoirs air-conditioning systems. One such example is *Exophiala jeanselmei*, a black yeast known as a pathogenic species associated amongst others with cutaneous infections, subcutaneous cysts, systemic and nosocomial infections (Nucci et al. 2001; Nucci et al. 2002; Wang et al. 2001; Zeng et al. 2007). This species, living in humid and oligotrophic environments, is observed in feed, sludge and stagnant water. In indoor environments, *E. jeanselmei* is currently detected in sludge pipings, water systems (pipes, bathroom, reservoir, sauna …) and water reservoirs of air-conditioning systems (Badali et al. 2012; Nishimura et al. 1987; Nucci et al. 2002; Sterflinger et al. 2009). For some other species, it has been postulated that the way of indoor air contamination is linked to these water reservoirs of air-conditioning units or pipings (Kelkar et al. 2005). However, although *E. jeanselmei* can be detected in humid indoor environments (e. g., water samples taken from water reservoirs of air-conditioning systems), it has never been found before in indoor air. It can be hypothesized that this is due to the disadvantages of the currently used monitoring methods.

Indeed, routine protocols for indoor air fungal monitoring are still based on culture, colony counting and microscopic visualization. Therefore, the results of these methods are dependent on the culture conditions such as the selection of the medium, species competition, growth conditions and differences in terms of incubation time (Pitkaranta et al. 2011; Vesper 2011). Moreover, culture-based protocols are not able to detect the uncultivable and/or dead fraction (i.e., fragments of mycelia or cell walls, dead cells…) which could be airborne and so, collected during the

sampling (Pitkaranta et al. 2011; Vesper 2011). However, this fraction could have an impact on health and should therefore also be monitored; otherwise, important elements are missing to establish the scientific link between fungal airborne contamination and health problems. This could especially be true for *E. jeanselmei*. The monitoring of this fungus in water samples is complicated, as it needs up to 21 days to grow in culture (Libert et al. 2016, Nolard et al. 2004), while being highly demanding in terms of culture conditions, i.e. growth medium chosen, competition between species, temperature and humidity. Especially this last factor could impede the detection of *E. jeanselmei* in indoor air, as the fact that it might occur in desiccated form will prevent its cultivation, and hence its detection, using the classical monitoring procedures.

The use of molecular methods such as the polymerase chain reaction (PCR), quantitative real-time PCR (qPCR) and sequencing could offer a solution to investigate the indoor airborne fungal community, including this uncultivable and/or dead fraction (Haugland et al. 2004; Hospodsky et al. 2010; Michealsen et al. 2006; Pitkaranta et al. 2011; Rebroux et al. 2009; Timothy et al. 2004; Vesper 2011). We have previously developed a qPCR-based assay for the detection of *E. jeanselmei* in water samples (Libert et al. 2016). In the present study, we have tried this method on air samples collected with the Coriolis® μ air sampler from different offices in contact with air-conditioning reservoirs contaminated with *E. jeanselmei*. The aim was to investigate the hypothesis that this species is occurring in indoor air as uncultivable and/or dead fraction, i.e. that it is not detectable with the classical monitoring methods based on culturing, but that it can be detected using molecular, i.e. DNA-based, technologies.

To demonstrate that massive parallel sequencing could improve our insight in the fraction of uncultivable fungi, the qPCR analysis was complemented with massive parallel sequencing of the DNA extracted from the air and water samples. Our results, with *E. jeanselmei* as a case study, not only demonstrated for the first time the presence of *E. jeanselmei* in air samples, but it also delivered a proof-of-concept for the added value of the use of molecular tools to monitor indoor airborne fungal contamination, including the dead and/or uncultivable fraction.

6.2 Material and methods

6.2.1 Sampling

All the samples (i.e., air and water samples) were collected between February 12th and March 13th, 2015.

The reservoir of water from 5 air-conditioning systems (Table 6.1), for which previous monitoring results had pointed to *E. jeanselmei* contamination, was investigated using the sampling protocol of water from air-conditioning reservoirs previously described (Nolard et al. 2004). In short, 1 L of water situated between 1 cm and 5 cm above the bottom of the water-reservoir situated in the pulse group (PG, i.e. the part of the air-conditioning system containing the machinery to pulse the air and the water-reservoir needed to maintain the required hygrometry level) was put into a sterile Duran bottle. All samples were kept at 4 °C until their analysis.

In addition to the water sampling, an air sampling was done in the offices connected to the 5 air-conditioning systems of which water samples were collected. For each of the air-conditioning systems, 3 offices were selected based on their distance to the PG i.e., the closest and most distantly located office relative to the PG and one office in between the two other offices. Each sample was taken in duplicate. The indoor air samples were collected with a Coriolis® μ air sampler following the sampling protocol reported by Libert and colleagues in 2015 (Libert et al. 2015).

Table 6.1: Detection of *E. jeanselmei* in indoor air and water in air-conditioning systems: Classical analysis and qPCR

Sample ID	Pulse group	Office	Sample type	Analysis method		
				Culture	qPCR	
				CFU/ml [1]	T_m (°C) [2]	C_q [3]
PG1 [4]	1		Water [5]	0	N/A	N/A
PG1-1	1	1	Air	0	N/A	N/A
PG1-2	1	2	Air	0	N/A	N/A
PG1-3	1	3	Air	0	N/A	N/A
PG2	2		Water [5]	10	79.53 ±0.12	28.00 ±1.35
PG2-1	2	1	Air	0	N/A	N/A
PG2-2	2	2	Air	0	N/A	N/A
PG2-3	2	3	Air	0	N/A	N/A
PG3	3		Water [5]	10	79.79 ±0.32	28.39 ±0.93
PG3-1	3	1	Air	0	N/A	N/A
PG3-2	3	2	Air	0	N/A	N/A
PG3-3	3	3	Air	0	N/A	N/A
PG4	4		Water [5]	100	79.93 ±0.05	20.32 ±1.98
PG4-1	4	1	Air	0	79.44 ±0.10	23.79 ±0.92
PG4-2	4	2	Air	0	79.49 ±0.03	24.49 ±0.23
PG4-3	4	3	Air	0	79.38 ±0.03	34.05 ±1.75
PG5	4		Water [5]	15	79.34 ±0.23	27.36 ±1.12
PG5-1	5	1	Air	0	79.39 ±0.02	27.62 ±0.63
PG5-2	5	2	Air	0	N/A	N/A
PG5-3	5	3	Air	0	N/A	N/A

[1] CFU/ml defined as the number of colonies of *E. jeanselmei* on plate after 21 days of incubation at 37 °C (Nolard et al. 2004)
[2] T_m defined as the average of the melting temperature (°C) observed for each qPCR amplicon obtained during the qPCR SYBR®Green analysis performed in duplicate in 2 independent runs.
[3] C_q defined as the average quantification cycle observed for each qPCR amplicon obtained during the qPCR SYBR®Green analysis performed in duplicate in 2 independent runs.
[4] PG1 was maintained before the sampling
[5] One sample per pulse group was analyzed
[4] Percentage obtained as the ratio between the number of reads grouped into the 10 OTUs and the total number of reads observed for each sample
[5] Number of reads corresponding to OTU identified as *E. jeanselmei*
[6] Percentage obtained as the ratio between the number of reads corresponding to the *E. jeanselmei* OTU and the total number of clustered reads
[7] PG1 maintained before the sampling
[8] One sample per pulse group was analyzed

6.2.2 Classical analysis

The protocol used for the classical analysis (i.e., culture and fungal determination) of the water and air samples, collected with the Coriolis® μ air sampler, has been previously described (Libert et al. 2015; Libert et al. 2016; Nolard et al. 2004). According to the protocols, the incubation time ranged between 5 and 21 days as recommended for the detection of major indoor air species and for the detection of *E. jeanselmei.* After 5 days of incubation the plate was analyzed as it was recommended for the detection of indoor air species. After this microscopic analysis, the plate was re-incubated during 16 days (total incubation time 21 days) as it is recommended for the detection of *E. jeanselmei.*

6.2.3 DNA extraction

The protocol used for the DNA extraction from water (Libert et al. 2016) and indoor air samples (Libert et al. 2015) was previously described. Genomic DNA amount and purity were evaluated with a Nanodrop® 2000 (Thermo Scientific, Wilmington, USA).

6.2.4 qPCR screening

The analyses of DNA extracted from air and water samples were performed in duplicate in 2 independent runs. The qPCR assay used was the SYBR®green *Ejeanselmei_ITS* assay earlier reported for the detection of *E. jeanselmei* in water (Libert et al. 2016). This assay uses the SYBR® green PCR Mastermix (Diagenode, Liège, Belgium). The sequences of the primers used (targeting the internal transcribed spacer region (ITS)) are provided by Libert and colleagues (Libert et al. 2016). The primers were purchased from Eurogentec (Liège, Belgium). Each run was done on a CFX Touch™ Real-Time PCR detection System equipped with CFX Manager software V. 2 (Biorad, Temse, Belgium) and was carried out with 5 µl of a positive control (PC) corresponding to 200 theoretical copies of gDNA of *E. jeanselmei* strain BCCM/IHEM 4740 purchased from the BCCM/IHEM collection of the Scientific Institute of Public Health in Brussels (Belgium). A “no template control” (NTC), i.e. using Gibco® DNase, RNase, Protease free pure water (Life Technologies, Gent, Belgium) as template, was also added in order to verify that no contamination occurred.

In order to confirm the identity of each qPCR amplicon, a Sanger sequencing analysis was performed on an ABI3130xl Genetic Analyzer apparatus (Applied Biosystem, Life Technologies, Gent, Belgium) with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer’s instructions. Each sequence was identified by comparison to the sequences available in the NCBI database using the BLASTn tool (http://blast.ncbi.nlm.nih.gov/).

6.2.5 Massive parallel sequencing

To confirm the identity of qPCR amplicons, but also to demonstrate that massive parallel sequencing could be useful for the investigation of uncultivable and dead species present in the air and water samples, all the samples were sequenced using an Illumina MiSeq instrument.

Because a massive parallel sequencing analysis using the Illumina MiSeq with a MiSeq reagent kit v3 reaches its optimal performance with fragments no longer than 600 bp and because the fungal ITS region has a length around 800 bp, the PCR amplicons for library preparation were prepared for both the ITS-1 and ITS-2 region using DNA extracted from different environmental (water and air) samples. Hereto, amplification with the universal forward (f) and reverse (r) primers ITS1f/ITS2r (White et al. 1990) for the amplification of the ITS-1 region and with the universal primers ITS3f/ITS4r (White et al. 1990) for the ITS-2 region was performed. The primers used were extended with the Illumina sequences needed for PCR-based library preparation. The PCR-reaction mix (25 µl final volume) contained 2.5 µlof high fidelity PCR buffer (10X), 0.5 µL of a mix of 0.2 mM of each dNTP, 0.5 µM of each primer, 0.1 µl of Platinum® Taq DNA Polymerase high fidelity enzyme (5 U/µl) (ThermoFischer Scientific, Life Technology, Gent, Belgium) and 15.90 µl of Gibco® DNase, RNase, Protease free pure water (Life Technologies, Gent, Belgium). At the end, 5 µl of gDNA (10 ng) were added. Each run was performed with following PCR protocol i.e., 1 cycle at 94 °C for 3 min, 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C and 1 min at 72 °C and at last, 10 min at 72 °C.

After the PCR-reaction, all samples were purified with the AMpure® XP PCR purification kit (Agencourt Biosciences corporation, Beverly, USA). The quality and amount of PCR fragments for massive parallel sequencing was verified on a Bioanalyzer 2100 (Agilent Technologies, Amstelveen, the Netherlands). ITS-1 and ITS-2 amplicons from a same sample were mixed together. Equimolar mixes were made according to the size of the peaks observed during the Bioanalyzer analysis. Sequencing was performed by BaseClear (Venlo, The Netherlands) with an Illumina MiSeq, yielding 2 x 300 bp paired-end reads.

6.2.6 Bio-informatics analysis

The FASTQ sequence files were generated using the Illumina Casava pipeline version 1.8.3 (BaseClear, Venlo, the Netherlands). The first quality assessment was based on the Illumina Chastity filtering. Reads with PhiX control signal and/or short reads below 20 bp (after adapter clipping) were removed using an in-house filtering protocol from BaseClear. Then, a second quality assessment control was done using the tool PRINSEQ-lite quality control v.0.20.4. Finally, an average quality score per sample was obtained with FastQC v.010.1. (Andrews et al. 2010). The sequences which did not pass this quality control were removed for the remainder of the

analysis.

To determine the presence of *E. jeanselmei* and other fungal species in indoor air and water samples, sequencing reads were analyzed with an operational taxonomic unit (OTU) classification approach with the Microbial Genomics Module from the CLC Genomic Workbench V8 software (Qiagen Benelux, B.V., KJ Venlo, the Netherlands). Briefly, in order to have comparable read lengths and to remove reads with low coverage, the paired-end reads were firstly treated with 3 different tools i.e., the "adapter trimming", the "fixed length trimming" and the "filter samples based on the number of reads". Thus, at the end of the workflow, the OTU clustering was performed only on reads with the same length and a low level of bias.

The OTUs clustering was made with the OTUs clustering tool. The database used as reference was the fungal rDNA sequences Database UNITE V7.1. (last updated January 31th, 2016) (Kõljag et al. 2005). Finally, all the OTUs were aligned with the MUSCLE tool and a Neighbor Joining tree was constructed according to the Jukes Cantor nucleotides model.

6.3 Results

6.3.1 Classical analysis by culturing

The sampling of the offices and water reservoirs of the air-conditioning systems was performed as elaborated in Materials and Methods. The water and indoor air samples were both incubated on plate in order to detect *E. jeanselmei*, as outlined in the classical monitoring procedures.

For the 5 water samples collected from the pulse group (PG) of the air-conditioning systems, *E. jeanselmei* was detected in 4 of them (Table 6.1). Expressed in colony forming units per ml (CFU/ml), the range of contamination of the water samples was determined to be between 10 and 100 CFU/ml (Table 6.1). *E. jeanselmei* colonies were not observed for the sample collected from the PG 1 (Table 6.1). In addition, 4 other determined taxa and 3 infertile mycelium or undetermined species were detected on plate i.e., *Aspergillus fumigatus, Aspergillus puulaeunsis* and *Penicillium chrysogenum* (Table 6.2).

As it was done for the water samples, the indoor air samples collected in each office were incubated according to the protocol developed for the detection of *E.* jeanselmei (Libert et al. 2015; Libert et al. 2016; Nolard et al. 2004). As shown in Table 1a, after 21 days of incubation, no *E. jeanselmei* was detected on plate for any of the offices in contact with the air-conditioning system. However, 4 other determined taxa and 4 infertile mycelium or undetermined species were observed on plate i.e., *A. alternata*, *Aspergillus* sp., *A. versicolor* and *P. chrysogenum* (Table 6.3).

Table 6.2: Fungal contamination in water samples, other than *E. jeanselmei*: Classical analysis and sequencing analysis comparison

Sample	Classical analysis[1]		NGS	
	Species	CFU/ml	Species	Abundance of reads per OTU[2] (%)
PG1	*P. chrysogenum*	5	*P. chrysogenum*	100
PG2	*P. chrysogenum*	5	*P. chrysogenum*	60.64
	A. puulaaeunsis	1	*A. puulaaeunsis*	11.27
	Infertile mycelium	1		
	A. fumigatus	4	*A. fumigatus*	36.34
PG3	*P. chrysogenum*	5	*P. chrysogenum*	30.15
	Infertile mycelium	2		
	Undetermined species	1		
PG4[3]	*A. fumigatus*	2	*A. fumigatus*	12.19
PG5	*A. puulaaeunsis*	0	*A. puulaaeunsis*	11.86
	P. chrysogenum	3	*P. chrysogenum*	23.30

[1] CFU/ml defined as the number of colonies observed on plate after 5 days and 21 days of incubation at 37 °C (Nolard et al. 2004), *E. jeanselmei* excluded.
[2] The massive parallel data were analyzed with the CLC Genomic Workbench software (Qiagen Benelux, B.V., KJ Venlo, the Netherlands) and the Microbial Genomics Module. The database used as reference was the UNITE database (Kõljag et al. 2005), last update January 31th 2016
The PG4 contained *E. jeanselmei* only.

Table 6.3: Fungal contamination in air samples: Classical analysis and sequencing analysis comparison

Samples	Classical analysis[1]		NGS[2]	
	Species	CFU/ml	Species	Abundance of reads per OTU[2] (%)
PG1-1	*A. alternata*	5	*A. alternata*	21.86
	A. versicolor	3	*A. subversicolor*	11.49
	P. chrysogenum	9	*P. chrysogenum*	66.65
PG1-2	*A. alternata*	3	*A. alternata*	24.72
	Aspergillus sp.	2	*A. monodii*	7.70
	Infertile mycelium	1	*A. rugulosus*	4.43
	P. chrysogenum	7	*P. chrysogenum*	62.40
PG1-3	*A. alternata*	2	*A. alternata*	31.28
	Undetermined species	1	*A. monodii*	15.93
	Infertile mycelium	1	*A. rugulosus*	12.47
	P. chrysogenum	7	*P. chrysogenum*	39.58
PG2-1	Undetermined sp.	2	*E. olivicola*	16.22
	P. chrysogenum	6	*P. chrysogenum*	71.44
			E. undulata	12.35
PG2-2	*A. alternata*	2	*A. alternata*	24.59
	P. chrysogenum	6	*P. chrysogenum*	75.41
PG2-3	*A. alternata*	4	*A. alternata*	32.28
	A. versicolor	5	*A. subversicolor*	32.09
	Aspergillus sp.	1	*A. monodii*	24.26
	Infertile mycelium	1	*A. puulaaeunsis*	11.37
PG3-1	*A. alternata*	4	*A. alternata*	53.50
	P. chrysogenum	5	*P. chrysogenum*	46.50

Table 6.3: *Continued*

Samples	Classical analysis[1]		NGS[2]	
	Species	CFU/ml	Species	Abundance of reads per OTU[2] (%)
PG3-2	*A. alternata*	5	*A. alternata*	71.96
	Undetermined sp.	2	*E. olivicola*	16.41
			E. undulata	11.63
PG3-3	*Aspergillus* sp.	2	*A. monodii*	11.18
			A. rugulosus	19.56
			A. subversicolor	20.40
	P. chrysogenum	6	*P. chrysogenum*	48.85
PG4-1	*A. alternata*	1	*A. alternata*	26.18
	A. versicolor	1	Undertermined sp.	13.07
	Infertile mycelium	1		
PG4-2	*A. alternata*	3	*A. alternata*	17.48
	P. chrysogenum	9	*P. chrysogenum*	40.88
	Undetermined sp.			
PG4-3	*A. versicolor*	4	*A. subversicolor*	29.12
	Infertile mycelium	1	*A. puulaaeunsis*	24.38
			A. monodii	7.17

Table 6.3: *Continued*

Samples	Classical analysis[1]		NGS[2]	
	Species	CFU/ml	Species	Abundance of reads per OTU[2] (%)
PG5-1	*P. chrysogenum*	7	*P. chrysogenum*	64.57
	Undetermined sp.	1	*E. undulata*	13.98
	Infertile mycelium	1	*E. olivicola*	10.46
PG5-2	*A. alternata*	4	*A. alternata*	31.33
	A. versicolor	4	*A. subversicolor*	32.55
			A. puulaaeunsis	36.12
	Infertile mycelium	2		
PG5-3	*A. versicolor*	6	*A. subversicolor*	39.24
	P. chrysogenum	8	*P. chrysogenum*	60.76

[1] CFU/ml defined as the number of colonies observed on plate after 5 days and 21 days of incubation at 37 °C (Nolard et al. 2004)
[2] The massive parallel data were analyzed with the CLC Genomic Workbench software (Qiagen Benelux, B.V., KJ Venlo, the Netherlands) and the Microbial Genomics Module. The database used as reference was the UNITE database (Kõljag et al. 2005), last update January 31th 2016.

6.3.2 qPCR detection

The qPCR screening was performed on DNA extracted from each air and water sample with the primers specific for the detection of *E. jeanselmei* only (Libert et al. 2016).

In each run, the PC was amplified with an average quantification cycle (C_q) value of 19.48 ±1.08 and a melting temperature (T_m) average at 79.58 ±0.27°C, corresponding to the expected values previously reported by Libert and colleagues (Libert et al. 2016). Four of the five water samples gave a positive signal for *E. jeanselmei* with a T_m average at 79.63 ±0.30 °C (Table 6.1). No signal was observed for the water sample collected from PG1.

Of the 15 indoor air samples tested, 4 of them gave a positive signal with the expected T_m, i.e., offices 1, 2 and 3 from PG4 and office 1 from PG5 (Table 6.1). All the NTC were negative. Although the specificity of the *E. jeanselmei* qPCR primers has previously been demonstrated (Libert et al. 2016), the identity of each amplicon (i.e., amplicons from air and water samples) was confirmed as *E. jeanselmei* with a BLASTn analysis. The identify scores obtained with BLASTn for the 8 positive samples ranged between 97 % for air sample PG4-2 and 100 % for the water samples from PG 2 and 4.

6.3.3 Massive parallel sequencing

In order to confirm the results observed in qPCR and to obtain a broader view on the uncultivable fraction in the air and water samples, a massive sequencing analysis was performed on all the DNA samples.

As shown in Table 6.4, the massive parallel analysis performed on all samples (water and air) has generated between 6,352 (sample PG1-3) and 29,666 reads (sample PG1-1). Among these reads, between 6,797 and 11,602 reads from water samples were clustered into OTUs present in the UNITE database, corresponding to 60.97 % (sample PG3) and 99.95 % (PG5) of the total reads.

For the PG 2, 3, 4 and 5 (water samples), between 2,727 and 5,700 reads were grouped into the OTU identified as *E. jeanselmei* strain AY156963 (Table 6.1, Fig. 6.1), the only representative sequence for *E. jeanselmei* included in the UNITE database. The percentage of reads identified as *E. jeanselmei* was between 28 % (sample PG2) and 53.69 % (sample PG4) of the total number of clustered reads. No reads from the PG1 were clustered into the *E. jeanselmei* OTU (Table 6.4).

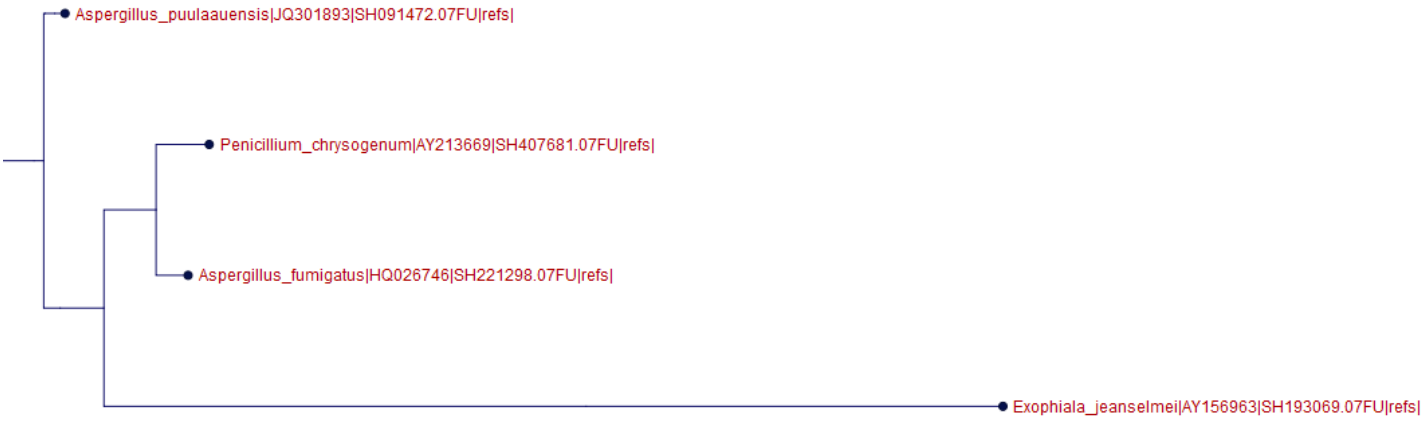

Figure 6.1: Neighbor Joining tree obtained with the water samples
The Neighbor Joining tree constructed according to a Jukes Cantor model for the reads obtained for all water samples grouped together.

The number of reads from air samples clustering into OTUs was between 6,305 (sample PG1-3) and 25,260 (sample PG2-3). The percentage of reads clustered into OTUs was recorded between 42.95 % (sample PG5-2) and 99.99 % (PG4-2). In 4 out of the 15 air samples, *E. jeanselmei* reads were found. Indeed, among the reads clustered into OTUs, between 1,248 (sample PG5-1) and 7,888 (sample PG4-3) reads were clustered into the *E. jeanselmei* AY156963 OTU (Table 6.4, Fig. 6.2). Expressed in %, between 10.99 % (sample PG5-1) and 60.74 % (sample PG4-1) of reads

grouped into OTUs were identified as *E. jeanselmei* OTU. These samples were collected in 4 offices connected to 2 PG i.e., the office 1, 2 and 3 of PG4 and the office 1 of PG5 (Table 6.4). For the samples of PG1, 2 and 3, no reads were correctly clustered into the *E. jeanselmei* OTU (Table 6.4).

In addition to *E. jeanselmei*, as shown in Tables 2 and 3, additional OTUs were detected besides *E. jeanselemei* i.e., 3 in water samples, *Aspergillus fumigatus, Aspergillus puulaaeunsis* and *P. chrysogenum* (Fig. 6.1) and 9 in air samples i.e., *A. alternata*, *Aspergillus monodii*, *Aspergillus puulaaeunsis*, *Aspergillus rugulosus*, *Aspergillus subversicolor*, *Aspergillus sp.*, *Emericella olivicola*, *Emericella undulata* and *P. chrysogenum* (Fig. 6.2).

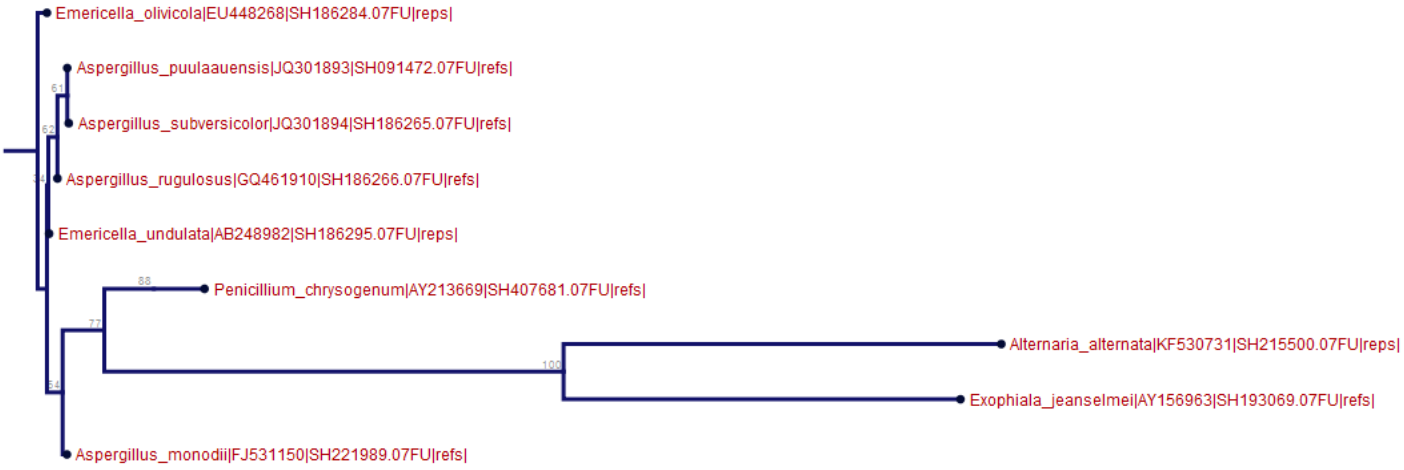

Figure 6.2: Neighbor Joining tree obtained with the air samples
The Neighbor Joining tree constructed according to a Jukes Cantor model for the reads obtained for all air samples grouped together.

Table 6.4: Detection of *E. jeanselmei* in indoor air and water in air-conditioning systems: sequencing data and clustering results

Samples ID	Pulse group	Office [1]	Sample type	Total number of reads [2]	Total of reads clustered [3]	Percentage of reads clustered (%) [4]	Abundance of reads into *E. jeanselmei* OTU [5]	Percentage of reads into *E. jeanselmei* OTU (%) [6]
PG1 [7]	1		Water [8]	13,124	11,602	88.40	0	0
PG1-1	1	1	Air	29,666	23,545	79.37	0	0
PG1-2	1	2	Air	19,753	19,603	99.24	0	0
PG1-3	1	3	Air	6,352	6,305	99.26	0	0
PG2	2		Water [8]	9,755	9,741	99.86	2,727	28.00
PG2-1	2	1	Air	28,487	20,928	73.47	0	0
PG2-2	2	2	Air	15,193	15,093	99.34	0	0
PG2-3	2	3	Air	28,338	25,260	89.14	0	0
PG3	3		Water [8]	11,148	6,797	60.97	2,278	33.51
PG3-1	3	1	Air	9,154	9,003	98.35	0	0
PG3-2	3	2	Air	9,474	9,434	99.58	0	0
PG3-3	3	3	Air	9,474	9,165	96.74	0	0

Table 6.4: *Continued*

Samples ID	Pulse group	Office [1]	Sample type	Total number of reads [2]	Total of reads clustered [3]	Percentage of reads clustered (%) 4	Abundance of reads into *E. jeanselmei* OTU [5]	Percentage of reads into *E. jeanselmei* OTU (%) [6]
PG4	4		Water [8]	10,675	10,616	99.45	5,700	53.69
PG4-1	4	1	Air	8,402	8,360	99.50	5,078	60.74
PG4-2	4	2	Air	16,838	16837	99.99	7,011	41.64
PG4-3	4	3	Air	20,286	20,059	98.88	7,888	39.32
PG5	4		Water [8]	9,302	8,458	90.93	4453	52.65
PG5-1	5	1	Air	12,288	11,355	92.41	1248	10.99
PG5-2	5	2	Air	16,337	7,016	42.95	0	0
PG5-3	5	3	Air	9,328	9,320	99.91	0	0

The massive parallel data were analyzed with the CLC Genomic Workbench software (Qiagen Benelux, B.V., KJ Venlo, the Netherlands) and the Microbial Genomics Module. The database used as reference was the UNITE database (Kõljalg et al. 2005), last update January 31th 2016.

[1] 3 offices per pulse group (PG) were sampled according to their distance to the PG. The offices 1 correspond to the closest located office to the PG, the offices 3 the most distantly located office to the PG and offices 2 correspond to the office located between the 2 others.

[2] The total number of reads observed for each sample

[3] Number of total reads grouped in the 10 OTUs detected i.e., *A. alternata*, *Aspergillus fumigatus*, *Aspergillus monodii*, *Aspergillus puulaaeunsis*, *Aspergillus rugulosus*, *Aspergillus subversicolor*, *Emericella olivicola*, *Emericella undulata*, *P. chrysogenum* and *E. jeanselmei*.

[4] Percentage obtained as the ratio between the number of reads grouped into the 10 OTUs and the total number of reads observed for each sample.

[5] Number of reads corresponding to OTU identified as *E. jeanselmei*.

[6] Percentage obtained as the ratio between the number of reads corresponding to the *E. jeanselmei* OTU and the total number of clustered reads.

[7] PG1 maintained before the sampling.

[8] One sample per pulse group was analyzed.

6.4 Discussion and Conclusion

E. jeanselmei is commonly found in moist environments, especially in water reservoirs of air-conditioning systems, but it has not yet been detected in indoor air (Beguin et al. 1994; Parat et al. 1996). This could be explained by the classical use of culture-based protocols for the monitoring and investigation of fungal indoor air contamination. Indeed, competition between species, incubation conditions and the presence of uncultivable taxa (dead or not) could impact the diversity observed on plate (Pitkaranta et al. 2011; Vesper 2011). Therefore, the results from these culture-dependent workflows could be biased. The non-detected part of the fungal diversity could however also have an impact on health and should therefore also be investigated. It could be hypothesized that *E. jeanselmei*, preferring humid environments and requiring up to 21 days of incubation to be able to be detected on plate (Libert et al. 2016), cannot be detected in indoor air samples using the classical culture-based monitoring methods. In this study it was investigated whether the presence of *E. jeanselmei* in indoor air could be demonstrated using molecular methods, for which the viability of the fungal contaminant is not required.

Hereto, a sampling was organized in different offices in contact with an air-conditioning system for which a contamination by *E. jeanselmei* was previously detected. First, water samples from the water-reservoir from 5 PG were collected in order to verify the presence of *E. jeanselmei* inside the system, i.e. in the water samples. These samples were analyzed using the classical protocol based on culture and microscopic identification (Nolard et al. 2004), and molecular methods including qPCR (Libert et al. 2016) and massive parallel sequencing analysis. All samplings were performed between February and March i.e., at the yearly restart of the air-conditioning systems, in the framework of the air-conditioning water monitoring. This monitoring is yearly carried out before the water decontamination (part of the maintenance of the system), except for the PG1 which had been maintained (including decontamination and draining) already before the time of sampling. The classical analysis of the water samples showed that all of the investigated water systems were contaminated with *E. jeanselmei*, except the one from PG1 (Table 6.1). The maintenance performed on PG1 explained the absence of *E. jeanselmei*. The results of the qPCR as well as of the massive parallel sequencing confirmed these positive detections of *E. jeanselmei* in water samples (Tables 6.1, 6.4) of PG2, 3, 4 and 5. Consistently, neither a signal nor a read was observed for the PG1 sample with qPCR or massive parallel sequencing, respectively.

In parallel, indoor air samples were collected in 15 offices in contact with the air-conditioning systems. For each system, 3 offices were selected based on their distance to the pulse group i.e., one close to the pulse group, one far from the pulse group and one in the middle of the 2 others.

This was done in order to define whether a distance effect could impact the detection of *E. jeanselmei* in air. As performed for the water samples, each air sample was analyzed with classical and molecular approaches. No *E. jeanselmei* colonies were observed for the air samples incubated on plates, even after 21 days of incubation. However, positive signals for *E. jeanselmei* were observed by qPCR in air samples from the offices 1, 2 and 3 linked to PG4, and in the office 1 of PG5. These positive detections were obtained for the air-conditioning systems where the highest water contamination was observed on plate (100 CFU/ml for PG4 and 15 CFU for PG5, compared to 10 for PG2 and PG3). In the PG5, *E. jeanselmei* was observed only in the office the closest located to the PG, not in office 2 and 3 of PG5. Moreover, according to the qPCR results, a gradient of distance from the closest to the most distantly located offices was observed for the PG4 with C_q values ranging between 23.79 ±0.92 for the PG4-1 and 34.06 ±1.12 for the PG4-3. The distance between the source of the contamination and the sampling site and the contamination load could probably affect the spreading of biological material originating from *E. jeanselmei* as it was observed for others species from indoor and outdoor environment (Afanou et al. 2015). It would be interesting to study the size and the form of the airborne biological compounds of *E. jeanselmei* involved to determine their aerosolizing distance in indoor air to verify this hypothesis.

The results of the massive parallel sequencing confirmed the ones obtained by qPCR. The obtained reads were clustered onto the UNITE database as reference which contained 6,825 reference ITS sequences at the date of research. Reads were clustered onto the *E. jeanselmei* OTU for samples PG4-1, PG4-2, PG4-3 and PG5-1. It was also for these samples that positive qPCR signals were obtained. The % of reads clustering into *E. jeanselmei* is decreasing with an increasing distance between the sampling site and the PG location. This is a similar trend as was observed with the C_q values in the qPCR analysis. This points towards a semi-quantitative nature of the massive parallel sequencing analysis.

The massive parallel sequencing was performed to obtain a broader view on the uncultivable species present in the water and air samples. Indeed, besides *E. jeanselmei* in indoor air and water samples, *A. alternata*, *A. fumigatus*, *A. puulaaeunsis*; *A. versicolor*, *P. chrysogenum*, as well as *Aspergillus* sp., an undetermined sp. and an infertile mycelium were detected on plate with the classical culture-based protocol. These taxa are commonly observed in indoor environment (Bellanger et al. 2009; Libert et al. 2015). In the water samples, the sequencing and classical approaches yielded the same species. However, besides *E. jeanselmei*, five additional OTUs were retrieved from air with the massive parallel sequencing which were not detected by the classical methods in the air samples, i.e., *A. monodii*, *A. puulaaeunsis*, *A. rugulosus*, *E. olivicola*, *E. undulata*. This means that of the species retrieved in the OTU analysis from air samples, only 2 were confirmed by the culturing approach, i.e. *A. alternata* and *P. chrysogenum*. *A. versicolor* was

found in the air samples with the classical methods, but not with the massive parallel sequencing. However, two species from the same phylogenetic species i.e., *Versicolores* were observed in the OTUs, i.e. *A. puulaaeunsis* and *A. subversicolor*. It should be noted that poor read quality could affect the correct determination of closely related species, e.g. for the species from the *Versicolores* complex and especially *A. versicolor* and *A. puulaauensis*, for which the ITS region differs only in a few nucleotides.

During the clustering, between 0.01 and 57.05 % of reads were not included in the OTUs. This can be linked either to reads that did not comply with the quality criteria or to reads that correspond to species that are not covered in the UNITE database or chimeric reads. This indicates that the massive parallel sequencing approach would benefit from improved and extended ITS databases in the future. The lower number of clustered reads obtained for some samples, especially for the samples PG3 and PG5-2, could have an impact on the results obtained during the diversity analysis. However, based on the qPCR results, these potential biases have not affected the detection of *E. jeanselmei*.

According to the literature available on *E. jeanselmei*, this study is the first to show the detection of *E. jeanselmei* in air samples. In 2010, Huang and his team reported a case of hypersensitivity pneumonitis caused by *E. jeanselmei* in a person who was a regular visitor of a specific sauna (Huang et al. 2010). According to their conclusion, the way of contamination was probably by the sauna steam (Huang et al. 2010), due to the presence of the black yeast in the water of the sauna visited by the patient. However, this black yeast was never detected before in dry indoor air from buildings as it was reported in this paper.

The detection of *E. jeanselmei* with molecular tools but not with the classical method confirms the hypothesis that *E. jeanselmei* could be present in the dead and/or uncultivable fraction of the indoor airborne fungal community. As shown in this study, qPCR but even more massive parallel sequencing analysis could improve the monitoring of indoor air, especially in the field of fungal contamination. Indeed, the use of universal primers amplifying the ITS region of a large panel of fungal species, increases the number of species that can be simultaneously detected during a monitoring. This is limited to one or a few a priori selected species with qPCR (Afanou et al. 2015). Another advantage of massive parallel sequencing is that the detection and identification are performed simultaneously, while the confirmation of qPCR results requires the sequencing of the amplicon if the tool is not fully specific. However, massive parallel sequencing is more time-consuming and requiring bio-informatics expertise for the data interpretation which is not the case for qPCR. In addition to the use of molecular tools, the detection of *E. jeanselmei* has probably also been facilitated by the use of this particular air collector, which is different from the one generally used for culture-dependent monitoring. The Coriolis® μ allows collecting a high volume

of air (1.5 m^3) directly into collection liquid which can be incubated on plate or analyzed with molecular tools without culturing step, as it was done in this study.

The results obtained in this study, taking *E. jeanselmei* as a case study, demonstrate that molecular methods, such as qPCR or massive parallel sequencing, combined with an appropriate air sampler can increase the data retrieved on the fungal community diversity in indoor air. More investigations on indoor air should be performed in order to improve the knowledge on indoor airborne fungi contamination, in particular on the dead/uncultivable fraction. Eventually, an improved insight on this diversity will contribute to the understanding of public health problems such as respiratory diseases (asthma, rhinitis or other chronicle respiratory infections) and sick buildings syndrome observed in many indoor environments and to taking the appropriate preventive measures.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 7

Exploring the indoor airborne fungal community with a metagenomic approach and next generation sequencing

7.1 Introduction

Over the years, the number of studies focusing on the load of micro-organisms or biological fragments in air has augmented, driven by the fact that air pollution is suggested to be a public health problem. Even if bacteria are the major group of organisms found in air, airborne fungi, especially from indoor environment, and their potential implication in health issues are increasingly studied (American Industrial Hygiene Association 2011; European Environment Agency 2013; Bernstein et al., 2008; Bruce 2000; Gots et al. 2003; Horner et al. 1995; Khan et al. 2012; Meheust et al. 2014; Mendell et al. 2011;Mudarri, 2007; Rebroux et al. 2010; United States Environmental Protection Agency 2016; Verhoeff et al. 1997; World Health Organization 2009; World Health Organization Regional Office for Europe 2004).

At first, studies on airborne organisms were based on conventional tools and phenotypical determinations (Beguin and Nolard 1994, Shelton et al. 2002). However, the complexity of microscopic determination and the problems linked to culturing such as species competition, influence of media selection, time of growth or the impossibility to cultivate some species, could lead to some bias in the diversity composition observed in some studies. So, to overcome the disadvantages of the culture-based methods, these enquiries are now increasingly performed with molecular methods, and mainly sequencing tools, which enlarge the diversity observed, including for applications in mycology (White et al. 1990; Pryce et al. 2003; Dannemiller et al. 2014). Not only do molecular tools facilitate the species determination, but they are also culture- and viability- independent. So, the use of polymerase chain reaction (PCR)–based tools as an alternative for microscopic-based determination permitted to have more insights in the indoor airborne diversity, including in those species that are uncultivable or dead. However, some studies considered that climate changes, new policies for energy saving and the increase of urbanization could favor the development of species not yet observed in our countries and so, not targeted by the current monitoring methods (Schenck et al. 2010; D'Amato et al. 2015; Vardoulakis et al. 2015). So, according to these observations and the fact that it is not always *a priori* known which fungi are present, fungal diversity investigations would benefit from an open approach, not limited to an *a priori* species selection as it is done with molecular tools such as PCR-based tools. In this context, next generation sequencing (NGS) technologies seem to be ideal tools to explore the fungal community and enlarge the knowledge about this diversity.

Therefore, metagenomics approaches, developed to understand biological activities or processes and then, used for the analysis of each genome present in an environmental sample (Venter et al. 2004; Thomas et al. 2012), appear to be a valuable alternative for the classical and targeted molecular tools used in the studies on fungal diversity. As it was done for most of the taxonomic

tools developed for fungi, most of the metagenomics studies were performed on the basis of the analysis of the internal transcribed spacer region (ITS region) included in the nuclear ribosomal gene cluster. The ITS region is comprised between the 18S (small subunit, SSU) and the 28S (large subunit, LSU), and contains the internal transcribed spacer 1 (ITS-1), the 5.8S subunit and the internal transcribed spacer 2 (ITS-2) (Cuadros-Oreliana et al. 2013) . Due to their interspecies variability, but also to their high copy number, ITS-1 and ITS-2 of fungal species are widely used for phylogenic studies. By the way, in 2012, the ITS region was appointed as a valuable barcoding region for the fungal kingdom (Schoch et al. 2012) which offers the possibility to inventarise the fungal diversity.

Performed at first with clone libraries and shotgun sequencing techniques, metagenomics approaches have been recently improved by the evolution and use of NGS technologies. Most of these NGS studies have been performed on pyrosequencing platforms such as the 454 Life Sciences NGS system (Cuadros-Oreliana, 2013). While this platform is relatively fast (i.e. between 10h for the GS Junior System and the GS FLX Titanium XLR70 and 23h for the GS FLX Titanium XL+), it has the disadvantage to produce a low amount of reads per run (i.e. 1,000,000 reads per runs). The 454 NGS system is also no longer available on the market. For these reasons, today, metagenomics studies use mainly NGS platforms provided by Illumina such as the HiSeq and the MiSeq platform. Indeed, these sequencing platforms can produce between 12,000,000 and 3,000,000,000 of reads during a single run. Increasing the number of reads allows increasing the amount of information delivered during the analysis. Despite this, the Illumina platform is restricted in terms of read length i.e., between 100 bp for the HiSeq and 300 bp for the MiSeq. Therefore, because the fungal ITS region has a length around 800 bp, fungal metagenomics analyses based on ITS region sequencing are most of the time limited to one part of the ITS region, i.e. ITS-1, which is considered as the most informative for fungal diversity. There exist publicly available databases of ITS sequences that can be used for the analysis of ITS-bases metagenomics analyses, such as the User-friendly Nordic ITS Ectomycorrhiza Database (UNITE) (Kõljag et al. 2005) and ITSoneDB (ITSone) (Fosso et al. 2012).

Although metagenomics were already applied to the fungal indoor air community (Shin et al. 2015; Tedersoo et al. 2015; Uroz et al. 2013), most of the studies were based on the use of a pyrosequencing platform and on the ITS-1 as fungal marker. The present study describes a metagenomics analysis of fungal airborne communities of houses where health problems, especially respiratory diseases (such as asthma or allergies) were detected. The NGS analysis was performed using an Illumina MiSeq 300 targeting the entire ITS region, i.e. a combination of the ITS-1 and ITS-2. The results were analysed with the CLC Microbial Module and CLC Genomics Workbench tools. Results obtained from NGS were also compared to those obtained for the same

samples analyzed with classical methods (i.e., culture and microscopic visualization) in order to verify whether this approach could be used to improve the knowledge on the indoor airborne diversity allowing to enlarge the scientific data on fungal contamination and public health issues.

7.2 Material and methods

7.2.1 Sample collection

Indoor air samples were collected, from February 2014 until June 2015, in houses or flats in Brussels (Belgium), in the framework of the indoor pollution investigation of the CRIPI (*Cellule Régionale d'Investigation en Pollution Intérieure* from Brussels Environment, Brussels, Belgium) (Table 7.1). Two sets of samples were independently collected i.e., one for classical analysis, and one for NGS analysis, at the height of a seated person, in different rooms i.e., the bedroom, the bathroom, the kitchen and the living room. To avoid any contamination from outside or from another part of the house, the sampling site was isolated by closing doors and/or windows and with no one inside.

The sampling workflow was previously described in (Libert et al. 2015). Briefly, 1.5 m³ (300 L/min, 5 min) of air were collected with a Coriolis®μ (Technology, Montigny-le-Bretonneux, France) into an sterile cone containing 15 ml of ultra-pure water and Tween 20® 0.01% (Sigma-Aldrich, St Louis, USA).

7.2.2 Classical analysis

The protocol used for the classical analysis (i.e., culture and fungal determination) of air samples collected by the Coriolis® μ sampler was previously described in (Libert et al. 2015). In short, after an incubation of 5 days at 25 °C for mesophilic and 2 days at 45 °C for thermophilic fungi, the species determination from the air samples was done by microscopic visualization (Nolard et al. 2004).

7.2.3 DNA extraction

The DNA extraction of the indoor air samples was done according to the protocol described in Libert et al. (2015). DNA quality and concentration was evaluated on a Nanodrop® 2000 (Thermo Scientific, Wilmington, USA).

7.2.4 Massive parallel sequencing

Because a massive parallel sequencing analysis using the Illumina MiSeq with a MiSeq reagent kit v3 reaches its optimal performance with fragments no longer than 600 bp and because the fungal

ITS region has a length around 800 bp, the PCR amplicons for library preparation were prepared for both the ITS-1 and ITS-2 region (with an overlapping region) using DNA extracted from the different air samples. Hereto, amplification with the universal forward (f) and reverse (r) primers ITS1f/ITS2r (White et al. 1990) for the amplification of the ITS-1 region and with the universal primers ITS3f/ITS4r (White et al. 1990) for the ITS-2 region was performed. The primers were extended with the Illumina sequences needed for PCR-based library preparation. The PCR-reaction mix (25 µl final volume) contained 2.5 µlof high fidelity PCR buffer (10X), 0.5 µL of a mix of 0.2 mM of each dNTP, 0.5 µM of each primer, 0.1 µl of Platinum® Taq DNA Polymerase high fidelity enzyme (5 U/µl) (ThermoFischer Scientific, Life Technology, Gent, Belgium) and 15.90 µl of Gibco® DNase, RNase, Protease free pure water (Life Technologies, Gent, Belgium). At the end, 5 µl of gDNA (2ng/µl) were added. Eachrun was performed with the following PCR protocol i.e., 1 cycle at 94 °C for 3 min, 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C and 1 min at 72 °C and at last, 10 min at 72 °C.

After the PCR-reaction, all the samples were purified with the AMpure® XP PCR purification kit (Agencourt Biosciences Corporation, Beverly, USA). The quality and amount of PCR fragments for massive parallel sequencing were verified on a Bioanalyzer 2100 (Agilent Technologies, Amstelveen, the Netherlands). ITS-1 and ITS-2 amplicons from a same sample were mixed together. Equimolar mixes were made according to the size of the peaks observed during the Bioanalyzer analysis. Sequencing was performed by BaseClear (Venlo, The Netherlands) with an Illumina MiSeq, yielding 2 x 300 bp paired-end reads.

It has to be remarked that initially 2 sets of 60 air samples from 20 residences (houses or apartments, and different rooms per residence) were collected all around Brussels (Belgium) in order to identify the fungal contamination in two ways: a classical culture-depend identification protocol and a metagenomics analysis based on massive parallel sequencing. However, due to some problems during the sequencing step that was performed by an external company, only 28 samples from 10 residencies gave usable sequencing data. Therefore, the number of samples analyzed by classical analysis was reduced to these 28 samples in order to have a valuable comparison between the classical and the metagenomics approaches.

7.2.5 Bio-informatics analysis

The FASTQ sequence files were generated using the Illumina Casava pipeline version 1.8.3 (BaseClear, Venlo, the Netherlands). The first quality assessment was based on the Illumina Chastity filtering. Reads with PhiX control signal and/or short reads below 20 bp (after adapter clipping) were removed using an in-house filtering protocol from BaseClear. Then, a second quality assessment control was done using the tool PRINSEQ-lite quality control v.0.20.4. Finally,

an average quality score per sample was obtained with FastQC v.010.1.40. The sequences which did not pass this quality control were removed for the remainder of the analysis.

Sequencing reads were subsequently analyzed with an operational taxonomic unit (OTU) classification approach with the Microbial Genomics Module from the CLC Genomic Workbench V8 software (Qiagen Benelux, B.V., KJ Venlo, the Netherlands). Briefly, in order to have comparable read lengths and to remove reads with low coverage, the paired-end reads were firstly treated with 3 different tools i.e., the "adapter trimming", the "fixed length trimming" and the "filter samples based on the number of reads". Thus, at the end of the workflow, the OTU clustering was performed only on reads with the same length and a low level of bias.

The OTUs clustering was made with the OTUs clustering tool using two different approaches. The first approach was performed using the ITSone database (Fosso et al. 2012) as the reference database for the clustering. ITSone is an ITS-1 database specifically developed for amplicon-based metagenomic identification of environmental fungi. All the sequences collected in ITSone come from the GenBank database of the National Center of Biotechnology Information (NCBI) from the United States (Fosso et al. 2012). The second approach was based on the use of the UNITE database V7.1. (last updated January 31th, 2016) (Kõljag et al. 2005) as the reference database. This database contains ITS sequences (i.e., sequences from ITS-1 and ITS-2 regions), available in the GenBank database of NCBI (Kõljag et al. 2005). Finally, all the OTUs were aligned with the MUSCLE tool and a Neighbor Joining tree was constructed according to the Jukes Cantor nucleotides model.

7.2.6 Statistical analysis

All the data obtained with the classical culture-based analysis and the two bio-informatics clustering approaches (UNITE and ITSone) were compared to each other during an analysis of variance (ANOVA). Moreover, ANOVA analysis was used to verify whether the houses' conditions (i.e., presence/absence of visible fungal colonies, presence/absence of water damage) were associated with the fungal diversity. Additionally, the impact of fungal diversity on the presence of healthy people/people with health problems was also investigated through the use of ANOVA analysis. During these tests, a difference was considered as significant for a p-value < 0.05 %. Each statistical analysis was performed with the R free software (https://www.r-project.org/).

7.3 Results

7.3.1 Description of sample collection

As shown in Table 7.1, 3 rooms per house were sampled. The bedroom, the bathroom and the kitchen (or the living room) - except in the “house”4 which was composed of only 2 rooms - were each time sampled according to the sampling place configuration. Out of the 10 sampled houses further investigated, 7 displayed visible molds in at least 1 sampled room and in 6 of them, water damage was observed (Table 7.1). Finally, 8 out the 10 sampled places housed at least one person affected by health problems, especially allergies and asthma (Table 7.1).

Table 7.1: Overview of air samples taken, sample and house characteristics and global fungal diversity

House ID	Sample ID	Room	Respiratory Problems in house	Visible Fungi	Water damage	Classical analysis Total # fungal taxa		Metagenomics analysis Total # fungal taxa			
								ITSone DB[1]		UNITE DB[2]	
						/ house	/room	/ house	/room	/house	/room
1	CR1-1	Kitchen	No	No	No	2	1	3	2	4	2
1	CR1-2	bathroom		No	No		2		2		3
1	CR1-3	bedroom		No	No		2		2		2
2	CR2-1	bedroom	Yes	No	No	3	2	6	2	5	2
2	CR2-2	bathroom		No	No		2		1		2
2	CR2-3	living room		No	No		4		4		5
3	CR3-1	bedroom	Yes	Yes	No	3	2	4	4	7	5
3	CR3-2	bathroom		Yes	No		3		3		5
3	CR3-3	living room		Yes	No		2		3		3
4	CR4-1[1]	bathroom	Yes	Yes	Yes	5	3	8	4	7	5
4	CR4-2	bedroom/kitchen[2]		Yes	Yes		4		6		6
5	CR5-1	bathroom	Yes	Yes	Yes	6	4	5	4	7	3
5	CR5-2	Bedroom		Yes	Yes		2		3		2
5	CR5-3	kitchen		Yes	Yes		2		3		3
6	CR6-1	Laundry room	Yes	Yes	Yes	3	2	4	3	3	2
6	CR6-2	bathroom		Yes	Yes		2		2		2
6	CR6-3	bedroom/kitchen[2]		No	Yes		2		2		2
7	CR7-1	Bedroom	Yes	No	Yes	5	2	7	4	9	6
7	CR7-2	bathroom		No	Yes		3		4		4
7	CR7-3	kitchen/living room[2]		Yes	Yes		3		5		8

Table 7.1: *Continued*

House ID	Sample ID	Room	Respiratory Problems in house	Visible Fungi	Water damage	Classical analysis Total # fungal taxa		Metagenomics analysis Total # fungal taxa			
						/house	/room	/house	/room	/house	/room
8	CR8-1	Bedroom	Yes	Yes	Yes	3	3	6	4	8	4
8	CR8-2	bathroom		Yes	Yes		3		4		3
8	CR8-3	bedroom/kitchen[2]		Yes	Yes		2		3		4
9	CR9-1	Kitchen	No	No	No	2	1	3	2	2	2
9	CR9-2	bathroom		Yes	No		2		2		2
9	CR9-3	Bedroom		No	No		2		3		2
10	CR10-1	Bedroom	Yes	Yes	Yes	3	2	6	4	4	3
10	CR10-2	bathroom		Yes	Yes		3		5		4
10	CR10-3	kitchen/living room[2]		Yes	Yes		3		4		2

The classical analysis was based on culture and microscopic determination. The metagenomics analysis was based on bio-informatics clustering performed on the massive parallel sequencing data and using 2 databases as reference i.e., ITSone (Fosso et al. 2012) and UNITE (Kõljag et al. 2005).
[1] Flat of 2 rooms i.e. a bathroom and a room containing the kitchen and bedroom.

7.3.2 Classical analysis: relative abundance and species richness

At first a classical protocol based on culture, microscopic identification and cell counting was performed according to the routine monitoring protocol (Nolard et al. 2004). As shown in Table 7.2, 4 genera were identified i.e., *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium*. Among them, the most abundant groups were *Penicillium chrysogenum* which corresponded to 44.15 % of relative abundance, *Aspergillus versicolor* (29.52 % of relative abundance), the *Cladosporium* sp. (15.69 % of relative abundance) and the *Alternaria alternata* (4.26 % of relative abundance). *Aspergillus nidulans* and *Aspergillus niger* were also observed but with a lower occurrence (< 2 % of relative abundance). Infertile mycelium and some undetermined yeast were also observed (Fig 7.1). The maximum level of colony forming units per plate was determined at 15 CFU/plate, and observed for *P. chrysogenum* in 4 samples (i.e., CR1-2, CR4-1, CR6-2 and CR10-2) (Appendix B – table B1).

As shown in Table 7.1, the species richness per house ranged between 2 (houses 1 and 9) and 6 (house 5), while the species richness per room ranged between 1 (CR1-1 and CR9-1 samples) and 4 (samples CR2-3, CR4-2 and CR5-1). The fungal contamination was mostly observed in the bathroom with 38.03 % of the relative abundance and in the bedroom (32.09 %), and reached 18.31 % in the kitchen. The remaining 11.27 % were shared between the laundry room and the living room.

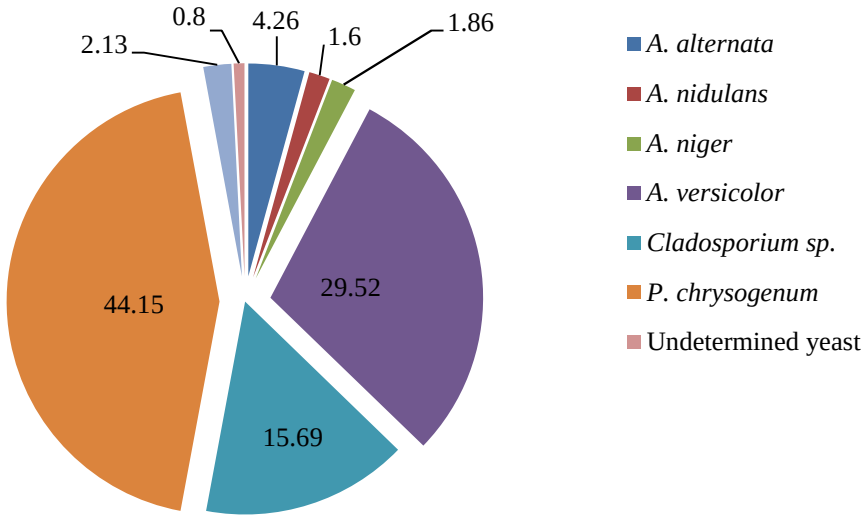

Figure 7.1: Classical analysis: Relative abundance per species
Relative abundance of each species observed on plate, calculation based on the total number of CFU per plate (Appendix B – Table B1).

Table 7.2: Classical and metagenomics analysis: Species determination and abundance

House ID	Sample ID	Room	Classical analysis		NGS				
						Metagenomic analysis			
						ITSone DB[1]		Unite DB[2]	
			Taxa	Abundance /room (%)[3]	Total number of reads	Taxa	Abundance /room (%)	Taxa	Abundance /rom (%)
1	CR1-1	Kitchen	*P. chrysogenum*	100	29,666	*P. chrysogenum*	16.65	*P. chrysogenum*	99.73
						Uncultured fungi	84.35	*A. puulaauensis*	0.27
	CR1-2	Bathroom	*Cladosporium* sp.	34.78	19,753	*C. cladosporioides*	30.35	*C. cladosporioides*	41.45
			P. chrysogenum	65.22		*P. chrysogenum*	69.65	*P. chrysogenum*	58.54
								C. halotolerans	0.01
	CR1-3	Bedroom	*Cladosporium* sp	42.86	13.124	*C. cladosporioides*	19.89	*C. cladosporioides*	10.42
			P. chrysogenum	57.14		*P. chrysogenum*	80.11	*P. chrysogenum*	89.58
2	CR2-1	Bedroom	*A. versicolor*	62.50	6,352	*A. versicolor*	80.27	*A. versicolor*	59.09
			Cladosporium sp.	37.50		*C. herbarum*	19.73	*C. herbarum*	40.91
	CR2-2	Bathroom	*A. versicolor*	59.09	6,526	*A. versicolor*	80.27	*A. versicolor*	64.46
			Cladosporium sp.	40.91		*C. herbarum*	19.73	*C. herbarum*	35.54
	CR2-3	Living room	*A. alternata*	10.00	5,163	*A. alternata*	25.76	*A. alternata*	20.44
			A. nidulans	20.00		*A. nidulans*	28.38	*A. nidulans*	15.61
			A. versicolor	60.00		*A. versicolor*	24.75	*A. versicolor*	42.28
			Cladosporium sp.	10.00		*C. herbarum*	21.12	*C. herbarum*	21.02
								Alternaria sp	0.64

Table 7.2: *Continued*

House ID	Samples ID	Room	Classical Analysis		NGS				
						Metagenomics analysis			
						ITSone DB[1]		Unite DB[2]	
			Taxa	Abundance /room (%)[3]	Total number of reads	Taxa	Abundance /room (%)	Taxa	Abundance /room (%)
3	CR3-1	Bedroom	*A. nidulans*	20.00	28,847	*A. nidulans*	18.79	*A. nidulans*	42.44
			A. versicolor	60.00		*A. versicolor*	49.98	*A. versicolor*	47.91
			Infertile mycelium	20.00		*Aspergillus* sp.	6.17	*Aspergillus* sp.	8.77
						Uncultured fungi	5.306	*E. undulata*	0.80
								E. olivicola	0.08
	CR3-2	Bathroom	*A. nidulans*	9.09	15,193	*A. nidulans*	5.05	*A. nidulans*	8.96
			A. versicolor	40.91		*A. versicolor*	23.83	*A. versicolor*	29.35
			P. chrysogenum	50.00		*P. chrysogenum*	71.11	*P. chrysogenum*	20.86
								E. undulata	0.75
								E. olivicola	0.25
	CR3-3	Living room	*A. nidulans*	20.00	28,338	*A. nidulans*	15.20	*A. nidulans*	0.28
			A. versicolor	80.00		*A. versicolor*	75.02	*A. versicolor*	90.31
						Aspergillus sp.	9.77	*A. subversicolor*	9.41
4	CR4-1[4]	Bathroom	*A. niger*	14.71	16,337	*A. niger*	6.62	*A. niger*	20.15
			Cladosporium sp.	35.29		*C. herbarum*	12.55	*C. herbarum*	20.06
			P. chrysogenum	44.12		*P. chrysogenum*	62.04	*P. chrysogenum*	53.25
						A. nidulans	0.57	*A. nidulans*	0.49
						Uncultured species	18.23	*Aspergillus* sp.	6.06

Table 7.2: *Continued*

House ID	Sample ID	Room	Classical analysis		NGS	Metagenomic analysis			
						ITSone DB[1]		Unite DB[2]	
			Taxa	Abundance /room (%)[3]	Total number of reads	Taxa	Abundance /room (%)	*Taxa*	Abundance /room (%)
2	CR4-2[4]	Bedroom/kitchen[5]	*A. alternata*	29.41	9,328	*A. alternata*	15.81	*A. alternata*	21.43
			A. niger	5.88		*A. niger*	12.61	*A. niger*	29.14
			Cladosporium sp.	23.53		*C. herbarum*	16.36	*C. herbarum*	10.88
			P. chrysogenum	41.18		*C. cladosporioides*	16.21	*C. cladosporioides*	10.69
						P. chrysogenum	37.44	*P. chrysogenum*	29.97
						Alternaria sp.	1.57	*Aspergillus* sp.	0.89
5	CR5-1	Bathroom	*A. versicolor*	5.41	12,324	*A. versicolor*	37.05	*A. versicolor*	11.70
			Cladosporium sp.	21.62		*C. herbarum*	3.37	*C. herbarum*	1.59
			P. chrysogenum	37.84		*P. chrysogenum*	59.58	*P. chrysogenum*	86.71
			Undetermined yeast	5.41					
	CR5-2	Bedroom	*P. chrysogenum*	87.50	27,066	*P. chrysogenum*	8.19	*P. chrysogenum*	80.00
			Infertile mycelium	12.50		*C. herbarum*	10.91	*C. herbarum*	20.00
						Uncultured species	7.19		
	CR5-3	Kitchen	*A. alternata*	66.67	19,753	*Alternaria* sp.	12.66	*Alternaria* sp.	72.44
			Infertile mycelium	33.33		*C. herbarum*	27.55	*C. herbarum*	27.47
						Uncultured species	6.42	*A. monodii*	0.09

Table 7.2: *Continued*

House ID	Sample ID	Room	Classical analysis		NGS				
						Metagenomics analysis			
						ITSone DB[1]		Unite DB[2]	
			Taxa	Abundance /house (%)[3]	Total number of reads	Taxa	Abundance /house (%)	Taxa	Abundance /house (%)
6	CR6-1	Laundry room	*A. versicolor*	36.36	24,937	*A. versicolor*	25.27	*A. versicolor*	27.93
			P. chrysogenum	63.64		*P. chrysogenum*	63.39	*P. chrysogenum*	72.07
						Aspergillus sp.	10.84		
	CR6-2	Bathroom	*A. versicolor*	42.31	18,153	*A. versicolor*	447.45	*A. versicolor*	62.64
			P. chrysogenum	57.69		*P. chrysogenum*	52.25	*P. chrysogenum*	37.36
	CR6-3	Bedroom/kitchen4	*A. alternata*	40.00	20,333	*Alternaria* sp.	62.06	*A. alternata*	56.56
			A. versicolor	60.00		*A. versicolor*	37.94	*A. versicolor*	43.44
7	CR7-1	Bedroom	*A. alternata*	16.67	13,673	*A alternata*	3.08	*A alternata*	48.71
			A. versicolor	83.33		*A. versicolor*	10.49	*A. versicolor*	14.43
						A. niger	14.07	*A. niger*	31.69
						uncultured species	45.36	*A. puulaauensis*	4.11
								E. undulate	0.58
								E. olivicola	0.77
	CR7-2	Bathroom	*A. nidulans*	16.00	9,335	*A. nidulans*	32.58	*A. nidulans*	32.98
			A. versicolor	36.00		*A. versicolor*	22.08	*A. versicolor*	19.23
			P. chrysogenum	48.00		*P. chrysogenum*	36.06	*P. chrysogenum*	41.01
						uncultured species	9.28	*A. fumigatus*	6.78

Table 7.2: *Continued*

House ID	Sample ID	Room	Classical analysis		NGS				
						Metagenomices			
						ITSone DB[1]		Unite DB[2]	
			Taxa	Abundance /room (%)[3]	Total number of reads	Taxa	Abundance /room (%)	Taxa	Abundance /room (%)
	CR7-3	Kitchen/living room[5]	*A. alternata*	6.25	11,024	*A. alternata*	23.55	*A alternata*	35.31
			A. niger	6.25		*A. niger*	6.29	*A. niger*	9.86
			A. versicolor	31.25		*A. versicolor*	38.21	*A. versicolor*	20.28
			P. chrysogenum	56.25		*P. chrysogenum*	28.82	*A. puulaauensis*	0.24
						Aspergillus sp.	3.14	*P. chrysogenum*	31.01
								A. fumigatus	3.03
								E. undulata	0.12
								E. olivicola	0.17
8	CR8-1	Bedroom	*A. alternata*	33.33	7,338	*A. alternata*	20.42	*A. alternata*	66.31
			A. versicolor	50.00		*A. versicolor*	59.35	*A. versicolor*	42.26
			Cladosporium sp.	16.67		*C. cladosporioides*	16.92	*C. cladosporioides*	52.74
						Uncultured species	3.31	*Aspergillus* sp.	12.11
8	CR8-2	Bathroom	*A. alternata*	21.05	15,373	*A. alternata*	32.04	*A. alternata*	30.94
			A. versicolor	42.11		*A. versicolor*	34.75	*A. versicolor*	501.38
			Cladosporium sp.	36.84		*C. cladosporioides*	13.17	*C. cladosporioides*	18.68
						Uncultured species	20.04		
8	CR8-3	Kitchen	*A. alternata*	50.00	8,126	*Alternaria* sp.	12.14	*A. alternata*	15.54
			A. versicolor	50.00		*A. versicolor*	45.42	*Alternaria* sp.	5.03
						Uncultured species	42.44	*A. versicolor*	61.35
								C. cladosporioides	18.08

Table 7.2: *Continued*

House ID	Sample ID	Room	Classical analysis		NGS				
						Metagenomics analysis			
						ITSone DB[1]		Unite DB[2]	
			Taxa	Abundance /room (%)[3]	Total number of reads	Taxa	Abundance /room (%)	Taxa	Abundance /room (%)
9	CR9-1	Kitchen	*Cladosporium* sp.	16.67	5,323	*C. herbarum*	100	*C. cladosporioides*	20.48
			P. chrysogenum	83.33				*C. herbarum*	79.52
	CR9-2	Bathroom	*Cladosporium* sp.	30.00	10,114	*C. herbarum*	30.48	*C. cladosporioides*	11.91
			P. chrysogenum	70.00		*P. chrysogenum*	47.69	*P. chrysogenum*	52.62
						A. alternata	21.84	*A. alternata*	31.47
	CR9-3	Bedroom	*Cladosporium* sp.	11.11	6,005	*C. herbarum*	18.45	*C. cladosporioides*	15.96
			P. chrysogenum	88.89		*P. chrysogenum*	52.23	*P. chrysogenum*	47.56
						A. alternata	29.32	*A. alternata*	36.48
10	CR10-1	Bedroom	*A. versicolor*	33.33	17,632	*A. versicolor*	26.34	*A. versicolor*	23.69
			Cladosporium sp.	8.33		*C. herbarum*	6.57	*C. herbarum*	6.63
			P. chrysogenum	58.33		*P. chrysogenum*	67.09	*P. chrysogenum*	69.68
	CR10-2	Bathroom	*A. versicolor*	5.88	23,829	*A. versicolor*	12.98	*A. versicolor*	9.61
			Cladosporium sp.	5.88		*C. herbarum*	9.35	*C. herbarum*	14.05
			P. chrysogenum	88.24		*P. chrysogenum*	51.98	*P. chrysogenum*	55.54
						Alternaria sp.	20.30	*Alternaria* sp.	20.80
						uncultured sp.	4.88		

Table 7.2: *Continued*

House ID	Sample ID	Room	Classical analysis		NGS				
						Metagenomics analysis			
						ITSone DB[1]		Unite DB[2]	
			Taxa	Abundance /room (%)[3]	Total number of reads	Taxa	Abundance /room (%)	Taxa	Abundance /room (%)
10	CR10-3	Kitchen/living room[5]	*A. alternata*	20.00	28,487	*Alternaria* sp.	20.45	*Alternaria* sp.	17.85
			A. versicolor	80.00		*A. versicolor*	19.77	*A. versicolor*	14.57
						P. chrysogenum	58.99	*P. chrysogenum*	67.58
						uncultured sp.	0.79		

[1]Fosso et al. 2012
[2] Kõljag et al. 2005
[3] Abundance/house (%) corresponds to the ratio between the abundance of each taxa (expressed in CFU or number of reads) in the samples and the total abundance observed per room.
[4]Flat of 2 rooms i.e. a bathroom and a room containing the kitchen and bedroom.
[5]Open space considered as one room.

7.3.3 Massive parallel sequencing data analysis

The data obtained with the massive parallel sequencing were analyzed as described in Materials and Methods using two different ITS databases, i.e. ITSone (Fosso et al. 2012) and UNITE (Kõljag et al. 2005).

7.3.4 Clustering: ITSone database

Alpha diversity: Relative abundance and species identification

The massive parallel sequencing results were firstly analyzed with the ITSone database containing ITS-1 fungal sequences. As shown in Table 7.2, the clustering performed with the ITSone database revealed in total 7 taxa belonging to 3 genera (i.e., *Alternaria*, *Aspergillus* and *Cladosporium*) (Table 7.2, Appendix B – Fig. B1). Corresponding respectively to 40.08 % and 25.98 % of the total relative abundance, *P. chrysogenum* and *A. versicolor* were the most abundant taxa (Fig.7.2), followed by *Alternaria* sp. (6.25 %) and *C. herbarum* (5.52 %), *A. nidulans* (3.16 %) and *C. cladosporioides* (3.08 %). The other taxa (i.e., *A. alternata*, *A. niger* and *Aspergillus* sp) reached together 4.34 %. Finally, 11.59 % of the total relative abundance corresponded to uncultured species.

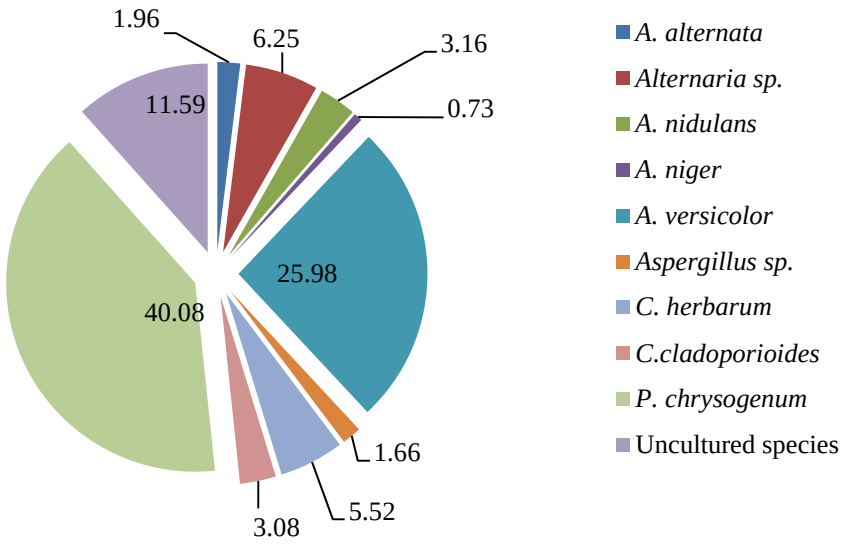

Figure 7.2: ITSone clustering: Relative abundance per OTU

Relative abundance of each species observed with the clustering performed with the ITSone database as reference (Fosso et al. 2012). The relative abundance of each species was calculated on the basis of the number of reads per OTU obtained during the clustering (Appendix B – Table B1).

Species richness repartition

The species richness observed among the 10 sampled residences ranged between 8 and 3, with an average per house of 5 species (Table 7.1). The maximum of species richness was observed for the "house" 4 and the minimum for the "houses" 1 and 9. The fungal relative abundance among the rooms was distributed as follows: 33.01 % in the bedroom, 30.10 % in the bathroom and 26.21 % in the kitchen. The remaining 10.68 % corresponded to the group the living room and the laundry room.

7.3.5 Clustering: UNITE database

Alpha diversity: Relative abundance and species identification

The massive parallel sequencing results were subsequently analyzed with the UNITE database containing ITS-1 and ITS-2 fungal sequences. In total 14 species belonging to 5 genera (i.e., *Alternaria*, *Aspergillus*, *Cladosporium*, *Emericella* and *Penicillium*) were observed in the indoor air samples (Table 7.2, Appendix B- Fig.B2). Indoor air samples were largely dominated by *P. chrysogenum* and *A. versicolor* which corresponded respectively to 35.11% and 31.66 % of the total relative abundance (Fig. 7.3) whereas species such as *A. alternata*, *A. nidulans*, *A. niger*, *C. cladosporioides* and *C. herbarum* have a relative abundance ranging between 2.75 % and 8.75 % (Fig 7.3). Finally, the remaining relative abundance (i.e., 11.51 %) included some *Aspergillus* species, *Cladosporium* species and *Emericella* species, but also undetermined *Alternaria* and *Aspergillus*.

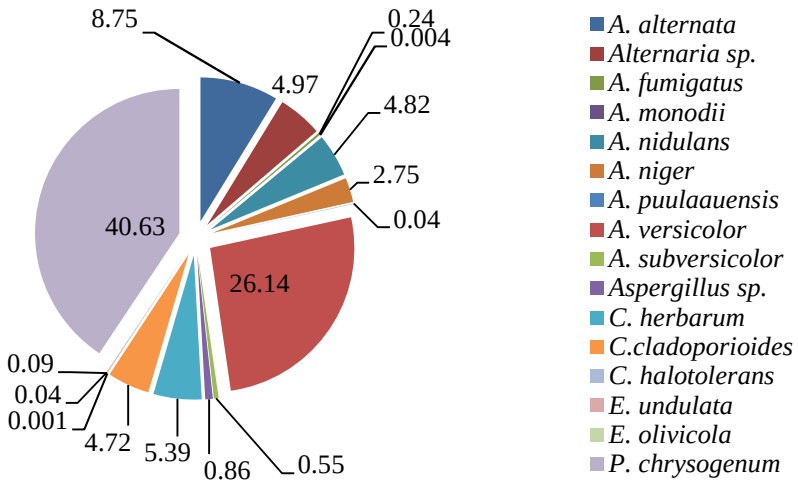

Figure 7.3: UNITE clustering: Relative abundance per OTU

Total relative abundance per OTU relative abundance of each species observed with the clustering realized with the UNITE database as reference (Kõljag et al. 2005).
The relative abundance of each species was calculated on the basis of the number of reads per OTUs obtained during the clustering (Appendix B – Table B1).

Species richness repartition

Among the 10 analyzed residences, the species richness per house was on average 6, with a maximum of 9 species observed in the ”house” 7 and the minimum of 2 species in “house” 9 (Table 7.1). Among the rooms, the fungal relative abundance was quite the same between the bathroom (31.73 %) and the bedroom (32.69 %). In the kitchen, this relative abundance reached 25.96 %.

7.3.6 ANOVA analysis

The ANOVA analysis performed between water damaged houses and non-water damaged houses revealed no significant difference concerning the fungal richness (P-value = 0.9871 for the classical analysis; P-value = 0.8725 for ITSone; P-value = 0.9245 for UNITE). Moreover, for the culture-based approach as well as for the 2 bio-informatic analysis, no significant differences in terms of fungal richness were detected between houses of people affected by respiratory problems and those of healthy people (P-value = 0.6042 for the classical analysis; P-value = 0.7326 for ITSone and 0.7219 for UNITE).

The richness per house observed with the classical analysis was significantly different to those observed for the 2 metagenomics-based clusterings, i.e., p-value = 0.0095 for the ITSone and p-value = 0.0040 for the UNITE. Between the two databases, this richness (uncultured species included) is not considered as significant different (P-value = 0.3583). However, the determination at the species level (i.e., uncultured species excluded) is significantly different between the 2 databases (p-value = 0.024).

7.4 Discussion

Based on their independence to cell viability and their high sensitivity, molecular methods seem to be a good alternative to the classical approach for the monitoring of fungal diversity. However, most of these tools are designed only for one or a small panel of species, i.e. those considered as the most important in terms of occurrence or in terms of public health impact. For some years, microbial diversity has been studied using metagenomics approaches. Thanks to the development of metagenomics tools based on NGS technologies, investigations on the airborne microbial diversity have largely improved, especially those focusing on the indoor airborne fungal diversity.

This study reports the analysis of the indoor airborne fungi from residences of people with respiratory problems (asthma, allergies, sick syndrome buildings…). Because most of the

investigation protocols are still culture-based, this study was carried out with two different approaches i.e., a classical approach based on culture and microscopic determination and an ITS-based metagenomics analysis coupled to a NGS performed directly on DNA extracted from samples without cultivation. The NGS data have been subsequently analysed using two different ITS databases.

In total, with all 3 types of analysis, five different genera were observed in this study i.e., *Alternaria, Aspergillus, Cladosporium, Emericella* and *Penicillium.* Apart from the *Emericella* genus, the 4 others are commonly observed in air from indoor environments (Beguinand Nolard 1994, Bellanger et al. 2009, Shelton et al. 2002) and are known to have some implications in respiratory diseases such as asthma and allergies. While *Aspergillus* and *Penicillium* species are considered as indoor species, *Alternaria* and *Cladosporium* species are considered as outdoor species coming inside across the ventilation system, windows or any other openings. In this study, these 4 genera were all detected on plate with the classical analysis. Easily cultivable, these 4 fungal groups are commonly observed with a classical protocol. As to *Emericella*, this is also an outdoor species but less observed in indoor air than the two others. *Emericella* species are considered as a sexual state of some *Aspergillus* species, such as *A. nidulans* or *A. niger,* and so, are genetically close to these *Aspergillus.* For this genus not much information is available yet on its allergenic potential. However, some species such as *Emericella nidulans,* are suspected to induce aspergillosis and hypersensitivity reactions (Fisher et al. 2006). *Emericella* species are also cultivable but require a longer time of growth - until 10 days of incubation - and are rarely observed on plate. In this study, the classical analysis was based on a culture-based protocol used in routine analysis which included an incubation of 5 days. So, the absence of detection of *Emericella* species (i.e., *E. undulata* and *E. olivicola*) by the classical approach could be due to a shorter incubation time used. Nevertheless, nothing indicated that the *Emericella* species were viable. Hence, these taxa could also belong to the dead fraction as well as *A. puulaauensis* and *A. monidii,* which were detected by the metagenomics approach, but were not detected either with the classical approach, while they should be able to grow in 5 days at 37 °C (Samson et al. 2014, Jurjevic et al. 2012).

Among those five detected genera, the common species such as *A. alternata*, *A. niger A. nidulans, A. versicolor* and *P. chrysogenum* were each time detected with the 3 approaches, except for *P. chrysogenum* in sample CR 9-1 which was not detected with the two NGS approaches and *A. nidulans* in sample CR4-1 which was not detected with the classical analysis. These 5 species are commonly observed in indoor environment. *A. alternata* is considered as an outdoor species, while the other species are ubiquitous indoor species, which are frequently associated to water-damaged buildings (Andersen et al. 2011). However, as shown in Table 7.1, 3 houses did not show

moisture or water damage (houses 1, 2 and 9) while their fungal community was dominated by *A. versicolor*, *Cladosporium* species and *P. chrysogenum* (Table 7.2). It has been hypothesized that fungal diversity is higher in water-damaged houses than in non-water damaged houses (Pitkaranta et al. 2011). However, in this study, based on the ANOVA results, the correlation between the fungal diversity and moister or water damage was not found. However, more houses should be sampled to increase the data need to confirm this observation.

In terms of diversity, as expected, the species richness observed with the massive parallel sequencing is superior to the one observed with classical tools whatever the database considered as reference for the clustering. Indeed, a maximum of 9 taxa per house was observed with the clustering with UNITE database, 8 with the ITSone, against 6 for the classical protocol. Despite these variations, the richness observed with the two bioinformatics approaches was not significantly different (P value = 0.3823). However, this absence of significance could be explained by the higher number of undetermined taxa observed with the ITSone database as compared with the UNITE database. When the comparison was made after exclusion of the 'undetermined' species, the metagenomics approach using the UNITE database yielded an observation of a significantly higher fungal diversity. The detection of 'undetermined' species ('uncultured' species) with the ITSone database could also partly explain why the identity of the house with the highest number of fungal species detected is different for both metagenomics approaches. These observations permit to suggest that the type of sequences included in the UNITE database allows a better taxa discrimination than that contained in the ITSone database. Indeed, the ITSone database contains only ITS-1 sequences (Fosso et al. 2012), which are known to be useful for fungal species discrimination (Nilson et al. 2008, Schoch et al. 2012). The UNITE database contains the entire ITS sequence i.e., the ITS-1, the 5.8S rDNA and the ITS-2 regions (Kõljag et al. 2005, Abarenkov et al. 2010). So, even if the 5.8S rDNA and the ITS-2 regions are known to be less informative for fungal species discrimination (less variable than the ITS-1), the results obtained in this analysis suggest that the combination of the 3 regions allows a better discrimination than the ITS-1 alone.

Among all the species identified with the classical and the NGS approaches, some taxa were undetermined. With the culture-based protocol, these taxa were not identified due to an absence of identifiable structures (e.g., reproductive structures) or to the presence of unknown species. They were called "infertile mycelium" or "undetermined yeast". In this study, this undetermined group corresponded to less than 3 % of the total relative abundance observed on plate. Uncultured taxa were also observed with the NGS approach, but as elaborated above, only for the clustering performed with the ITSone database and corresponded to 14.30 % of the total relative abundance observed in this analysis.

In addition to the reads corresponding to "uncultured taxa" in the ITSone database, some reads were not attributed to an OTU. These undetermined reads reached 37. 29 % of the reads with the ITSone and 28.86 % of the reads with UNITE (Appendix B – Fig. B3). This poor identification could be due to different drawbacks i.e., an insufficient quality of the reads (and therefore they were excluded from the clustering analysis, or were giving no match with the database because of sequencing errors, or low quality overlap between the ITS-1 and ITS-2 region) and insufficient ITS sequences in the database. Therefore, it is needed for the future to extend the ITS database with more sequences of other and new species.

Additionally, according to some studies, the use of the ITS region could be not sufficiently adequate for the correct identification of some fungal taxa up to the species level (Bellemain et al. 2010; Schoch et al. 2012). This observation would favor the use of a genetic marker combination to identify fungal taxa at the species level, such as ITS, actin genes, beta-tubulin gene, elongation factors genes, Small Subunit rRNA genes (SSU), Large Subunit rRNA genes (LSU)… However, the use of such other markers requires adapted bioinformatics tools (combination of the markers, and linking them to the same isolate) and the development of additional databases, as it was suggested for the fungal barcoding (Schoch et al. 2012).

Despite the higher discrimination observed with the NGS approaches as compared to the classical method, some results could be explained by the low quality of the reads. This quality issue could explain why *P. chrysogenum* was not found with the 2 bio-informatics analysis performed on sample CR9-1 sequencing data, while it was observed on plate during the classical analysis. Similarly, in sample CR3-3, *A. subversicolor* was retrieved only with the UNITE database (while the OUT is present in the ITSone database), while *A. versicolor* was detected by the three workflows. Knowing the close genetic relationship between *A. versicolor* and *A. subversicolor* (Jurjevic et al. 2012) and the few nucleotide variations existing between their ITS regions, the detection of *A. subversicolor* could be attributed to a low quality of reads which could have inferred an incorrect determination. This hypothesis could support the fact that a high read quality is required to perform metagenomics analysis in order to avoid as many determination biases as possible (Thomas et al. 2012, Tonge et al. 2014). This also underlines once more the recommendation that additional markers as well as complete database should be used for correct fungal determination, up to species level.

All along the study, the sampling focused specifically on rooms frequently visited by the patient during everyday life. So, most of the samples were collected in the bathroom, the bedroom, the kitchen and to a lesser extent in the living room. As observed in other studies, the bathroom and bedroom were the most contaminated rooms with a relative abundance higher than 30 % retrieved with each approach. There the most frequently observed fungal contaminants were *P.*

chrysogenum and *A. versicolor*. However, it is important to note that the 2 rooms were also the most frequently sampled rooms. Indeed, depending on the house configuration, kitchen and living rooms were not each time sampled. Thus, even if the observations match with others observed in other studies, it seems interesting to increase the number of sampling places in order to confirm these observations.

As previously elaborated, with the exception of *Emericella*, the 4 other genera observed during this study are all common in indoor environments. Yet, these genera are known to produce allergens and were associated in some studies to respiratory diseases such as allergies or asthma (Garrett et al. 1998), especially the *Alternaria* and the *Cladosporium* species. Interestingly, *Cladosporium* species were the major contaminants together with *P. chrysogenum* in the house 1 and 9 which were the only sampling residences where no health problems were detected. In this study, however, according to the ANOVA analysis, no significant association between species richness and health problems was observed. Increasing the number of sampled houses is however needed to confirm this observation.

However, these environmental data are not sufficient to assess the causal link between fungal exposure and health problems. Immunological tests are needed to confirm fungal sensitivity. However, these are still limited to few species commonly observed in indoor environment and detected by culture based-methods such as *A. fumigatus*, *A. versicolor*, *A. niger*, *Cladosporium* species and *P. chrysogenum*. But as it was observed in this study, some other species are present in indoor air such as *A. puulaauensis*, *A. monodii*, *Cladosporium halotolerans* and *Emericella* species. So, to improve the understanding about the fungal impact on health, it is important to include these species in studies on fungal allergy and fungal sensitivity. Moreover, as it was observed for pollen, the absolute quantification of each fungal allergen should be determined to confirm their impact on health (Kaarijeveld et al. 2015). In this study, because NGS analysis requires a PCR amplification step and because of the problem of the copy number of the ITS region (see chapter 2), no absolute quantification of the fungi present could be performed with this technology. However, based on the number of reads observed for each taxon, and comparing these with the abundance of colonies found for each species (Table 7.2) we can consider a "semi-quantitative" estimation of their contribution in each cluster and so we can obtain an estimation of their contribution to the diversity observed in each sample. Nevertheless, in the future, more immunological qualitative and real quantitative data are needed to clearly understand the causal link existing between indoor airborne fungi and health.

In conclusion, this study describes an analysis of the fungal diversity performed by 3 different ways: a classical culture based protocol and two bio-informatics analyses based on massive parallel sequencing data. The comparison between the classical protocol and the NGS approaches

reveals a higher diversity detected with the non-culture based methods. When comparing the two bio-informatics approaches (i.e., based on the UNITE and ITSone database) both differed on the level of discrimination. Indeed, the analysis performed with the ITSone database showed some undetermined species that could be identified with the UNITE database, such as *A. monodii, A. puulaaunesis, A. fumigatus, C. halotolerans, E. undulata* and *E. olivicola.* According to their growth conditions, the detection of these taxa also suggests that current culture protocols used in monitoring should be adapted in order to detect a larger diversity on plate. Moreover, this study demonstrates that the use of NGS analysis as an open approach, multiplex detection tool could increase the number of species detected during one single analysis compared to the culture-based approach. This could reduce the turn-around-time required to detect fungal species and thereby could improve the treatment of contamination. Additionally, our results suggest that the use of NGS analysis performed on the entire ITS region allows a better species discrimination than a culture based method or metagenomics analysis based on the ITS-1 only, although some improvements of current ITS databases are needed. Therefore, NGS could be promising and could be more informative to understand the fungal diversity in indoor air and the link with the human health.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 8

General conclusions and future perspectives

Even if associations between indoor fungal contamination, indoor air pollution and health problems, especially respiratory diseases (asthma, allergies, rhinitis, SBS…), have been established, the scientific evidence for the causal link between indoor airborne fungal contamination and public health issues is still poorly documented. This could be partly attributed to the routine analysis protocols which are yet based on classical culturing methods. Although culture-based methods are useful and have increased the knowledge in the field of fungal contamination, some drawbacks have been observed such as an underestimation of the diversity, the impossibility to detect the "unknown" fraction (dead cells or uncultivable species), etc. Moreover, commercial extracts currently used for immunological tests and which are needed to make the causal link between fungal contamination and health issues, are limited to a few species such as *Alternaria alternata*, *Aspergillus fumigatus*, *Candida albicans*, *Cladosporium herbarum* or *Penicillium chrysogenum* (O'Driscoll et al. 2005). Indeed, eventually, a direct exposure ('intake') of an affected person to the detected fungal species should be demonstrated by immunological tests where specific antibodies recognizing those allergens are present in the serum of that person. These studies are hampered by the lack of representative, commercial extracts, containing the allergens of species that are frequently found indoor. In this context, the aim of the present PhD research was to develop and implement a molecular platform that allows to collect more data about the indoor airborne fungal diversity and especially the composition of its "unknown fraction". Through the future use of these tools in routine analyses, the collected data could be used to develop immunological tests required to improve the scientific evidence to assess the link between indoor airborne fungi and public health issues which will contribute to the definition of the most clinically relevant indoor airborne fungal species.

The first part of this PhD research had a technological aim and consisted of the development of different molecular tools, from simplex (qPCR SYBR®green methods) to multiplex (HRM, Luminex technologies and massive parallel sequencing) methods, focused on indoor airborne fungi. Through the development of molecular methods, the goal was to improve the detection of fungal contaminants, including the uncultivable and/or dead fraction, as compared to the currently used classical monitoring methods. A special focus was put on the feasibility of the developed methods to be used in a routine environment. This is required for the Scientific Institute of Public Health (WIV-ISP), the location where this PhD research has been performed, to be able to apply these tools to fulfill its mission and tasks in support of a proactive public health policy. Moreover, the molecular tools (qPCR, HRM, Luminex xMAP® and the NGS metagenomics approach) as well as the sampling protocol implemented during this PhD work could be used in the future in the framework of the monitoring and studying of other airborne bio-contaminants such as pollen, outside airborne fungi and spores, bacteria or viruses.

The second part of this PhD research had a scientific aim, with the application of the developed

tools to specific case studies to improve the current knowledge on fungal contamination, including on the indoor airborne fungal diversity present in Brussels. The insights obtained could eventually lead to the development of new immunological tests, to allow a better understanding of the link existing between indoor airborne fungi and public health.

The fungal monitoring starts with the air sampling. This must be representative for the species present in the indoor air at the time of sampling. All environmental sampling performed during this PhD work was done with an efficient protocol based on the use of the Coriolis®μ air sampler. Through the use of a special collection liquid compatible with classical culture-based and molecular protocols, but also thanks to the high volume of air sampled, which is limited with other air samplers, this apparatus has improved the sampling of indoor airborne fungi, including that of dead and/or uncultivable species, and species present at low concentration. So, the use of this air sampler in future routine analysis could improve the indoor air monitoring and could increase the data available on these airborne contaminants. During this PhD work, the accompanying protocols for the extraction of high quality DNA, free of inhibitors for downstream molecular reactions, have been successfully developed, starting from the collection liquid obtained from the Coriolis® μ sampler.

The development and application of qPCR tools (chapters 2 and 3), as well as the Luminex xMAP® tool (chapter 5), have demonstrated that molecular tools could improve the routine analysis by a decrease of the turn-around-time (TAT), while detecting at least the same fungal species as was done with the classical culture-based methods. As molecular tools are faster than classical culture-based protocols, their use in a monitoring framework of targeted species, could improve the chain of communication of the results to the involved team, e.g. in the framework of the CRIPI investigations. By advancing the communication to the medical team, the treatment of the patient could be improved as well. For example the qPCR tool developed for the detection of *E. jeanselmei* (chapter 3) could enhance the monitoring of water from air-conditioning systems by a drop of the TAT from 21 days for the classical culture based-protocol to 3 days for a molecular assay. The *E. jeanselmei* qPCR assay also allowed demonstrating for the first time the presence of this pathogenic species in indoor air samples (chapter 6). As it was not found by the classical monitoring methods, this indicates that the molecular methods not only have the advantage of reducing the TAT, but also of detecting the dead and/or uncultivable fungal fraction, that could also be allergenic.

Although the chosen qPCR chemistry (i.e. SYBR®green) has advantages in terms of assay costs, the discrimination of fungal species is not always as evident to do with qPCR SYBR® green because numerous species are genetically close and differ from each other by a few nucleotides only. This issue was addressed during the development of the qPCR tool for the identification of *A. versicolor* (chapter 2). A solution was provided through the development of a HRM tool for the

discrimination of *A. versicolor* and the two genetically close species *A. creber* and *A. sydowii* (chapter 4). In this PhD work, the discrimination of the 3 *Aspergillus* was used as a study case to demonstrate that HRM could be easily implemented in routine analysis for the monitoring of "problematic" (i.e., genetically closely related) species or in the framework of the BCCM collection in order to confirm the identification of new strains introduced in the collection. Moreover, this tool will make it possible in the future to investigate the differential impact of the three genetically closely related species, *A. versicolor*, *A. creber* and *A. sydowii*, on public health.

Nevertheless, because of the number of targets that can be detected during a single run is limited, qPCR technologies did not appear to be the most appropriate alternative in the framework of the monitoring of indoor airborne fungal contamination. Indeed, environmental samples could contain more than one species. Today, ten species are commonly considered as the most frequently occurring fungal species in indoor air (i.e., *A. alternata*, *A. creber*, *A. fumigatus*, *A. sydowii*, *A. versicolor*, *C. cladosporioides*, *C. herbarum*, *P. chrysogenum*, *S. chartarum* and *U. botrytis*), although a dozen of others have been detected as well but with less occurrences. So, if a molecular tool has to be used in the context of a monitoring programme, for the sake of time and sometimes scarce sample availability, it would be preferable that this tool could detect at least the most frequently occurring species in a single analysis. In this context, the Luminex xMAP® assay developed in this PhD study is a valuable alternative to the culture based-protocols (chapter 5). Another advantage of the Luminex xMAP® is that it could detect up to 50 targets in a single run (when using the MagPix instrument), so additional targets could be easily added to the current assay in the future. The fact that the developed fungal Luminex® assay is based on a PCR using universal ITS primers, will facilitate the extension of the assay, i.e. only additional probes (specific for the other species to be targeted), should be included. Additionally, as already elaborated above, the TAT is highly reduced, as compared to the culture-based methods which could take between 5 to 21 days depending on the targeted species.

During the development of these molecular tools, it was noticed that the guidelines or the minimum of requirement for the development of molecular tools are poorly documented in the field of fungal detection. Until now, no document defined exactly the levels of specificity, the PCR efficiency or the thresholds of the LOD required to assess the performance of a new molecular tool specific for fungal detection in environmental samples. Additionally, no recommendation exists on the number of positive and negative strains which should be used to validate the specificity of molecular tools for these applications. Therefore, in this PhD study, it was decided to assess the performance of the developed tools according to the recommendations available in other domains such as GMO detection. If the resort to these recommendations was helpful to confirm the validity of tools, it was also observed that these criteria were not fully adapted to fungal detection. As guidance documents are required to verify the performance of

molecular methods, but are also vital to attest the quality of a molecular method, it is essential in the future to develop specific guidelines to harmonize the molecular tools available for fungi detection in environmental samples and subsequently, to improve the quality of the tools used in routine.

Considered as an open, non-targeted multiplex assay, the use of massive parallel sequencing, coupled to a metagenomics analysis targeting the ITS region (considered as the most informative region for fungal determination), offered the advantage to subsequently investigate the cultivable fraction as well as the non-cultivable fraction, without knowing *a priori* the composition of the fungal community.

This NGS-based approach was developed on air samples collected in contaminated houses using the ITS region. However, because of the size of ITS region is estimated to be around 800 bp and because of the NGS sequencer most likely available in routine labs (benchtop model, MiSEQ) delivers at maximum 2x 300bp reads, the ITS region was amplified in two parts, i.e. ITS-1 and ITS-2 using universal primers, and assuring an overlap between both PCR fragments. The sequencing data was analyzed by clustering using two databases as reference i.e., ITSone (Fosso et al. 2012) containing only sequences form the ITS-1 region and UNITE (Kõljag et al. 2005) containing sequences from ITS-1 and ITS-2 regions. During this analysis, a higher diversity in the samples was observed after the bio-informatics analysis performed with the UNITE database. This observation suggested that the use of the entire ITS region is more informative for the fungal determination than the ITS-1 alone (chapter 7).

It should be noted that poor read quality could affect the correct determination of closely related species, e.g. for the species from the *Versicolores* complex and especially *A. versicolor* and *A. puulaauensis*, for which the ITS region differs only in a few nucleotides. Therefore, the determination of these species could be considered as suspicious if the quality of the reads is too low, especially in the region of the overlap between ITS-1 and ITS-2. It might be interesting to put a quality threshold or 'certainty' when performing the bioinformatics analysis, and to only define the genus level, if the quality is below this threshold. Indeed, the use of this threshold could filter the reads with a high quality level which could be used to determine until the species level from those with low quality for which the determination should be limited at the genera level. The application of this type of filter will increase the trueness of the results. Alternatively, this problem could also be partly overcome by the use of the PacBio sequencing technology. Indeed, as the PacBio platform is able to deliver large read lengths (until 14 kb), the sequencing of the entire ITS region could be performed in one single read, which could improve the identification based on sequencing comparison with the databases. This proposal will be investigated in the future to improve the metagenomic analysis performed during this PhD study.

During this analysis, also many unidentified reads were observed. This could be due to a low

quality of the reads which could impact their identification based on the reference sequences available in the databases. The completeness of the database is also an issue which could explain the number of unidentified reads. Indeed, some reads could be considered as 'unidentified" because of the absence of a reference sequence in the database. In the future, the metagenomics analysis would benefit of extended databases with ITS sequences of more fungal species.
Finally, in some cases the use of ITS markers (ITS-1 and ITS-2) is not sufficient to delineate the species from the reads. Additional markers, e.g. the beta-tubulin gene, the calmoduline gene or gene encoding the elongation factor, might be needed for full species identification as it was observed for fungal barcoding (Schoch et al. 2012) and as it is also currently done for the BCCM/IHEM collection using Sanger sequencing. So, new databases containing fungal markers other than ITS-1 and ITS-2 should be created, together with adapted bioinformatics analysis pipelines. However, it might be difficult to link the data of several markers (all in separate reads) in a metagenomics approach to the same 'isolate' present in the sample. Hereto, more advanced metagenomics approaches might be needed, not involving targeted sequences (PCR fragments per marker), but relying on shotgun metagenomics and full genome reconstruction. At the moment, this is not yet completely feasibly in terms of coverage (and hence cost per sample) or in available user-friendly data analysis tools. Alternatively, it should be investigated whether PacBio sequencing might be helpful to resolve this issue. Anyway, the use of the whole genome sequencing applied on pure strains from fungal collections, such as the BCCM/IHEM, might be interesting to increase the information on 'discriminative' markers for species level determination.

The NGS analyses performed during this PhD study have demonstrated that more information on the "dead fraction" and the uncultivable species could be collected, as compared to the classical monitoring tools. And as these species could have an impact on health, the use of NGS to investigate the entire fungal diversity could allow filling the gap in the link between fungal contamination and public health issues. Based on the obtained results (chapter 7), it might be interesting to extend the incubation time for the classical analysis in order to be able to detect also species like *Emericella undulate* or *Emericella olivicola.*

The application of the metagenomics approach to the water and air samples collected in the offices of the European Union commission, not only allowed to obtain a broader view of the diversity of fungi present in those samples, but also confirmed what had already been suggested by qPCR, i.e. the first detection of *E. jeanselmei* in indoor air (chapter 6). By comparing the presence of *E. jeanselmei* in water and air samples, taken at different distances from the air-conditioning system, also allowed hypothesizing that the distance between the source of the contamination and the sampling site and the contamination load could probably affect the spreading of biological material originating from *E. jeanselmei* as it was observed for other species from indoor and outdoor environment.

The NGS approach was also applied to investigate the indoor airborne fungal diversity in residences in Brussels. This study confirmed that classical protocols underestimate the fungal diversity existing in indoor air (chapter 7), and that NGS-based metagenomics approaches would be a valuable alternative tool to be used to increase the insight in the fungal diversity in air. However, as during this study the sample size was too limited to make significant associations between the airborne fungal species present and the reported health issues, it would be important to repeat the analysis in the future with additional houses (with people with and without reported health issues) included. Moreover, as these analyses were restricted to the Brussels area, it would be interesting to enlarge the sampling to the complete Belgium territory. Increasing the number of data available on indoor airborne fungi by enlarging the sampling area, will contribute to verify the observations made for Brussels and to improve the understanding between fungal contamination and health issues. However, it seems interesting to include some of the species from the "unknown" fraction observed in this study (i.e., *A. puulaauensis, A. monodii, C. halotolerans, Emercilla* species and *E. jeanselmei*) to immunological studies in order to define their implication in health problems. Additionally, the allergenic potential of these species could be investigated through whole genome sequencing performed with a metagenomics shotgun method, where all DNA content is sequenced (not only the ITS marker). In this context, the data collected during this PhD study, as well as those which will be collected in the future through the protocols developed during this work, will be useful in the framework of the bioSENS project (O. Denis, WIV-ISP) in order to develop new immunological testing for fungal allergies assessment. With the development of new tests, which include fungal species from the unknown fraction, the gap in the link between fungal contamination and public health could be filled. Indeed, if immunological tests are more representative to the fungal diversity, more accurate correlations (i.e., direct exposure) between the fungal diversity of indoor air and the patient's sensitivity could be made and the clinical relevance of each species could be established. This information would also allow to adapt the multiplex tools such as the xMAP® tools to detect the clinically relevant species in order to generate data usable by clinicians.

The technological objective of this PhD research was to develop molecular tools usable in a monitoring framework. This means that the tools should be qualitative (i.e. identification of the correct species), but also quantitative (i.e., the load of the fungal contamination). However, harmonized guidelines or norms do not exist to define a standard protocol in order to quantify the airborne fungal contamination or to define a threshold of indoor air contamination. Today, most of the microbial quantification tools are expressed as colony forming units (CFU) or in CFU/L air. Because these tools are culture-dependent, competition factors, difference in speed of growth, the presence of dead or uncultivable species could induce biases giving estimation errors as it was observed for the fungal detection.

As all the molecular tools developed during this project are based on the ITS region, an exact

quantification is not possible, as elaborated in chapter 2. Indeed, the copy number of the ITS region is variable, between species but also between strains from a same species. Therefore, molecular tools based on the ITS marker should be considered as semi-quantitative, not as quantitative, which was illustrated in this work (by each time comparing with the number of CFUs found on plate). Moreover, tools such as the Luminex xMAP® or massive parallel sequencing contain at least one step of PCR amplification. So, due to the exponential amplification of the DNA, the quantification cannot be done rigorously, i.e. the detected fluorescence or the number of reads does not translate directly to the number of DNA molecules in the sample. These methods are therefore only semi-quantitative, as elaborated in chapter 5 and 7. To develop quantitative molecular tools, more studies would have to be made to find a marker that is very well conserved all along the fungal kingdom, and with a constant copy number, preferably a single copy gene, and which could be used for the quantification. Hereto, there is a need for more whole genome sequence data of fungal species (and this for multiple strains per species) to screen the fungal genome in order to find a conserved single copy marker or a unique single copy marker per species, as was previously found for A. fumigatus (Herrera at al. 2009).

Some solution could also be found in the use of new technologies such as digital PCR where each targeted DNA is separated from the others and amplified individually, as was done for *Candida albicans* in blood (Schell et al. 2012). The quantification of each PCR reaction is then estimated based on the emission of fluorescence during the amplification. So, through the use of digital PCR, the copy number of ITS could be determined and then, a quantification system could be set up. This will involve the use (and the development if needed) of specific primers targeting the species to quantify. However, as digital PCR requires an *a priori* selection of the fungal species, an absolute quantification of each contaminant cannot be made without a prior screening of the diversity. This screening step, performed before the quantification step, could be done through a metagenomics NGS analysis.

These considerations on NGS and fungal quantification could be an interesting topic for a follow-up project of this PhD study focused on the detection and identification of the most clinically relevant species.

In the future, the combination of molecular detection, such as xMAP® tools or massive parallel sequencing, with an efficient quantitative tool (which will involve the whole genome sequencing of several important fungal contaminants as elaborated above) will improve greatly the monitoring of indoor air pollution. These tools applied to a significant high number of sampled contaminated and non-contaminated control houses will increase the amount of data available on indoor airborne fungal contaminants. This information can then be used to develop the specific immunological tests to demonstrate the direct exposure to fungal contaminants. Eventually, this will contribute to filling the gap in the causal link between indoor airborne fungi and public health issues.

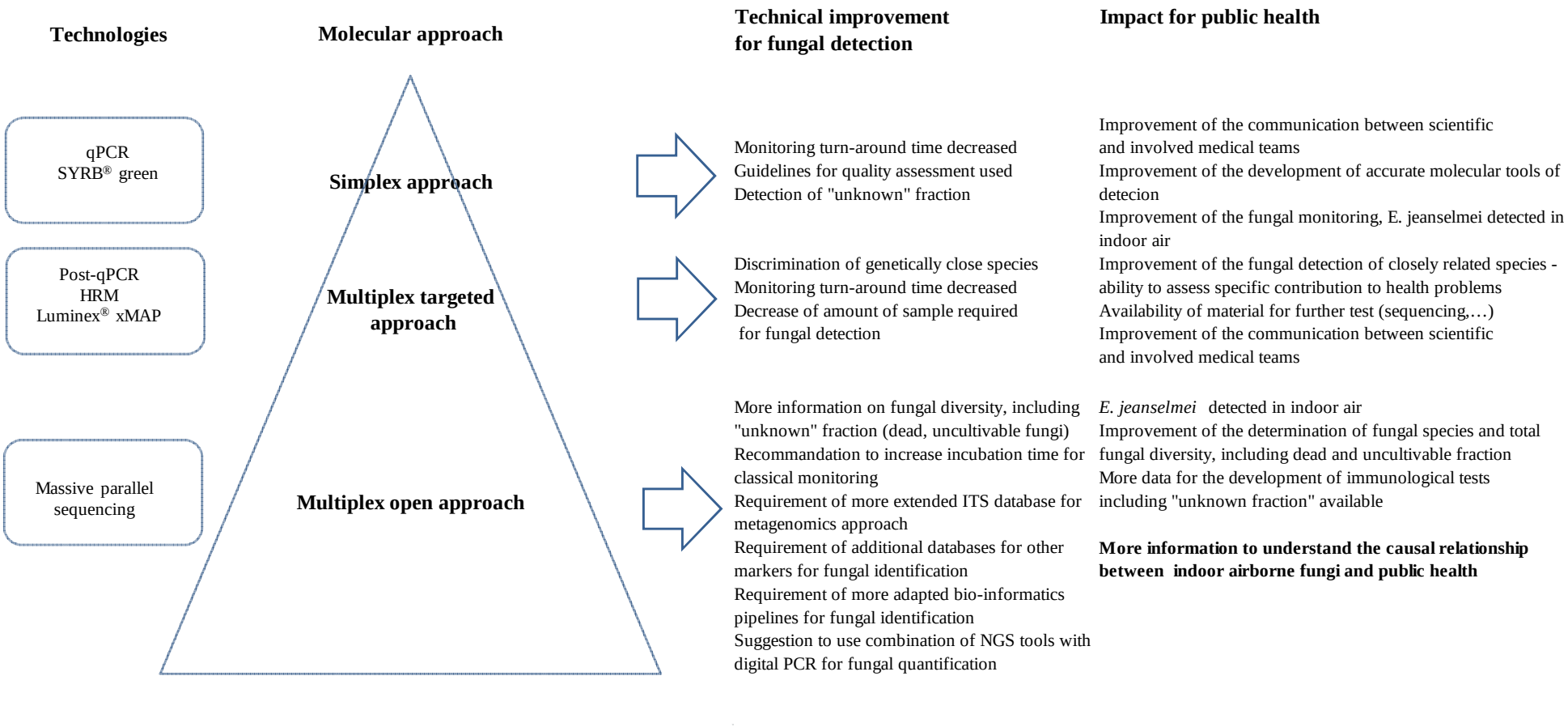


Figure 8.1: Schematic representation of the main results of the thesis and their implication for public health

Appendix A

Supporting information for Chapter 4

Table A1: Spike test results

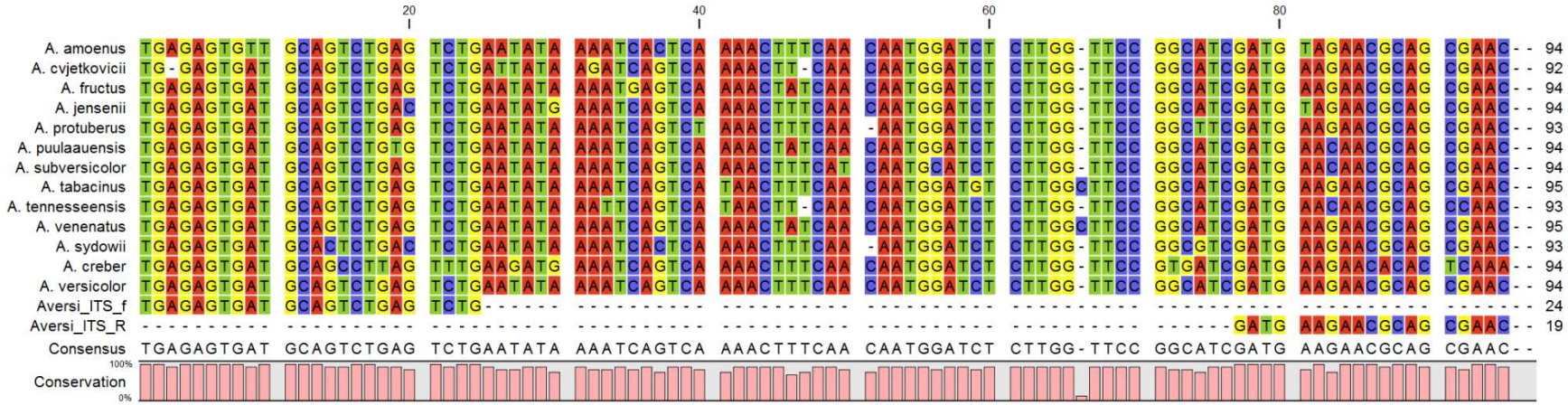
Sample	Sample type	Species [a]	Amount of DNA/HRM analysis (ng) [b]	Salmon Sperm [c]	Cluster [d]	Percentage of confidence (%) [d]	Number of positive detections [e]
Control							
NTC water	water only	water only	0	No	N/A	/	
NTC salmon sperm	Salmon sperm only	Salmon sperm only	10	Yes	N/A	/	
Positive control	Pure culture	*A. creber*	25	No	3	95.74 ±7.07	4/4
Positive control	Pure culture	*A. creber*	25	Yes	3	97.32 ± 3.90	4/4
Positive control	Pure culture	*A. sydowii*	25	No	2	97.91 ± 3.26	4/4
Positive control	Pure culture	*A. sydowii*	25	Yes	2	98.89 ± 0.97	4/4
Positive control	Pure culture	*A. versicolor*	25	No	1	99.16 ± 0.49	4/4
Positive control	Pure culture	*A. versicolor*	25	Yes	1	97.99 ± 2.38	4/4
Spiked environmental samples							
Sample 3	Spike	*A. creber*	25	No	3	99.08 ± 1.14	8/8
Sample 3	Spike	*A. creber*	25	Yes	3	98.52 ± 1.41	8/8
Sample 3	Spike	*A. creber*	LOD [e]	No	3	97.97 ± 1.57	5/8 [$]
Sample 3	Spike	*A. creber*	LOD [e]	Yes	3	98.22 ± 1.57	7/8
Sample 3	Spike	*A. sydowii*	25	No	2	96.43 ± 2.29	7/8[$]
Sample 3	Spike	*A. sydowii*	25	Yes	2	98.47 ± 1.51	8/8

Table A1: *Continued*

Sample	Sample type	Species [a]	Amount of DNA/HRM analysis (ng) [b]	Salmon Sperm [c]	Cluster [d]	Percentage of confidence (%) [d]	Number of positive detections [e]
Spiked environmental samples							
Sample 3	Spike	*A. sydowii*	LOD [e]	No	2	98.65 ± 0.99	6/8
Sample 3	Spike	*A. sydowii*	LOD [e]	Yes	2	99.07 ± 0.18	7/8
Sample 3	Spike	*A. versicolor*	25	No	1	98.25 ± 1.12	7/8
Sample 3	Spike	*A. versicolor*	25	Yes	1	98.38 ± 0.85	8/8
Sample 3	Spike	*A. versicolor*	LOD [e]	No	1	97.70 ± 1.56	5/8[$]
Sample 3	Spike	*A. versicolor*	LOD [e]	Yes	1	98.98 ± 0.93	8/8

[a] Determined with classical methods i.e. plate culture and microscopic analysis (determination and counting) in Libert et al. 2015.
[b] Amount of DNA extracted from air samples of 1.5 m³; DNA amount determined with a Nanodrop® 2000; 5 µl of extracted DNA (5ng/µl) were used in a 20 µl - HRM analysis.
[c] Salmon sperm (salmon sperm) DNA carrier added (10 ng).
[d] Cluster and % of confidence ± standard deviation (SD) defined with the Biorad Precision Melt Analysis software 1.2 (Temse, Belgium) .
[e] A sample is defined as positive for a specific species, if an amplicon is obtained, if the observed T_m corresponds to the Tm defined by Libert et al. (2015) for *A. versicolor* (i.e., 76.5 ±0.18 °C) and if the sample is classified in the same cluster as the cluster defined for its respective positive control with a confidence > 95%.
[f] LOD defined as limit of detection i.e. 0.1 pg for A. versicolor and 0.5 pg for *A. creber* and *A. sydowii*.
[$] At least one repetition is considered as negative due to a confidence below the 95 % threshold.

Figure A1: Alignment of the *9 Versicolores* species and the *Aversi_ITS* primers



This alignement was made using with the consensus sequences of all publicly available ITS sequences of *Versicolores* species (i.e., *Aspergillus amoenus*, *Aspergillus austroafricanus*, *Aspergillus creber*, *Aspergillus cvjetkovicii*, *Aspergillus fructus*, *Aspergillus jensenii*, *Aspergillus protuberus*, *Aspergillus puulaauensis*, *Aspergillus subversicolor*, *Aspergillus sydowii*, *Aspergillus tabacinus*, *Aspergillus tennesseensis*, *Aspergillus versicolor* and *Aspergillus venenatus*) and the Aversi_ITS primers (Aversi_ITS_f and Aversi_ITS_r).
Consensus (last lign of the alignment corresponds to a consensus sequence defined by the software. The conservation level among each sequence (0 to 100 % of conservation) is reprensented in the pink rectangles at the bottom of the figure.
The alignment was made and visualized with the CLC sequence viewer 7 (Qiagen Benelux, B.V., KJ Venlo, the Netherlands).

Table A2: Melting temperature obtained for the species from the *Versicolores* group

Species	T_m (°C)
A. amoenus	76.3
A. cvjetkovicii	76.8
A. fructus	76.4
A. jensenii	76.7
A. protuberus	76
A.puulaauensis	76.4
A. subversicolor	76.9
A. tabacinus	77.1
A. tennesseensis	76.1
A. venenatus	76.3
A. sydowii	76.6
A. creber	76.2
A. versicolor	76.5

Melting temperature calcultated with OligoAnalyzer V3.1 software (IDT,https://eu.idtdna.com/calc/analyzer).

Appendix B

Supporting information for Chapter 7

Table B1: Full data obtained during the classical culture-based and the bio-informatics approaches

House	Sample ID	Room	Classical analysis		NGS						
						Metagenomics analysis					
						ITSone DB[1]			Unite DB[2]		
			Taxa	CFU/plate	Total # of reads	Taxa	Reads in OTUs	Abundance/ OTUs	Taxa	Reads in OTUs	Abundance/ OTUs
1	CR1-1	kitchen	*P. chrysogenum*	8	29,666	*P. chrysogenum*	22,359	3,500	*P. chrysogenum*	25,187	25,120
						Uncultured fungi		18,859	*A. puulaauensis*		67
	CR1-2	bathroom	*Cladosporium* sp.	8	19,753	*C. cladosporioides*	16,543	5,020	*C. cladosporioides*	17,550	7,274
			P. chrysogenum	15		*P. chrysogenum*		11,523	*P. chrysogenum*		10,274
									C. halotolerans		2
	CR1-3	bedroom	*Cladosporium* sp.	3	13,124	*C. cladosporioides*	10,976	2,183	*C. cladosporioides*	11,469	1,195
			P. chrysogenum	4		*P. chrysogenum*		8,793	*P. chrysogenum*		10,274
2	CR2-1	bedroom	*A. versicolor*	5	6,352	*A. versicolor*	5,843	4,690	*A. versicolor*	4,903	2,897
			Cladosporium sp.	3		*C. herbarum*		1,153	*C. herbarum*		2,006
	CR2-2	bathroom	*A. versicolor*	13	6,526	*A. versicolor*	4,956	3,978	*A. versicolor*	4,809	3,100
			Cladosporium sp.	9		*C. herbarum*		978	*C. herbarum*		1,709
	CR2-3	linving room	*A. alternata*	1	5,163	*A. alternata*	4,158	1,071	*A. alternaria*	4,823	986
			A. nidulans	2		*A. nidulans*		1,180	*A. nidulans*		753
			A. versicolor	6		*A. versicolor*		1,029	*A. versicolor*		2,039
			Cladosporium sp.	1		*C. herbarum*		878	*C. herbarum*		1,014
									Alternaria sp.		31

Table B1: *Continued*

House	Sample ID	Room	Classical analysis		NGS						
						Metagenomics analysis					
						ITSone DB[1]			Unite DB[2]		
			Taxa	CFU/plate	Total number of reads	Taxa	Reads in OTUs	Abundance/ OTUs	Taxas	Reads in OTUs	Abundance/ OTUs
3	CR3-1	bedroom	*A. nidulans*	1	28,847	*A. nidulans*	21,175	3,978	*A. nidulans*	22,662	9,618
			A. versicolor	3		*A. versicolor*		10,584	*A. versicolor*		10,857
			Infertile mycelium	1		*Aspergillus* sp.		1,307	*Aspergillus* sp.		1,987
						Uncultured fungi		5,306	*E. undulata*		181
									E. olivicola		19
	CR3-2	bathroom	*A. nidulans*	2	15,193	*A. nidulans*	13,848	700	*A. nidulans*	14,514	5,365
			A. versicolor	9		*A. versicolor*		3,300	*A. versicolor*		5,526
			P. chrysogenum	11		*P. chrysogenum*		9,848	*P. chrysogenum*		3509
									E. undulata		87
									E. olivicola		27
	CR3-3	living-room	*A. nidulans*	1	28,338	*A. nidulans*	18,417	2,800	*A. nidulans*	22,702	63
			A. versicolor	4		*A. versicolor*		13,817	*A. versicolor*		20,503
						Aspergillus sp.		1,800	*A. puulaauensis*		2,136
4	CR4-1[3]	bathroom	*A. niger*	5	16,337	*A. niger*	16,084	1,064	*A. niger*	15,421	3,107
			Cladosporium sp.	12		*C. herbarum*		2,019	*C. herbarum*		3,093
			P. chrysogenum	15		*P. chrysogenum*		9,978	*P. chrysogenum*		8,211
						Uncultured species		2,932	*A. nidulans*		76
						A. nidulans		91	*Aspergillus* sp.		934
	CR4-2[3]	bedroom/kitchen[4]	*A. alternata*	5	9,328	*A. alternata*	6186	978	*A. alternata*	9,009	1,931
			A. niger	1		*A. niger*		780	*A. niger*		2,625
			Cladosporium sp.	4		*C. herbarum*		1,012	*C. herbarum*		980
			P. chrysogenum	7		*C. cladosporioides*		1,003	*C. cladosporioides*		963
						P. chrysogenum		2,316	*P. chrysogenum*		2,430
						Alternaria sp.		97	*Aspergillus* sp.		80

Table B1: *Continued*

House	Sample ID	Room	Classical analysis		NGS						
						Metagenomics analysis					
						ITSone [DB1]			Unite DB[2]		
			Taxa	CFU/plate	Total number of reads	Taxa	Reads in OTUs	Abundance/ OTUs	Taxa	Reads in OTUs	Abundance/ OTUs
	CR5-1		*A. versicolor*	13	12,324	*A. versicolor*	7,728	2,339	*A. versicolor*	11,182	1,378
			Cladosporium sp.	8		*C. herbarum*		486	*C. herbarum*		189
			P. chrysogenum	14		*P. chrysogenum*		8,590	*P. chrysogenum*		10,190
			Undetermined yeast	2							
	CR5-2	Bedroom	*P. chrysogenum*	7	27,066	*P. chrysogenum*	24,153	19,782	*P. chrysogenum*	26,809	21,578
			Infertile mycelium	1		*C. herbarum*		2,634	*C. herbarum*		5,231
						Uncultured species		1,737			
	CR5-3	kitchen	*A. alternata*	2	19,753	*Alternaria* sp.	19,174	12,661	*Alternaria* sp.	18,708	13,552
			Infertile mycelium	1		*C. herbarum*		5,282	*C. herbarum*		5,140
						Uncultured species		1,231	*A. monodii*		16
6	CR6-1	Laundry room	*A.versicolor*	4	24,937	*A. versicolor*	23,495	5,937	*A. versicolor*	22,305	6,229
			P. chrysogenum	7		*P. chrysogenum*		15,012	*P. chrysogenum*		16,076
						Aspergillus sp.		2,546			
	CR6-2	bathroom	*A. versicolor*	11	18,153	*A. versicolor*	17,516	8,364	*A. versicolor*	15,968	10,003
			P. chrysogenum	15		*P. chrysogenum*		9,152	*P. chrysogenum*		5,965
	CR6-3	bedroom/kitchen[4]	*A. alternata*	2	20,333	*Alternaria* sp.	19,626	11,800	*A. alternata*	18,922	10,703
			A. versicolor	3		*A. versicolor*		7,826	*A. versicolor*		8,219

Table B1: *Continued*

House	Sample ID	Room	Classical analysis		NGS						
					Metagenomics analysis						
						ITSone DB[1]			Unite DB[2]		
			Taxa	CFU/plate	Total number of reads	Taxa	Reads in OTUs	Abundance/ OTUs	Taxa	Reads in OTUs	Abundance/ OTUs
7	CR7-1	Bedroom	*A. alternata*	1	13,673	*A alternata*	9,325	2,805	*A alternata*	12,665	6,169
			A. versicolor	5		*A. versicolor*		978	*A. versicolor*		1,789
						A. niger		1,312	*A. niger*		4,014
						uncultured species		4,230	*A. puulaauensis*		521
									E. undulata		74
									E. olivicola		98
	CR7-2	bathroom	*A. nidulans*	4	9,335	*A. nidulans*	8,459	2,756	*A. nidulans*	9,029	2,978
			A. versicolor	9		*A. versicolor*		1,868	*A. versicolor*		1,736
			P. chrysogenum	12		*P. chrysogenum*		3,050	*P. chrysogenum*		3,703
						uncultured species		785	*A. fumigatus*		612
	CR7-3	kitchen/living room[4]	*A. alternata*	1	11,024	*A. alternata*	11,008	2,690	*A alternata*	10,165	3,589
			A. niger	1		*A. niger*		800	*A. niger*		1,002
			A. versicolor	5		*A. versicolor*		4,060	*A. versicolor*		2,061
			P. chrysogenum	9		*P. chrysogenum*		3,058	*A. puulaauensis*		24
						Aspergillus sp.		400	*P. chrysogenum*		3,152
									A. fumigatus		308
									E. undulata		12
									E. olivicola		17

Table B1: *Continued*

House	Sample ID	Room	Classical analysis		NGS						
						Metagenomics analysis					
						ITSone DB [1]			Unite DB[2]		
			Taxa	CFU/plate	Total number of reads	Taxa	Reads in OTUs	Abundance/ OTUs	Taxa	Reads in OTUs	Abundance/ OTUs
8	CR8-1	bedroom	*A. alternata*	2	7,338	*A. alternata*	6,039	1,233	*A. alternata*	6,965	1,998
			A. versicolor	3		*A. versicolor*		3,584	*A. versicolor*		3,013
			Cladosporium sp.	1		*C. cladosporioides*		1,022	*C. cladosporioides*		1,589
						Uncultured species		200	*Aspergillus* sp.		365
8	CR8-2	bathroom	*A. alternata*	4	15,373	*A. alternata*	14,975	4,798	*A. alternata*	14,439	4,468
			A. versicolor	8		*A. versicolor*		5,204	*A. versicolor*		7,274
			Cladosporium sp.	7		*C. cladosporioides*		1,972	*C. cladosporioides*		2,697
						Uncultured species		3,001			
8	CR8-3	Kitchen	*A. alternata*	1	8,126	*Alternaria* sp.	6,426	780	*A. alternata*	7728	1,201
			A. versicolor	1		*A. versicolor*		2,919	*Alternaria* sp.		389
						Uncultured species		2,727	*A. versicolor*		4,741
									C. cladosporioides		1,397
9	CR9-1	Kitchen	*Cladosporium sp.*	1	5,323	*C. herbarum*	5,040	5,040	*C. cladosporioides*	4,131	846
			P. chrysogenum	5					*C. herbarum*		3,285
	CR9-2	bathroom	*Cladosporium sp.*	6	10,114	*C. herbarum*	9,187	2,800	*C. cladosporioides*	10,085	1,201
			P. chrysogenum	14		*P. chrysogenum*		4,381	*P. chrysogenum*		5,710
						A. alternata		2,006	*A. alternata*		3,174
	CR9-3	bedroom	*Cladosporium sp.*	1	6,005	*C. herbarum*	5,702	1,052	*C. cladosporioides*	4272	1,300
			P. chrysogenum	8		*P. chrysogenum*		2,978	*P. chrysogenum*		3,874
						A. alternata		1,672	*A. alternata*		2,972

Table B1: *Continued*

House	Sample ID	Room	Classical analysis		NGS						
						Metagenomics analysis					
						ITSone DB [1]			Unite DB[2]		
			Taxa	CFU/plate	Total number of reads	Taxa	Reads in OTUs	Abundance/ OTUs	Taxa	Reads in OTUs	Abundance/ OTUs
10	CR10-1	bedroom	*A. versicolor*	4	17,632	*A. versicolor*	15,021	3,956	*A. versicolor*	16,034	3,798
			Cladosporium sp.	1		*C. herbarum*		987	*C. herbarum*		1,063
			P. chrysogenum	7		*P. chrysogenum*		10,078	*P. chrysogenum*		11,173
	CR10-2	bathroom	*A. versicolor*	1	23,829	*A. versicolor*	20,033	2,601	*A. versicolor*	22580	2,169
			Cladosporium sp.	1		*Alternaria* sp.		4,067	*Alternaria sp.*		4,697
			P. chrysogenum	15		*C. herbarum*		1,973	*C. herbarum*		3,173
						P. chrysogenum		10,414	*P. chrysogenum*		12,541
						Uncultured sp.		978			
	CR10-3	kitchen/living room[4]	*A. alternata*	1	28,487	*Alternaria* sp.	25,181	5,149	*Alternaria* sp.	25345	4,523
			A. versicolor	4		*A. versicolor*		4,978	*A. versicolor*		3,694
						P. chrysogenum		14,854	*P. chrysogenum*		17,128
						uncultured sp.		200			

[1]Fosso et al. 2013
[2]Abarenkov et al. 2010
[3]Flat of 2 rooms i.e. a bathroom and a room containing the kitchen and bedroom.
[4]No separation between the rooms. Open space considered as one room.

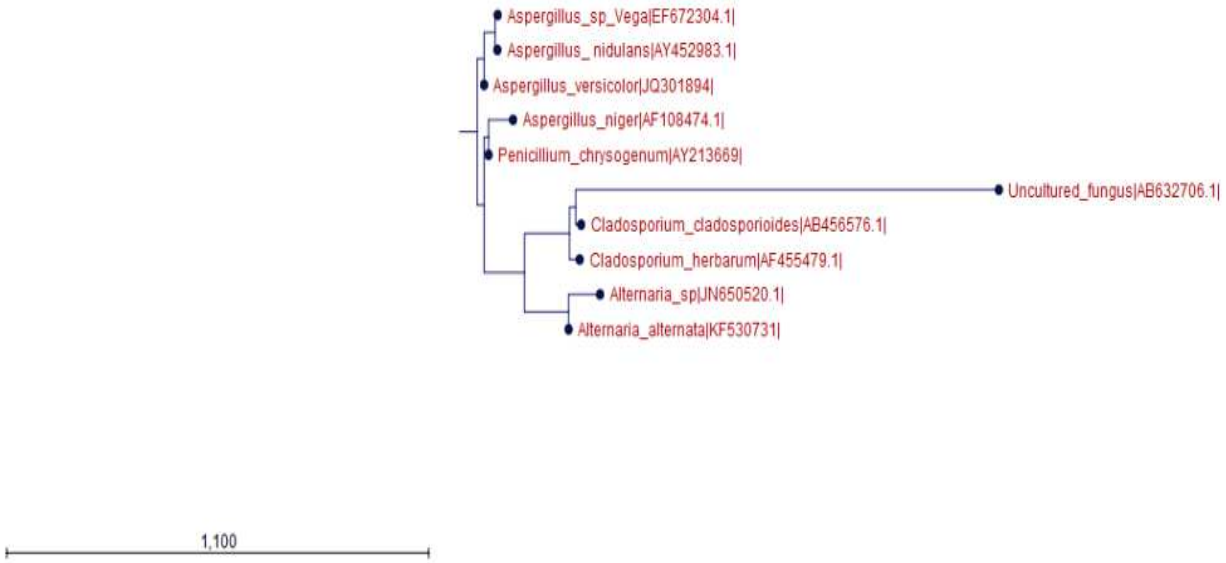


Figure B1 General phylogenetic tree obtained during the clustering analysis of all air samples using the ITSone database as reference

The phylogenetic tree of all OTUs is a Neighbor joining tree constructed with a Jukes Cantor nucleotides model and generated with MUSCLE according to the CLC bio recommendations. All the parameters used during these analyses were those recommended by the software manufacturer.

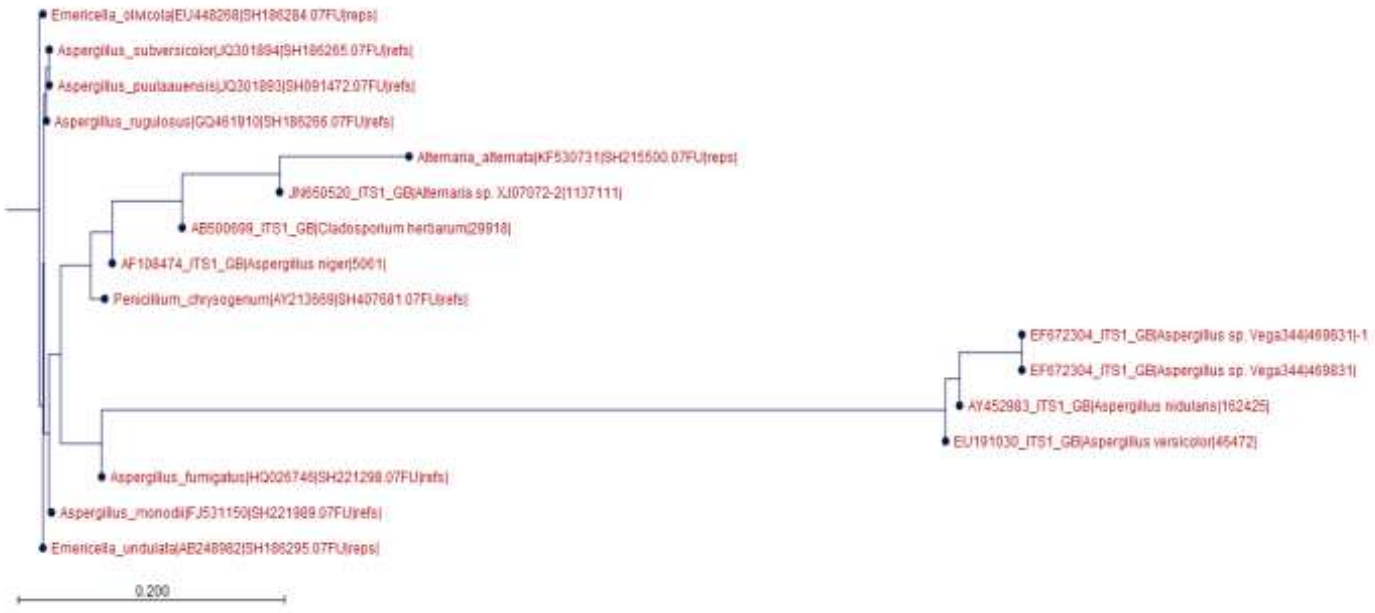


Figure B2 General phylogenetic tree obtained during the clustering analysis of all air samples using the UNITE database as reference

The phylogenetic tree of all OTUs is a Neighbor joining tree constructed with a Jukes Cantor nucleotides model and generated with MUSCLE according to the CLC bio recommendations. All the parameters used during these analyses were those recommended by the software manufacturer.

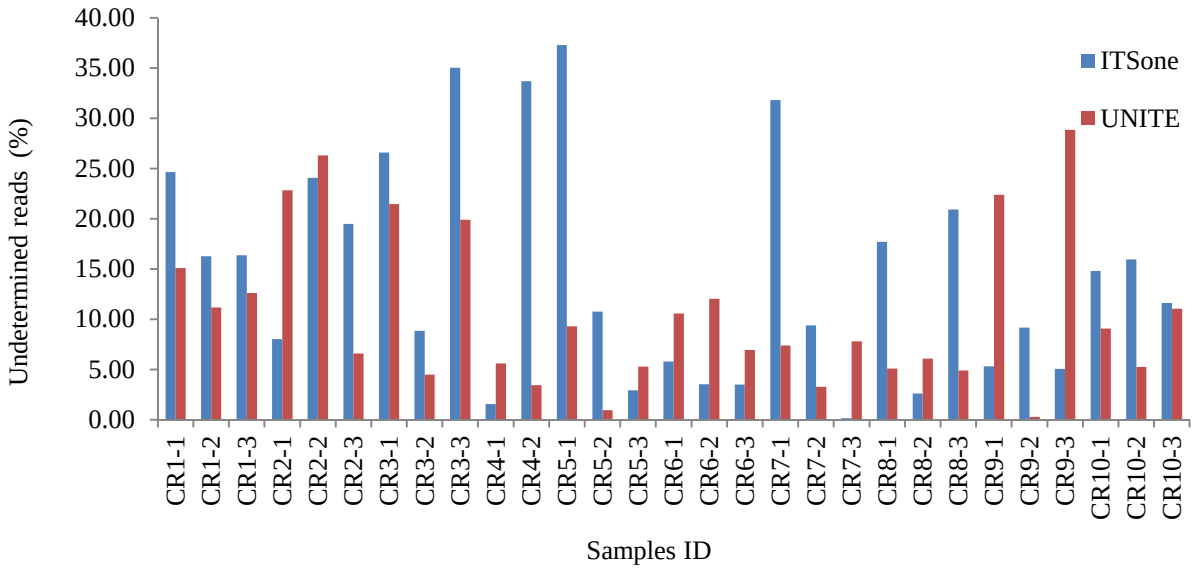


Figure B3: Percentage of undetermined reads per sample
Percentage per sample of the reads with a poor quality or not found in the databases (ITSone or UNITE).

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Curriculum vitae

Xavier Libert was born on August 28th, 1985 in Malmedy, Belgium. In 2009, at the University of Namur (Belgium), he obtained a bachelor's degree in Biological Sciences, followed by a master's degree in Biology of Orgnanisms and Ecology. Since then, he worked at the ISTREA (Institut national de recherche en sciences et technologies pour l'environnement et l'agriculture) in Bordeaux (France) under the supervision of Dr. Soizic Morin. Then, he has teached biology at the Collège Notre Dame de Bellevue at Dinant (Belgium). In November 2012, he started on his PhD project mycoMOLAIR at the Scientific Institute for Public Health (WIV-ISP, Brussels, Belgium) under the supervision of Dr. Ir. Sigrid De Keersmaecker (WIV-ISP) and with the support of Dr. Ir. Ann Packeu (WIV-ISP), Dr. Ir. Nancy Roosens (WIV-ISP) and Prof. Fabrice Bureau (University of Liège) as additional promoters. During his PhD he has also collaborated with Dr. Camille Chasseur (WIV-ISP) and the CRIPI team from Brussels Envirnonment (Brussels, Belgium) led by Dr. Sandrine Bladt.

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