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Human exposure to antifungal resistant fungi across working environments and time

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Contents

1. Preface

The project “Human exposure to antifungal resistant fungi across working environments and time”, was carried out by The National Research Centre for the Working Environment (NFA). The study is based on a collection of bioaerosol samples from the inhalation zone of 510 workers during whole workdays from 2000 to 2023. We thank all participating workers and companies included in this study.
We would like to acknowledge Pil Uthaug Rasmussen and Margit Wagtberg Frederiksen for their contribution to this project. Furthermore, we thank the authors for their diligent work on the scientific publication of the results in Kofoed et al. (2024).
The project was funded by the Danish Environmental Protection Agency, and we would like to extend our gratitude for the opportunity to carry out this work. Thank you to the advisory board for comments and suggestions during our meetings, and special thanks to Martin Laage Kragh, Louise Thorup Mundt and Birte Vogel for thoroughly reviewing the report draft and providing constructive and relevant feedback.

2. List of terms

Antibiotic: Broad term covering compounds targeting bacteria and fungi.
Antifungal: This term is commonly associated with medications or compounds that are specifically designed to treat fungal infections in humans, animals, or plants. Here this term is used for medical contexts to combat fungal pathogens.
Fungicide: This term typically refers to substances, often chemicals that are used to kill or inhibit the growth of fungi. Fungicides are commonly used in agriculture to protect crops from fungal infections. They can also be used in various other settings to control fungal growth.
Aspergillus: A fungal genus that can cause aspergillosis, mainly in immunocompromised patients.
Aspergillosis: Common term for infections caused by the *Aspergillus* genus.
Azoles: A class of antifungals targeting the production of ergosterol, an important component of the fungal membrane. Azoles are used in clinical settings, agriculture, and as preservatives.
Clinical breakpoint: A threshold concentration of e.g. an antifungal drug. If an isolate can exhibit visible growth at this concentration, it indicates resistance. Clinical breakpoints take into account how the drug acts in the body and can be used to guide treatment.
Cryptic species: Species that are morphologically indistinguishable from their non-cryptic counterparts, but may harbor different attributes and susceptibility profiles. For example, *Aspergillus lentulus* is a cryptic species of *Aspergillus fumigatus*.
Epidemiological cutoff value (ECOFF): A threshold concentration of e.g. an antifungal drug. If an isolate can exhibit visible growth at this concentration, it indicates resistance. In contrast to clinical breakpoints, ECOFFs do not consider the drug pharmacokinetics.
Exposure: The concentration of e.g. fungi, to which people are exposed given as CFU (colony forming units)/m^3 air. This is measured using air samplers attached directly to workers. Potential exposure can be measured using samplers placed stationary in the height of 1.5 m. The exposure to resistant fungi in a working environment is given as (exposure to species measured in sample × frequency of resistance for species in the specific environment).
Minimum inhibitory concentration (MIC): The lowest concentration of e.g. an antifungal to which an isolate exhibits no growth visible to the naked eye.
Para-occupational exposure: The exposure to e.g. fungi, which does not occur at work, but as an indirect consequence of work. An example is work clothes, which can carry fungal spores and fragments and spread these to the surroundings, thus constituting a source of exposure.
Resistance prevalence: We have estimated the prevalence of resistant fungi in an environment, as the number of resistant isolates out of the number of tested isolates for that environment.
Yeast: Single celled fungi, often associated with brewing and baking. However, yeasts can also cause infections, for example yeasts of the genus *Candida*.

3. Summary

The main purpose of this study was to measure workers exposure to antifungal resistant fungi across various working environments. The increase in resistance towards antifungal drugs, especially azole drugs, is an emerging health concern, as therapeutic options for fungal infections are becoming increasingly limited. The difficulties of clinical treatment of fungal infections lead to substantial human and financial costs (Geddes-McAlister and Shapiro, 2019).

Fungi are ubiquitous in the environment, and humans are continually exposed to fungal spores and fragments through air, with the working environment being no exception. High fungal exposure levels have been observed in working environments such as greenhouses, waste management and hospitals, and is associated with allergic disease and decreased lung health. Work is a significant part of most people's lives and for many the working environment can facilitate exposure to pathogenic fungi and may constitute a blind spot in relation to exposure to antifungal resistant fungi. Resistance monitoring of fungi have generally focused on clinical isolates of known pathogens, such as *Aspergillus fumigatus*, and data on the drug susceptibility of other species is limited. Thus, little is known about the human exposure to resistant fungi in the environment.

This project sought to answer the following **research questions**:

1. To which fungal species and at what concentrations are workers exposed in different environments.
2. What are the levels of exposure to antifungal resistant fungi in the working environment and are there working environments with a higher occurrence of antifungal resistance?
3. Can an overall increase in antifungal resistant fungi be observed in the working environment over time?
4. Is the prevalence of antifungal resistance higher for certain fungal species?
5. Do we find more resistance towards certain types of antifungals?

To address these research questions, a retrospective study was conducted on a unique collection of personal exposure samples, originating from Danish working environments in sectors including, agriculture (conventional and organic food- and crop production), animal handling, waste management, and healthcare, all obtained over a 20-year period. Samples were cultivated on DG18 at 37°C, with isolates identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Several species of infectious fungi were selected for resistance testing against clinically relevant triazole drugs (Itraconazole, Voriconazole, Posaconazole), and one polyene drug (Amphotericin B). Resistant isolates were further identified by sequencing *calmodulin* and *beta-tubulin,* to elucidate the presence of cryptic fungal species of *Aspergillus*. Furthermore, resistant *Aspergillus fumigatus* isolates had the *cyp51A* promoter and gene sequenced to determine underlying resistance mechanisms.

Overall, by characterizing 669 samples, the study demonstrated:

- Working environments varied in the in the exposure to resistant fungi and prevalence of antifungal resistance, and in addition, varied in the overall fungal exposure and species composition.
- Resistance was observed in 34 isolates, the majority of which belonged to the *Aspergillus niger* complex; however resistance was also observed among isolates of *Aspergillus fumigatus*, *A. nidulans*, *A. lentulus*, and *A. versicolor*.
- Resistance was most frequently observed against the drug Itraconazole.
- The majority of resistant isolates were re-identified as various cryptic species of the *A. niger* complex upon sequencing of *beta-tubulin* and *calmodulin*.

- Sequence analysis of the *cyp51A* gene in resistant *Aspergillus fumigatus* isolates, revealed the well-characterized TR_{34}/L98H mutation.

4. Introduction

4.1 Project description - Aim of project and research questions

Resistance towards antifungal drugs has become a major health concern, with resistance developing both in clinical settings and in the environment. The use of antifungal drugs and agricultural fungicides has been postulated to contribute to the development and spread of resistant fungi. Antifungal resistance in clinical isolates is routinely monitored, and generally focuses on known fungal pathogens such as *Aspergillus fumigatus*. The well described resistance in clinical settings, contrasts with a limited knowledge regarding antifungal resistance in occupational environments. Despite the potential of numerous fungal species to cause infections in humans, investigations of resistance have primarily been extensively studied for a select few species. Furthermore, studies on environmental fungal isolates often do not consider human exposure.

Humans are continually exposed to fungi, and the working environment is no exception. Since work is a significant part of people's lives, the working environment can constitute a potential source of exposure towards pathogenic resistant fungi. In addition, the work exposure to fungi is in several occupational settings more than 100 times higher than in normal home or outdoor environments (examples given in **Figure 1**).

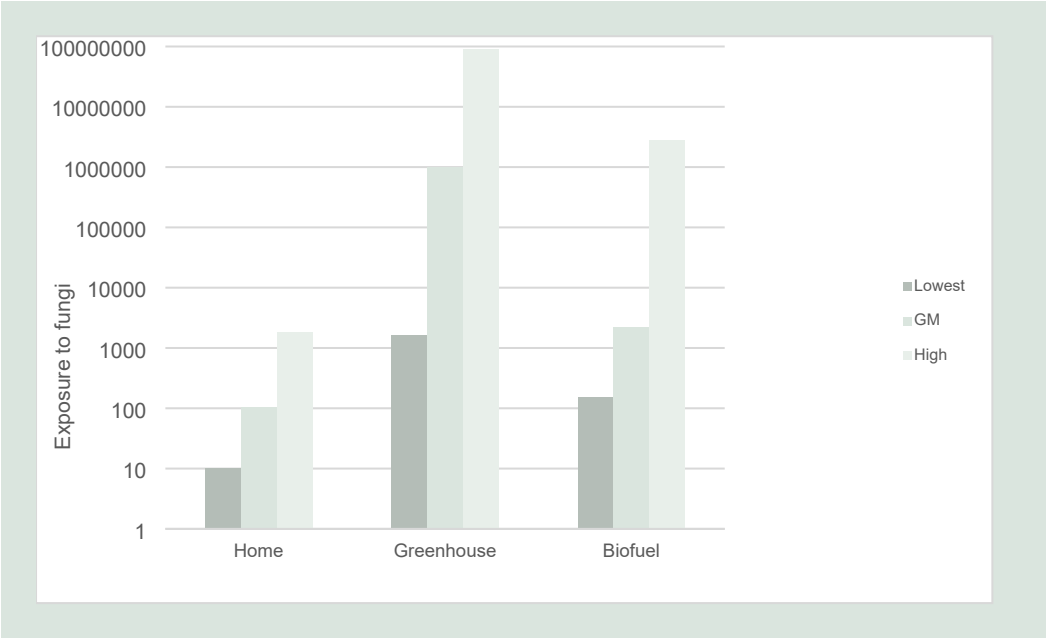

FIGURE 1. Examples of personal exposure (CFU/m^3) to fungi in three environments, GM = geometric mean (Frankel et al., 2012; Madsen, 2006; Madsen et al., 2021b)

The aim of this project is to obtain knowledge about whether workers are exposed to antifungal resistant human pathogenic fungi across a wide range of working environments: *agriculture and biofuel* (biofuel plants using straw and woodchips, cabbage fields, a grass seed plant, greenhouses), *animal handling* (pigeons and pig farms), *waste management* (biowaste plants, waste collection, wastewater treatment plants (WWTP)), and *healthcare and homes* (homes, hospital, nursing homes). Antifungal resistance is investigated in relation to fungal species, occupational environment, and time (categories of years). The data are presented as exposure through air (colony forming units (CFU)/m^3) and as a proportion of fungal isolates that are resistant. Thus, the project will elucidate the prevalence of antifungal

resistance and the exposure to resistant fungi in various working environments as well as changes over time. Additionally, the findings from this project can serve as a foundational basis for conducting risk assessment of occupational exposure to antifungal-resistant fungi. The research questions addressed in this project are as follows:

1. To which fungal species and at what concentrations are workers exposed in different environments?
2. What are the levels of exposure to antifungal resistant fungi in the working environment and are there working environments with a higher occurrence of antifungal resistance?
3. Can an overall increase in antifungal resistant fungi be observed in the working environment over time?
4. Is the prevalence of antifungal resistance higher for certain fungal species?
5. Do we find more resistance towards certain types of antifungals?

To address these questions, this project utilized a unique collection of exposure samples (n = 669), obtained from environments with anticipated variations in exposure levels to fungal pathogens. The samples were collected over a time span of 23 years (2000-2023), and they have mainly (n = 510) been sampled using personal samplers that are mounted on workers and sample air from the inhalation zone. The samples cover the exposure of a full workday of individual workers, in contrast to e.g. surface or material samples that might only give a snapshot view of potential exposure (Madsen et al., 2020; Schmithausen et al., 2015; White et al., 2021). As the samples are taken from the inhalation zone of the workers, constituting the inhalable fraction, they serve as a measure of personal exposure. The focus on airborne fungi in workers' inhalation zone was chosen, as inhalation may pose the highest health risk to the exposed worker. In addition to personal samplers, air samples were collected using stationary samplers in work areas (137) and in homes (22 samples) representing potential exposures for individuals in these areas. Apart from inhalation, airborne fungi may deposit on skin, hair, and clothing. Thus, exposed workers and their clothes may be vectors for the resistant fungi, potentially transporting them to other environments (para-occupational exposure). Finally, airborne fungi may be transported over long distances by wind.
Fungal species that can pose a risk to humans, such as fungi from the genus *Aspergillus,* was tested for resistance to clinically relevant antifungals (three azoles; Itraconazole, Voriconazole, Posaconazole and the polyene; Amphotericin B).

4.2 Fungi in occupational exposure, infections, treatment and implications of antifungal resistance

4.2.1 Fungal infections and occupational exposure

Exposure to fungal pathogens such as certain species of *Aspergillus* or *Candida* can lead to various infections and diseases. It is estimated that fungal infections affect approximately 300 million people each year, causing 1.6 million deaths globally (Bongomin et al., 2017; Denning, 2017; Geddes-McAlister and Shapiro, 2019). While many fungal infections manifest as superficial infections of e.g. skin, hair shafts, and nails (Geddes-McAlister and Shapiro, 2019), more serious invasive infections can develop, especially in immunocompromised individuals. ***Aspergillus* spp.** cause infections, commonly termed aspergillosis. The invasive form of aspergillosis is associated with high mortality rates (Latgé and Chamilos, 2019). In addition, *Aspergillus* spp. are the cause of ~3 million cases of chronic lung disease, and are associated with fungal-associated asthma, which affects around 10 million people per year globally (Bongomin et al., 2017). The genus *Aspergillus* consists of commonly present saprophytic mold species, occurring worldwide in a variety of environments and over a broad range of temperatures. *Aspergillus* spp. readily sporulate and their conidia disperse and become

airborne. Many human *Aspergillus* infections, are caused by inhalation of airborne fungal conidia[1] (Amaike and Keller, 2011; Latgé, 2001).
In occupational settings, exposure to *Aspergillus* species has caused infections of the airways. A case of pulmonary aspergillosis has been reported for a poultry farmer with *Aspergillus flavus* as the causal agent (Pal and Torres Rodríguez, 1990). Allergic bronchopulmonary aspergillosis has been reported in a person working with garden waste with *A. fumigatus* as the causal agent (Poole and Wong, 2013) and acute invasive pulmonary aspergillosis has been reported for a water pipeline maintenance worker – also caused by *A. fumigatus* (Pilaniya et al., 2015).

4.2.2 Occurrence and pathogenicity of key fungal species

Of all the *Aspergillus* species, ***Aspergillus fumigatus*** most often causes invasive aspergillosis as well as allergic disease and chronic pulmonary aspergillosis (Van De Veerdonk et al., 2017). The species is able to grow and sporulate efficiently at high temperatures, which likely contributes to its' pathogenicity (Mortensen et al., 2010; Rhodes, 2006). This species has caused occupational health problems in several settings (Madsen and Crook, 2021; Madsen et al., 2021a; Mousavi et al., 2016).
The species ***Aspergillus niger***, distinguishable by its black pigments, is described to be safe for industrial fermentation (Varga et al., 2011). However, *A. niger* can produce the mycotoxin ochratoxin A, which can contaminate food products (Schuster et al., 2002), and can cause infections in immunocompromised individuals such as pulmonary infections or infection of the ear (Gautam et al., 2011). Only a few cases of occupational infections by this species have been published, one of which is a case of occupational otitis (Bünger et al., 2000). The species has been found in high concentrations in waste workers' exposure (Madsen et al., 2020), but it is found worldwide (Madsen and Crook, 2021).
Aspergillus flavus is known to infect agricultural crops and to grow and sporulate in crops post-harvest and contaminate by producing aflatoxin (Frisvad et al., 2019). Aflatoxin is a causal agent of human and animal disease and is associated with the development of liver cancer (Amaike and Keller, 2011). In addition, *A. flavus* is one of the leading causes of invasive aspergillosis, second only to *A. fumigatus* (Krishnan et al., 2009). The species has caused occupational infections in different settings (Rudramurthy et al., 2019).
Aspergillus versicolor is mainly associated with allergenic effects, whereas it rarely causes invasive infections (Géry et al., 2023). However, cases of invasive pulmonary aspergillosis and onychomycosis[2], caused by *A. versicolor*, have been reported (Charles et al., 2011; Torres-Rodriguez et al., 1998).
Aspergillus nidulans is rarely involved in infections of humans overall. However, patients suffering from chronic granulomatous disease, are particularly prone to infections by this species (Bastos et al., 2020).
Aspegillus terrus causes a variety of infections in humans, including invasive aspergillosis. Infections by this species, is often associated with dissemination and high mortality rates (Balajee, 2009).
Fungi within the order of **Mucorales** include species of *Rhizopus*, *Rhizomucor*, and *Lichthemia* and are the most common cause of non-*Aspergillus* fungal infections in humans by filamentous fungi, commonly termed mucormycosis (Geddes-McAlister and Shapiro, 2019; Slavin et al., 2015).

4.2.3 Antifungal options

Only few classes of antifungal drugs are available for the treatment of fungal infections (azoles, polyenes, echinocandins, allylamines, and antimetabolites), with azoles and polyenes

[1] Fungal spores.

[2] Fungal infection of the toe nail, also termed tinea unguium.

constituting the first-line drugs of defense for most fungal infections (Gintjee et al., 2020; Schwartz and Patterson, 2018).
For treatment of aspergillosis, azoles are first-line drugs and greatly improve clinical outcome in patients by targeting the *cyp51* gene product, thereby inhibiting the synthesis of ergosterol, an essential component of the fungal cell wall. Azole containing drugs can be classified into three generations (Shafiei et al., 2020). The clinically approved triazoles[3] which include drugs such as Itraconazole (second generation azole), Voriconazole (third generation azole), and Posaconazole (third generation azole). The polyene class drug, Amphotericin B, which was the first approved drug (1958) for systemic treatment of invasive fungal infections (Anderson et al., 2014), exhibits broad spectrum activity against human fungal pathogens. Amphotericin B was replaced as first choice treatment, after the introduction of triazole antifungals Itraconazole and Fluconazole in the 1980s and is now a last-line drug, less preferred due to dose-limiting toxicity concerns (Shafiei et al., 2020).

4.2.4 Antifungal resistance and monitoring

Unfortunately, resistance towards antifungals, especially azole drugs has risen in the last decades. Infections caused by antifungal resistant fungi are harder to treat, leading to longer hospital admissions, treatment failure and increased mortality (Howard et al., 2009; van der Linden et al., 2011a). Today, azole resistant *A. fumigatus* have emerged worldwide, and have been classified by the WHO as a threat to global health (WHO, 2019). In Denmark, monitoring programs have existed since 1996, which examine the use of antibacterial agents and occurrence of antibacterial resistance in bacteria from humans, livestock, and food (DANMAP, 2019). For fungi, a program was introduced in 2018 which examines azole resistance in *A. fumigatus* in humans (Miljø- og Fødevareudvalget, 2019; Statens Serum Institut, 2019), and other studies in Denmark have investigated azole resistance in *A. fumigatus* in soil samples (Astvad et al., 2014; Mortensen et al., 2010). Numbers from SSI (2019, October) suggest about 500 people are infected with *Aspergillus* each year. Recently SSI (Statens Serum Institut, 2019) conducted a national monitoring of azole resistance in *A. fumigatus* (October 2018 to March 2019). From 319 patients infected with *A. fumigatus*, azole resistant isolates were recovered in 19 cases (Danmarks Radio, 2019; Statens Serum Institut, 2019).

4.2.5 Antifungal drug resistance mechanisms in *Aspergillus fumigatus*

Azoles are not themselves described as mutagenic (Verweij et al., 2016b). However, the presence of azoles can create a selective pressure, favoring mutations that lead to resistance (Rogers et al., 2022).
The primary mechanisms of azole resistance in *A. fumigatus* involve mutations in the *cyp51A* gene and in its' promoter region (Latgé and Chamilos, 2019). Mutations in the *cyp51A* gene are believed to affect azole access to the Cyp51 enzyme's active site (Zhang et al., 2019), while mutations in the *cyp51A* promoter, lead to up-regulation of gene expression (Snelders et al., 2011).
The mutations TR_{34}/L98H and TR_{46}/Y121F/T289A involve a tandem repeat (TR) insertion in the promoter region, as well as amino acid substitutions, such as L98H (substituting a leucine for a histidine) in the gene. These mutations are reported to confer multidrug-resistance (MDR) towards several tested clinical triazoles (Itraconazole, Voriconazole, Posaconazole) , but do not appear to confer cross-resistance towards other classes of antifungals including Amphotericin B (van Ingen et al., 2015).

[3] Azole drugs containing a triazole ring.

Several non-*cyp51A* associated resistance mechanisms have also been reported *in A. fumigatus*, involving mutations in the *hapE* gene[4] (Camps et al., 2012a), the *hmg1* gene[5] (Hagiwara et al., 2018), gene regulators (Blosser and Cramer, 2012), and overexpression of the *cyp51B* gene (Buied et al., 2013), and of efflux pumps (Slaven et al., 2002).

4.2.6 Antifungal drug resistance in other key fungal species

As *A. fumigatus* is responsible for most infections caused by this genus, less effort is placed on drug resistance in other *Aspergillus* species. Susceptibility to antifungals is highly variable between species, with some species being intrinsically resistant.

Mutations in the *cyp51A* gene, which lead to triazole resistance, have been reported in ***Aspergillus terreus*** (Arendrup et al., 2012). Furthermore, *A. terreus* has been reported to exhibit reduced susceptibility or intrinsic resistance against Amphotericin B (Lass-Flörl et al., 2009).

Azole drugs are generally effective against ***A. flavus*** (Denardi et al., 2018), however both *cyp51A, cyp51B* and *cyp51C* mutations have been reported in triazole-resistant isolates (Lucio et al., 2020), along with mutations in genes encoding efflux pumps and regulation factors (Choi et al., 2019). The species *A. flavus* exhibits reduced susceptibility towards Amphotericin B, which may indicate intrinsic resistance towards the drug (Rudramurthy et al., 2019).

Aspergillus niger exhibits variable susceptibility pattern with reduced susceptibility towards azoles (Van Der Linden et al., 2011b).

Aspergillus versicolor exhibits variable susceptibility towards azoles and reduced susceptibility towards Amphotericin B (Van Der Linden et al., 2011b).

Aspergillus nidulans has reduced susceptibility towards Amphotericin B (Van Der Linden et al., 2011b).

Species of **Mucorales** exhibit reduced susceptibility and possibly have intrinsic resistance towards triazole drugs Voriconazole and Fluconazole (Almyroudis et al., 2007; Espinel-Ingroff et al., 2015; Vitale et al., 2012).

4.2.7 Cryptic species and antifungal resistance

In recent years, the use of molecular tools for taxonomic studies has led to an increase in identification and characterization of cryptic fungal species, which by morphologic characterization are indistinguishable, leaving classic techniques such as microscopy inadequate. Studies have shown that MALDI-TOF MS[6] is able to identify cryptic species, but is limited by the quality and size of the database (Imbert et al., 2019; Vidal-Acuña et al., 2018).

With the introduction of the cryptic species concept, common *Aspergillus* species, including *A. niger*, *A. fumigatus*, and *A. flavus*, constitute complexes with several cryptic species (Gautier et al., 2016). Cryptic species can exhibit distinct susceptibility patterns, with high rates of resistance (Van Der Linden et al., 2011b). In addition, cryptic species such as *A. tubingensis* and *A. lentulus* are known to cause serious invasive infections (Imbert et al., 2021). The combined effect of species misidentification, distinct susceptibility patterns, and sparse susceptibility data, can lead to unfavorable clinical outcomes (Perlin et al., 2017; Van Der Linden et al., 2011b). This highlights the importance of accurate identification and susceptibility data, in order to prescribe correct antimicrobial regimen.

4.3 An environmental route of azole resistance development

Resistance towards antifungals is found in isolates of both clinical and environmental origin, and it is known that resistance can develop both in patients treated with antifungals, as well as in the environment (Dalhoff, 2018; Geddes-McAlister and Shapiro, 2019; Hendrickson et al.,

[4] CCAAT-bind factor complex subunit HapE.

[5] 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA), also involved in the ergosterol biosynthesis pathway.

[6] matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

2019). Azole compounds are commonly used in both clinical Triazoles (e.g. voriconazole, itraconazole, and posaconazole), veterinary (e.g. clotrimazol, ketonazol, enilconazol, miconazol, ronidazol, thiamazol) and agricultural (tebuconazol, epoxiconazol, difenocoazol) settings, where the use of azole in agricultural settings generally exceeds azole use in clinical settings (Verweij et al., 2009). Environmental hotspots for presence of resistant *A. fumigatus* on materials have previously been identified, and are linked to certain factors, including the presence of fungicide residues and favorable growth conditions (Schoustra et al., 2019; Verweij et al., 2020).

Regarding triazole resistance in *A.fumigatus*, it is generally accepted that resistance can develop in clinical settings through patient treatment (Verweij et al., 2016a), and resistance development has been documented in patients in response to azole therapy (Camps et al., 2012b). However, an increasing number of observations supports the notion that resistance in *A. fumigatus* towards clinical triazoles also originate from the environment, through the use of azole fungicides in agriculture, and as material and food preservatives (Dunne et al., 2017; Ren et al., 2017; Vaezi et al., 2018; Verweij et al., 2009).

Notably, the type of resistance mechanism may reflect the origin of the resistant fungal isolate. Azole resistant *A. fumigatus* isolates originating from patients treated with azoles, commonly contain point mutations in the *cyp51A* gene (Buil et al., 2019). By contrast, the common resistance mechanisms associated with environmentally derived isolates involves tandem repeats (TR) combined with point mutations (TR_{34}/L98H, TR_{53}, TR_{46}/Y121F/T289A) (Snelders et al., 2009; Verweij et al., 2009). A study from the Netherlands reported the dominating resistance mechanism in genetically distinct clinical isolates contained the same mutation (TR_{34}/L98H), despite originating from different patients and hospitals (Snelders et al., 2008). Azole resistant isolates from azole-naive patients have also been reported to carry this mutation (van der Linden et al., 2013; van der Linden et al., 2011a; Verweij et al., 2007). Furthermore, studies have demonstrated that azole fungicides (DMIs[7]: **propiconazol, tebuconazol, difenoconazol, epoxiconazol, bromuconazol, metconazole**) targeting plant pathogens also exhibit activity against wild-type *A. fumigatus*, but not against strains harboring the TR_{34}/L98H mutation. Moreover, azole fungicides exhibit similar docking structures (for binding to the Cyp51 enzyme) to medical triazoles, possibly explaining the observed cross-resistance (Snelders et al., 2012; Snelders et al., 2009).

4.4 Fungicide use in Denmark

Plant pathogenic fungi affect agriculture worldwide, decreasing food yield. In Denmark the agricultural industry relies on the use of fungicides to combat plant pathogens, such as *Zymoseptoria tritici* (*Z. tritici*) causing the leaf disease septoria tritici blotch (Torriani et al., 2015), and *Fusarium* spp. responsible for causing Fusarium head blight (Bongomin et al., 2017).

Azole fungicides are used extensively in agriculture, with sales in Denmark estimated at 165 tons in 2018 (Burks et al., 2021).The most common fungicides are demethylase inhibitors (DMIs) and succinate dehydrogenase inhibitors (SDHIs). DMI fungicides include azoles and are used extensively in Danish Agriculture (**Table 1**). In Denmark DMI fungicides are commonly used for control of septoria tritici blotch and has been used for decades (Jørgensen and Heick, 2021). However, a significant increase in DMI resistance, has been reported over the last decades (Heick et al., 2020). Recently, the European Commission, has taken action to reduce the overall use of chemical pesticides by 50% by 2030, as part of the European Green Deal[8]. In Danish conventional farming approximately 60% of fungicides are used for wheat production (Bekæmpelsesmiddelstatistikken 2019[9]). It is therefore relevant to study the presence of azole resistant fungi in environments where large amounts of straw are handled.

[7] Demethylase inhibitors.

[8] https://food.ec.europa.eu/horizontal-topics/farm-fork-strategy_en?prefLang=da

[9] https://www2.mst.dk/Udgiv/publikationer/2021/03/978-87-7038-279-3.pdf

TABLE 1. Overview of the sold amount (kg) of DMI (demethylase inhibitors) fungicides in Denmark, as found in Bekæmpelses-middelstatistikken 2021 (Miljøstyrelsen, 2021).

Fungicide	Main use	2012	2014	2016	2018	2019	2020	2021
Propiconazol[PB 1)]	Grain	17,125	12,837	7,627	5,916	5,624	5,325	13,872
Tebuconazol[PB]	Grain	58,833	36,583	60,685	43,021	65,985	75,668	80,414
Difenoconazol[P]	Potato	577	3,698	9,126	8,572	10,363	11,885	12,247
Epoxiconazol[P]	Grain	52,076	55,565	13,259	15,664	12,613	13,695	*
Metconazol[P]	Raps	572	2,390	4,464	871	501	370	771
Bromuconazol	-	-	-	-	-	-	-	-
Prothioconazol[P]	Grain	36,098	83,473	104,437	78,866	68,764	91,611	91,936
Imazalil[P]	Other[2)]	6,180	1,022	4,650	3,129	1,800	1,000	1,000
Paclobutrazol[P]	Horticulture	14	28.3	12.5	20.5	26	7	24

[1)] [PB] Aproved as pesticide(P) and/or as biocide(B). *: fungicide not approved for the year [2)]Other – use in public and private roads, parks, facilities, houses, gardens, golf course etc. and "seed treatment agents for export and seed treatment in closed systems". Main use: production of crops or other purposes for which the fungicide was primarily used.

4.5 The working environments

Occupational exposure is of particular interest for different reasons. First, the exposure to fungi is considerably higher in several occupational settings as compared to non-working hours exposure (**Figure 1**). Furthermore, the workforce is ageing and old age is associated with a higher risk of fungal infections. Work exposure is also associated with para-occupational exposure with work clothes as the vector. As an example, a recent study shows that waste collection workers accumulate up to 3.6×10^5 CFU *Aspergillus* spp./m^2 work clothes/ working hour (Møller et al., 2022). This forms the basis for a for take-home exposure both via hand contact with the clothes and via inhalation (as some of the fungi are released into the air during handling) (Madsen et al., 2022a; Møller et al., 2022). Hence, pathogenic microorganisms may be taken home and vulnerable people may be exposed in a home environment.

Agriculture and biofuel: The use of pesticides such as antifungals and antibacterials in agriculture (incl. greenhouses and animal farms) has led to a high occurrence of antifungal and antibacterial resistant organisms in these environments, which poses a risk to workers. Straw is considered a renewable energy wedge and is referred to as both biofuel and biomass. Over 66% of energy consumption in Denmark is considered renewable (Energistyrelsen, 2022). The use of straw and wood chips for energy (Fjernvarme, 2022) and the number of employees are anticipated to increase over the coming years. In biofuel plants, large amounts of straw is handled indoors, and in older studies, we have found that workers in biofuel plants are exposed to high concentrations of airborne *A. fumigatus* (Madsen, 2006). Whether work with biofuel is also associated with exposure to other human pathogens is not known. Straw is a side product from cereal (barley and wheat) production. Triazoles are used in the fields to prevent and combat fungal diseases. From 2006 to 2015 the use of triazoles in winter cereals increased from 0.07 kg of the active component of triazoles to 0.14 kg and also in spring cereals an increase was found in the same period (Nistrup). Therefore, it is relevant to study the exposure to azole resistant fungi in biofuel plants.
At the European level, Denmark has a large production of grass seeds and approximately 5000 people are involved in production of various types of seeds (LF). Several azoles are

used against fungal pathogens in amenity grass (reviewed in Jørgensen and Heick (2021)). Fungal pathogens also constitute a problem in grass seed production and a fungicide without azoles has been approved by Miljøstyrelsen (the Danish EPA), while another one containing azoles was rejected in 2023 by the Danish EPA (LF, 2023). In the process of grass seed production, some companies are specialized in treating seeds post-harvest. The seeds are handled in large amounts indoors, and this is associated with occupational exposure to human pathogens such as *A. fumigatus*, *A. niger,* and *A. versicolor* (Madsen et al., 2015).

Waste and wastewater: A growing part of the population will at one time or another be undergoing treatment against bacteria and fungi, some of which may be resistant to antibacterials and antifungals. Thus, there is a risk that both residual antibiotics and resistant microbes can be transferred from hospitals and homes into the wastewater. Indeed, studies have found both resistance genes and resistant bacteria in hospital outlets, wastewater plant inlets, river bank-filtered drinking water from waterworks, and wastewater from residential areas (Bahl et al., 2009; Jakobsen et al., 2008; Schwartz et al., 2003). Similarly, researchers have found fungi resistant to different azoles in treated wastewater (Assress et al., 2020).

An objective set forth by the "European Green Deal" aimed at bolstering material recycling (European-union, 2023). In tandem with this shift, European Union citizens in 2021 recycled 49% of domestic waste (Eurostat, 2021). A substantial (44%) increase in the number of employees working with waste has occurred between 2008 and 2021(Statistics-Denmark, 2021). Waste consists of many components, which may have been processed several times. With regards to biological waste, studies indicate that antibacterial resistance genes are present in the waste and remain after the waste recycling process (Cui et al., 2020; He et al., 2019; Liang et al., 2020), however, it appears this has not been studied for fungi (Cui et al., 2020). Interestingly, the number of antimicrobial resistance genes has been found to differ among meat, vegetable and fruit waste (Liang et al., 2020). In the waste sector (waste collection, waste reception and pretreatment), we have shown that workers are exposed to human pathogenic fungi (Madsen et al., 2020). Previous studies on waste workers and workers in composting facilities have shown that the high exposure to microorganisms can lead to health-related problems of the lungs and skin, and occupational infections (Bünger et al., 2000; Grüner et al., 1999; Poole and Wong, 2013). Nonetheless, in the Danish recycling of biowaste, if antifungal resistant fungi survive the steps until they are ultimately present in fertilizer used on agricultural lands, they may lead to the dissemination of resistance throughout the food chain (Cui et al., 2020; Rasmussen et al., 2021).

Healthcare and homes: In the healthcare sector, such as in hospitals or nursing homes, patients are exposed to antibacterial resistant, and even multidrug resistant bacteria (Diekema et al., 2004; Mulvey and Simor, 2009). We previously found MRSA in the air and on surfaces in the rooms of MRSA-colonized residents in Danish nursing homes, thereby constituting an exposure risk to the nursing home staff (Rasmussen et al., 2020).

The occurrence of resistant fungi in the healthcare and nursing homes for elderly people has been sparsely studied (e.g. Panagopoulou et al., 2007). Age-related changes in the immune system, along with the presence of other health issues, can make older individuals more vulnerable to infections in general. Thus, *Candida* infections among the elderly are an expanding clinical problem, and along with this the use of antifungals and antifungal resistance may increase (Barchiesi et al., 2017; Dekkers et al., 2018). While most people are exposed to *Aspergillus* spp. without developing serious infections, individuals with compromised immune systems, such as the elderly, may be more susceptible to infection and are consequently more often treated with antifungals. However, whether antifungal resistant *Candida* and *Aspergillus* species are present in Danish nursing home staff's exposure has not been studied.

More people are now treated for e.g. infections in their homes rather than at hospital, making the home relevant to consider for antifungal resistance. However, residential homes would, all together, be an environment with low expected exposure to antifungal resistant fungi.

Animal handling: Airborne antibacterial resistant *Staphylococcus aureus* (MRSA) has been found in a hobby pigeon exhibition (Madsen et al., in prep. nov. 2020). Furthermore, in a study from Turkey with cloacal swabs from pigeons kept for hobby breeding, researchers found

resistance genes and resistance in the bacteria *Escherichia coli* and *Enterococcus* species towards many antibacterials (Aslantaş and Gövce, 2020; Ghanbarpour et al., 2020). However, these studies were restricted to bacteria. It is therefore relevant to examine whether the pigeon keepers are exposed to antifungal resistant fungi. Antibacterial and antifungal resistant microbes in pigeons may have arisen from the use of drugs in the treatment or prophylaxis of pigeons. Alternatively, the spread of infections with resistant microbes may happen during national and international exhibitions where many pigeons are kept together in large numbers (Madsen et al., 2022b). Resistance in hobby pigeons can therefore pose a risk for the persons keeping the pigeons, and potentially neighbors, as the pigeons are often kept in residential areas. These persons handle the pigeons themselves and are often also the ones administering the drugs. This might lead to an elevated dosing and potentially the use of antibacterials and antifungals not approved in Denmark.

Pig farmers have a risk of colonization or infection with the bacteria MRSA, and are put in isolation when admitted to hospitals due to their occupation (The Danish Health Authority, 2016). Thus, volunteers visiting pig farms carry MRSA in their noses when exiting the stables, showing that they inhale the airborne antibacterial resistant bacteria during their work, risking transmission to their homes and surroundings (Angen et al., 2017). Pig farmers are exposed to a range of fungi, which can give work related infections, (e.g. *A*. *fumigatus* and *A. niger*) (White et al., 2019), and a resistance prevalence of 30% to at least one of the four tested clinical drugs (in the three classes echinocandins, polyenes, azoles) has previously been observed (White et al., 2021). Luckily, resistance to antifungals is class specific, meaning that resistance in e.g. azoles does not confer resistance to echinocandins and vice versa (Perlin, 2007).

5. Materials and methods

5.1 Research design and sample collection

For this study, a large collection of unique exposure samples (n = 669) taken from different working environments was utilized (**Table 2**). The working environments were categorized as either "*Agriculture and biofuel"*, "*Animal handling"*, "*Waste management"* or "*Home and healthcare"*. The samples had been collected between 2000 and 2023 (**Table 2**). *Agriculture and biofuel* (n = 155) were samples obtained from biofuel plants, greenhouses, cabbage fields and grass seed production. *Animal handling* (n = 69) were samples obtained from a pig farm and "pigeon coops". *Waste management* (n = 285) covered samples from biowaste plants, waste collection industry, and wastewater treatment plants (WWTP). *Home and healthcare* (n = 146) covered samples obtained from projects involving hospital- and nursing homes and domestic homes. Finally, outdoor air samples (n = 14) were included as reference. These environments cover a gradient of antifungal use, from healthcare and agricultural sites with high antifungal use, to lower levels in hobby animal keeping, with none in organic agricultural sites and with low expected antifungal use in residential homes (Kofoed et al., 2024).

5.2 Samples

The majority of samples (n=647/669) included in the study, were taken with gesamtstaubprobenahme samplers (GSP), collecting air with a velocity of 1.25 m/s at a flow rate of 3.5 L/min. Most GSP samplers were attached directly to workers (n = 510), and were used as a proxy of human inhalation during a workday. The remaining GSP samplers were stationary placed (n = 137). In addition to GSP samples, 22 samples were collected using electrostatic dust cloth (EDC) samplers. GSP samplers (personal and stationary) carried 37 mm polycarbonate filters (pore size of 0.8/1 µm; SKC or CIS by BGI, Inc, Cambridge, MA, USA respectively) or 37 mm Teflon filters (pore size 1.0 µm; Merck). Biological material from samples was extracted by suspending filters in 0.85 % NaCl and 0.05 % Tween80 and shaking at 500 rpm for 10 min. Subsequently, 1 mL of samples were vortexed with 500 µl 87 % Glycerol and stored at -80 °C until further use (for nursing home samples 20 % glycerol was added to the sample). For a more detailed description of samplers and measurement conditions, we refer to individual publications from which samples originate (**Table 2**).

TABLE 2. Overview of samples included in this study.

		Year	Total samples	Total GSP	GSP Person	GSP Stationary	EDC	References[1]
	Agriculture and biofuel							
1	Biofuel plants (n2) = 16)	2000	26	26	25	1	0	(Madsen, 2006)
		2004	34	34	34	0	0	(Madsen, 2011; Schlünssen et al., 2011)
		2022	7	7	7	0	0	
2	Free range farming, cabbage (n = 3)	2007	20	20	19	1	0	(Hansen et al., 2012)
3	Grass seeds (n = 1)	2009	3	3	2	1	0	(Madsen et al., 2015)

4	Greenhouse-flower (n =1)	2010-2012	26	26	26	0	0	(Thilsing et al., 2015)
	Greenhouse-tomato (n = 1)	2007-2012	39	39	39	0	0	(Hansen et al., 2012; Madsen et al., 2021b)
	Animal handling							
5	Pig farms (n = 1)	2016	40	40	40	0	0	(Angen et al., 2017; White et al., 2019; White et al., 2020)
6	Pigeon coops (n = 21)	2019-2020	29	29	0	29	0	(Madsen et al., 2022b; Madsen et al., 2023)
	Waste management							
7	Biowaste plants (n = 7)	2019-2020	22	22	7	15	0	(Rasmussen et al., 2021)
		2021-2022	62	62	45	17	0	(Rasmussen et al., 2023a)
8	Waste Collection (n = 5)	2015	18	18	13	5	0	(Madsen et al., 2016a)
		2018	14	14	14	0	0	(Madsen et al., 2020)
		2022	17	17	17	0	0	
9	WWTP (n = 7)	2010	6	6	6	0	0	(Uhrbrand et al., 2011)
		2015	46	46	46	0	0	(Lu et al., 2020a)
		2021	100	100	91	9	0	
	Home and Healthcare							
10	Homes 2010	2010	29	29	0	29	0	(Madsen et al., 2016b; Madsen et al., 2018)
	Homes 2021	2019-2022	22	0	0	0	22	
11	Hospital (n = 1)	2015	16	16	0	16	0	(Würtz et al., 2017)
12	Nursing homes (n = 5)	2017-2018	79	79	79	0	0	(Lu et al., 2020b), (Rasmussen et al., 2023b)
13	Outdoor air	2007-2023	14	14	0	14	0	
	Total Unique Samples		**669**	**647**	**510**	**137**	**22**	

GSP = gesamtstaubprobenahme samplers, EDC = Electrostatic dust cloth samplers. 1) References of which samples originate. 2) n is the number of companies or workplaces included for each category. Table from Kofoed et al. (2024)

5.3 Culturing and identification

In this study, we have used an open approach by using a fungal selective agar medium supporting growth of many different fungal species, Dichloran Glycerol agar (DG18; Thermo Fisher Scientific Oxoid, Basingstoke, UK), supplemented with chloramphenicol. This open approach was chosen, as different environments and work tasks support growth and spore aerosolisation of different species (Andersen et al., 2011; Madsen et al., 2006; White et al., 2019). The DG18 agar medium was used in this study as it supports growth of many species, and colonies are restricted in growth (Verhoeff et al., 1990; Wu et al., 2000), making it possible to identify and isolate species. Furthermore, previous studies in very different occupational settings show that human pathogens such as *A.fumigatus*, *A. niger*, and *A. versicolor* and yeast species can be found using DG18 agar (Madsen et al., 2020; White et al., 2019). An exception was with samples obtained from pigeon coops, where samples in addition to DG18 agar plates, were plated on Sabouraud agar plates (n = 18). All plates were incubated at 37 ℃ for up to 7 days. The ability of fungi to grow at 37 °C is a virulence factor (Karkowska-Kuleta et al., 2009), and therefore this study focuses on fungi able to grow at this temperature. Isolates were identified using MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry), using the Bruker library BDAL Filamentous Fungi library version 4 (MaldiBiotyperDBUpdate_V4_Fungi-856(RUO)), containing both human pathogens and environmental fungi.

5.4 Selecting fungal isolates for susceptibility testing

Fungal species with determined clinical breakpoints, epidemiological cutoff values (ECOFFs) and published MIC distributions from EUCAST, were isolated for susceptibility testing. In addition, a number of species classified as risk group 2 organisms according to GESTIS Biological Agents Database were isolated. An exception was samples from WWTPs (2021) where only *A. fumigatus, A. lentulus,* and *A. flavus* were isolated. Fungal colonies were isolated by culturing respective colonies on DG18, after which a square of approximately 5 mm^2 was extracted and stored at -80 °C in a freezing buffer (8.5 g NaCl, 1.0g Peptone, 1000 ml MilliQ water, and 500 ml Glycerol 87 %). Up to three individual isolates of the same species per sample were included. Thus, more than one isolate of the same species can originate from the same exposure sample.

5.5 Screening for azole resistance

Screening agar plates from VIPcheck™ was used to screen *A. fumigatus* isolates for resistance towards common medical triazoles - Itraconazole, Voriconazole, and Posaconazole. The VIPcheck™ screening method uses azole-containing and azole-free agar with inoculations of *A. fumigatus*, for a visual reading to assess possible resistance. The VIPcheck™ screening was carried out according to manufacturer's instructions using 4-well plates (VIPcheck™, MediaProducts, Groningen, the Netherlands) with wells supplemented with Itraconazole (4 mg/L), Voriconazole (2 mg/L) or Posaconazole (0.5 mg/L) as well as one non-supplemented well for growth control. *A. fumigatus* strain SSI-4524 was used for quality control. The screening method has been described in detail in EUCAST E.DEF. 10.2 June 2022 (Guinea J, 2022a).

5.6 Testing for antifungal resistance

Fungal isolates underwent susceptibility testing against four antifungal drugs, Amphotericin B, Itraconazole, Voriconazole, and Posaconazole, following EUCAST guidelines for filamentous fungi and yeasts, described in EUCAST E.DEF 9.4 March 2022 (Guinea J, 2022b), and EUCAST E.DEF 7.3.2 April 2020 (Guinea J, 2020). Isolates of *A. fumigatus* from samples from nursing homes (n = 40) and the pilot biowaste plant (n = 26), were not tested against Posaconazole. Concentrations ranged from 0.0016 to 8 mg/L for Amphotericin B, Itraconazole, and Voriconazole, and from 0.0008 to 4 mg/L for Posaconazole. Growth was visually assessed, and the minimum inhibitory concentration (MIC) values were determined. For

purposes of analysis, MIC values >8 mg/L were classified as 16 mg/L, and MIC values ≤0.016 mg/L were classified as 0.016 mg/L. The optical density at 600 nm and 540 nm for molds and 530 nm for yeasts was measured using an Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA).
Quality control was carried out using the strains *A. flavus* CNM-CM 1813, *Candida krusei* CNM-CL 3403, and *A. fumigatus* ATCC 204305, and isolates were classified as resistant or susceptible based on EUCAST Clinical breakpoints. When unavailable, Epidemiological cut-off values (ECOFFs) were used. Breakpoints or ECOFFs from *A. fumigatus*, and *A. niger* were applied to cryptic species within the same section. In the case of *A. versicolor*, published EUCAST MIC-distributions (European Committee on Antimicrobial Susceptibility Testing. Data from the EUCAST MIC distribution website, last accessed 2nd January 2024. http://www.eucast.org) were consulted for resistance characterization against triazoles only.

5.7 Sequencing of *camA* and *benA*

Isolates showcasing resistance of species *A. fumigatus* (n = 4), *A. niger*, *A. nidulans*, and *A. lentulus*, as well as nine susceptible isolates of *A. niger*, underwent amplicon sequencing of the genes for Calmodulin and Beta-tubulin (*camA* and *benA*) for identification of potentially cryptic species. Fungal isolates were inoculated in 10 ml sabouraud broth and grown over night at room temperature. DNA was extracted using the MasterPure Yeast DNA Purification Kit (Biosearch Technologies, UK) according to manufacturer's instructions. *Beta-tubulin* was amplified using forward primer Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC) and reverse primer Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC) (Hubka and Kolarik, 2012), with the following PCR conditions: Initial denaturation at 95 °C for 1 min followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 55.9 °C for 30 s, and elongation at 72 °C for 1 min. For *calmodulin* forward primers CF1L (5'-GCCGACTCTTTGACYGARGAR) and CF1M (5'-AGGCCGAYTCTYTGACYGA) and reverse primer CF4 (5'-TTTYTGCATCATRAGYTGGAC), were used (Sklenář et al., 2017), along with the following PCR conditions: Initial denaturation at 95 °C for 1 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 15 s and elongation at 72 °C for 1 min. PCR products were purified using Clean NGS SRPI-beads (Clean NA, The Netherlands) with a bead to sample ratio of 4:5. DNA concentrations were measured using Qubit™ 1X dsDNA high-sensitivity (HS) Assay kit (Invitrogen, USA), and were quality checked with Agilent 4150 Tapestation using ScreenTapeD5000 (Agilent Technologies, USA).
PCR products were barcoded and pooled equimolarly. The pooled library was prepared for sequencing using ligation sequencing kit SQK-LSK114 and barcoding expansion EXP-PBC096 in accordance with manufacturer's recommendations, protocol version PBC_9182_V114_REVG_ 07MAR2023. Approximately 50 fmol of the prepared library was loaded onto a FLO-MIN114 (Oxford Nanopore Technologies, UK). The library was sequenced for 3.5 h, with live-basecalling enabled in high-accuracy mode. Processing of sequencing data and generation of OTU-tables were carried out as described by Eskildsen (2024).

5.8 Sequencing of *cyp51A*

The complete cyp51A gene and its promoter region was amplified using primers 1F (5'-GTGCGT AGCAAGGGAGAA GGA) and 4R (5'-CCTATTCCGATCACACCAAA) and sequenced with Sanger sequencing using primers 1R (5'-CATTGAGCAAGATTGCCG), 2R (5'-GGTGAATCGCGCAGATAGT), 3R (5'-GTCAAGATCCTTGTACTGGAGC), 2F (5'-CGGCAATCTTGCTCAATG), 3F (5'-ACTATCTGCGCGATTCACC) and 4F (5'-CTCCAGTACAAGGATCTTGAC) (Mellado et al., 2001; Mortensen et al., 2011; Risum et al., 2022). Sequences were compared to an azole-susceptible A. fumigatus obtained from GenBank (Accession no. AF338659). Alignment was performed using the BioEdit software and the CLUSTAL O(1.2.4) software (Kofoed et al., 2024).

5.9 Statistical analyses and data treatment

Data was visualized using R v. 4.2.3 (R Core Team, 2013), and R-package “tidyverse” (Wickham et al., 2019). Statistical analysis was carried out using SAS software, Version 9.4. A generalized linear model was used assess differences in exposure levels between working environments. Logistic regression with a likelihood ratio test was used to examine the effect of working environment and time on the prevalence of resistance given as a binary outcome variable (resistant or susceptible). A generalized linear model was used to assess the effect of working environment and time on the occupational exposure to resistant fungi, given as: (species-specific sample exposure × the rate of resistance for that species). Time was given as the sample year split in periods, which encompassed several different working environments within each (1) 2000 – 2009, 2) 2010 – 2018, 3) 2019 – 2023).

6. Results

6.1 Total exposure levels to fungi across working environments

The concentrations (including both stationary and personal GSP) of fungi (here fungi able to grow on agar media at 37 ℃) differed significantly between work environments ($p < 0.0001$), and ranged between below the level of detection (2.6 CFU/m^3) and 6.7 ×10^6 CFU/m^3 (**Figure 2**). The highest geometric mean (GM) exposure levels were measured in the grass seed plant (GM = 3.7 × 10^5 CFU/ m^3), followed by biofuel plants (GM = 807 CFU/m^3), biowaste plants (GM = 632 CFU/m^3), and greenhouses (311 CFU/m^3). By contrast, the lowest exposure levels were measured in the WWTPs (GM = 12 CFU/m^3). Low exposure levels were also observed for nursing homes, domestic homes and at the hospital, with GM exposure levels between 16 and 24 CFU/m^3; these levels were comparable to exposure levels observed for outdoor air (17 CFU/m^3). As the EDC samples make use of a flat surface for sampling over several days, the concentration of fungi from these samples are given as CFU/m^2/day. The EDC samples from homes (n= 22), had a concentration (GM) of 185 CFU/m^2/day.

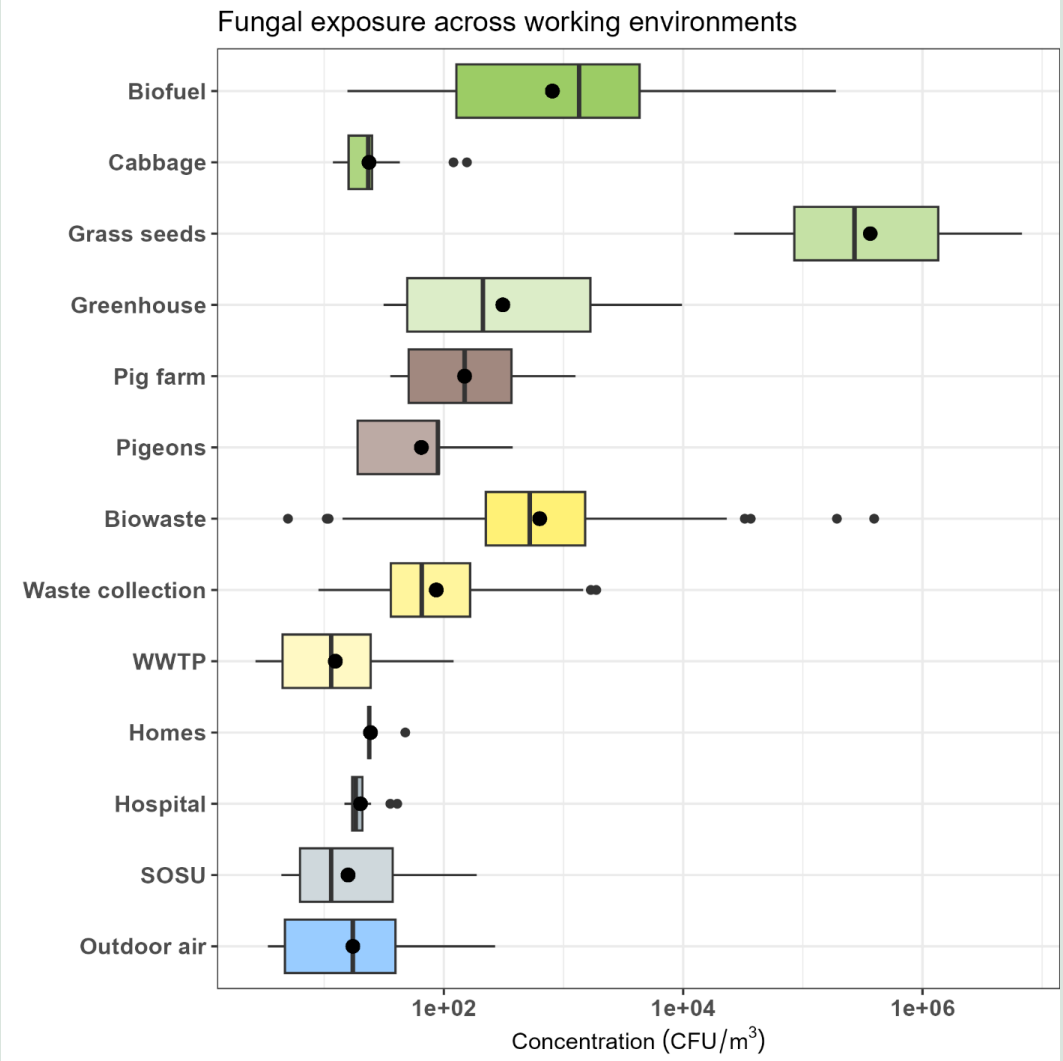

FIGURE 2. Exposure to fungi (CFU/m^3) across working environments. X-axis is log10-transformed. Only active air samplers (Personal GSP samples, n = 510, and stationary GSP, n = 137) included. The large black dot corresponds to the geometric mean value of each environment. Each small black dot is an outlier. Figure from Kofoed et al. (2024).

6.2 Fungal species composition across working environments

Across all GSP samples, 53 different fungal species were identified with MALDI-TOF MS (**Figure 3**). A total of 10 species were identified from EDC samples, including *A. fumigatus, A. montevidensis, A. niger, A. terreus, A. versicolor, Byssochlamys spectabilis, Lichtheimia ramosa, Penicillium chrysogenum, Rhizopus oryzae,* and *Talaromyces ruber*. Biowaste plant workers were exposed to the highest number of individual species followed by workers in greenhouse facilities, while the exposure of people working in cabbage fields and outdoor air, had the lowest number of species. Some species were only found in a single environment, *A. candidus* and *A. ustus* were only present in samples from nursing homes (SOSU), and similarly, several species of *Penicillium* were exclusive for greenhouse facilities. The composition of fungal species varied between working environments (**Figure 3**).

The species most frequently identified across working environments was *A. fumigatus*, which was found in 183 samples with an exposure of 207 CFU/m^3 (GM of positive samples). The species *A. fumigatus* was abundant in biofuel plant workers' exposure, where it was found in 45/67 samples, at a concentration of 1516 CFU/m^3 (GM), as well as in biowaste plants with

62/84 samples (GM = 368 CFU/m^3). Work on the grass seed plant caused the highest exposure to *A. fumigatus* (GM=2.60 × 10^5 CFU/m^3) and the species was present in 3/3 samples. The species *A. niger* was the second most frequently observed species, found in a total of 87 samples causing an exposure of 141 CFU/m^3 (GM of positive samples), and was most abundant in biowaste plants, where it was found in 51/84 samples (GM = 269 CFU/m^3). Species of *Penicillium* were found in most working environments, but none of them are classified as human pathogens. While greenhouse workers were exposed to multiple species of *Penicllium*, several workers were also exposed to *A. versicolor* in this environment. In general, yeasts were rare across environments but were most frequently observed in samples taken from pig farms, where *Diutina catenulata* (*Candida catenulata*) was found in 23 out of 40 people's exposure (**Figure 3)**. From the total of 53 different species found across environments, 23 species were selected for resistance testing, comprising 561 fungal isolates (**Table 3**).

TABLE 3. Species selected for resistance testing, n = 23. Species names are listed as identified by MALDI-TOF MS[1])

	Class 2 according to			**Pathogenicity and examples of cases of infections.**
	GESTIS 2)	AT 3)	Resistance data	
Aspergillus candidus	RG1	RG2	-	Different infections such as: Skin and brain (Kaur et al., 2021; Linares et al., 1971)
Aspergillus flavus	RG2	RG2	CPBs4) + ECOFFs5)	Frequent cause of invasive aspergillosis and has previously caused occupational infections (Krishnan et al., 2009; Rudramurthy et al., 2019).
Aspergillus fumigatus	RG2	RG2	CPBs + ECOFFs	Species that most frequently causes invasive aspergillosis (Van De Veerdonk et al., 2017)
Aspergillus lentulus	RG1	RG2	-	Has caused aspergillosis. Can be antifungal resistant (Nematollahi et al., 2021)
Aspergillus montevidensis	-	RG2	-	Has caused aspergillosis.(Fernandez-Pittol et al., 2022)
Aspergillus nidulans	RG1	RG2	CPBs + ECOFFs	Associated with infections in patients suffering from chronic granulomatous disease (Bastos et al., 2020).
Aspergillus niger	RG2	RG2	CPBs + ECOFFs	Has caused occupational otitis (Bünger et al., 2000)
Aspergillus ochraceus	RG1	RG2	-	Has caused aspergillosis. (Hakamifard et al., 2021)
Aspergillus terreus	RG2	RG2	CPBs + ECOFFs	Can cause a variety of infections including invasive aspergillosis (Balajee, 2009).
Aspergillus tritici	RG1	RG2	-	Has caused non-dermatophytic onychomycosis.(Hubka et al., 2012)
Aspergillus versicolor	RG1	RG2	MIC-distributions (EUCAST)	Has caused aspergillosis and onychomycosis (Charles et al., 2011; Torres-Rodriguez et al., 1998).
Byssochlamys spectabilis (Paecilomyces variotii)	RG2	-	-	Has caused several types of infections including: otitis media, pneumonia, onychomycosis, endophthalmitis, sinusitis.
Candida orthopsilosis	RG1	-	-	Rare case of septic arthritis (Heslop et al., 2015)
Candida parapsilosis	RG2	RG2	CPBs + ECOFFs	One of the leading causes of invasive candidiasis (Trofa et al., 2008).

Lichtheimia corymbifera	RG2	-	-	Has been associated with human Mucormycosis.(Garcia-Hermoso et al., 2009)
Lichtheimia ramosa	RG2	-	-	Several types of infections are described e.g.: cutaneous, rhinocerebral, pulmonary. Mucormycoses caused by Lichtheimia species.
Paecilomyces variotii (Byssochlamys spectabilis)	RG2	-	-	Has caused several types of infections including: otitis media, pneumonia onychomycosis, endophthalmitis, sinusitis,
Pichia kudriavzevii	RG1	-	CPBs + ECOFFs	Has caused fungaemia in neonates (Nagarathnamma et al., 2017).
Rhizomucor miehei	RG1	-	-	Rare cases of pulmonary infections (Maatallah et al., 2022)
Rhizomucor pusillus	RG1	-	-	Sinus, pulmonary, and skin infections (Menzinger et al.)
Rhizopus microspores	RG2	-	-	Lung infections. (Chen et al., 2021)
Scopulariopsis brevicaulis	RG1	-	-	Has caused infections, including occupational. (de Miguel-Martinez et al., 2017)
Trichoderma longibrachiatum	RG2	-	-	Different cases of infections are reported (Sautour et al., 2018)

1)MALDI_TOF MS identified the species P. variotii and B. spectabilis but in fact different sexual stages of the same fungi.

2)GESTIS (Unfallversicherung, 2017) 3)Danish working environments authority (Arbejdstilsynet, 2020):, 4)EUCAST clinical breakpoints,5)EUCAST epidemiological cutoff values.

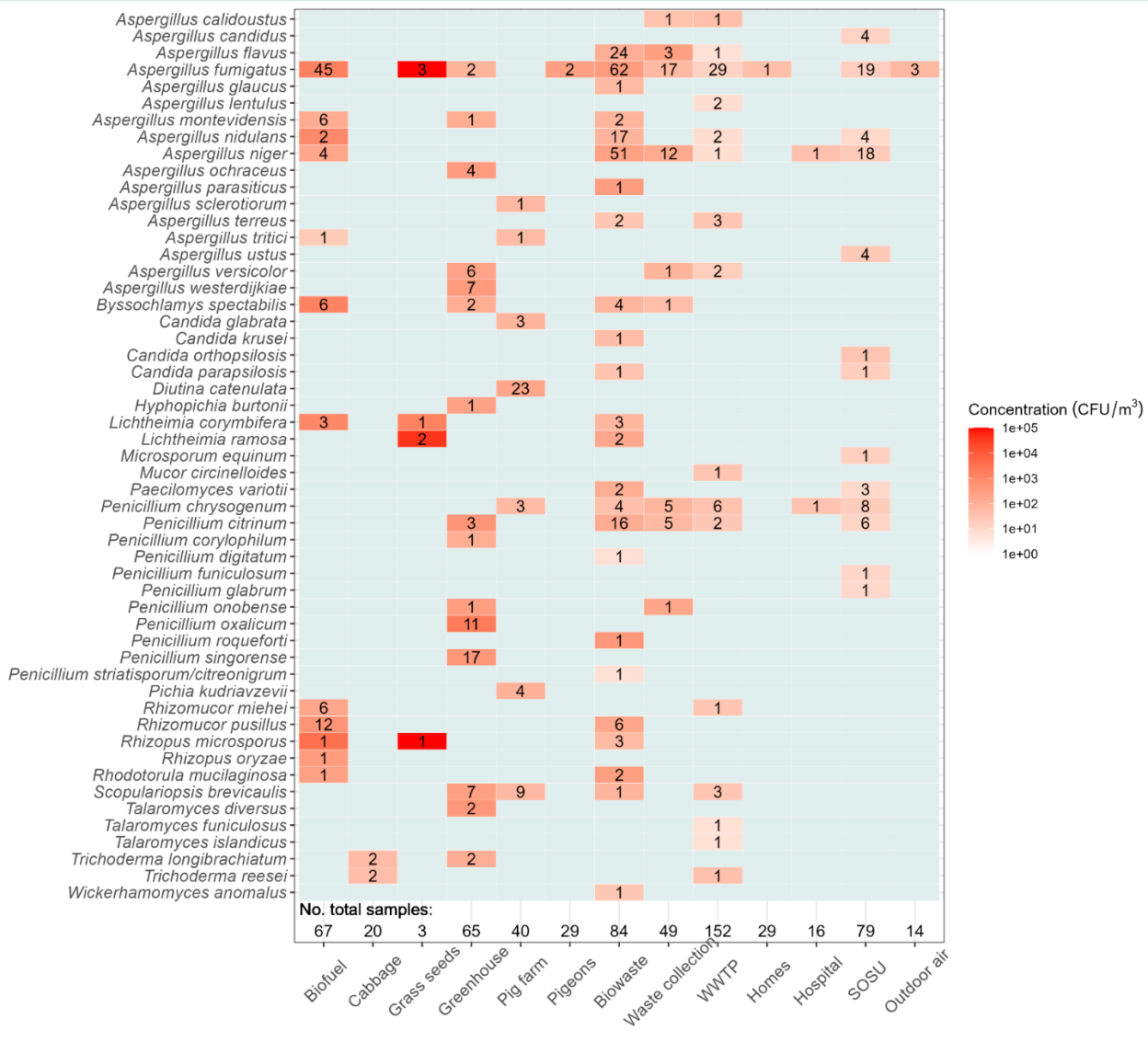

FIGURE 3. Heatmap of fungal species across working environments. Numbers with red background correspond to no. of positive samples. Color scale (log10 transformed), corresponds to concentration as GM of positive samples with a cutoff-value for the color scale at 1 ×10^5 CFU/m^3. No. total samples is the total number of analyzed samples for respective working environments. Figure from Kofoed et al. (2024).

6.3 Resistance and MIC distributions among fungal isolates

In the triazole resistance screening of 243 *A. fumigatus* isolates, 72 isolates demonstrated growth in the presence of at least one triazole, and 24 isolates yielded inconclusive results, possibly indicating minimal growth. Screening-positive and inconclusive isolates were selected for full susceptibility testing (broth microdilution). In addition, 74 isolates of *A. fumigatus* were directly tested without prior screening.

Antifungal susceptibility testing by EUCAST broth microdilution was carried out for 415 fungal isolates (**Figure 4**). From these, 34 isolates were characterized as resistant according to clinical breakpoints, ECOFFs or MIC-distributions from EUCAST, belonging to the species *A. niger* (n = 19), *A. fumigatus* (n = 8), *A. versicolor* (n = 3), *A. nidulans* (n = 2), and *A. lentulus* (n = 2) as identified with MALDI-TOF MS. The highest frequency of resistance was observed among isolates of *A. niger*, with resistance in 19 of 131 isolates (14.5 %). In addition, five isolates of *A. niger* exhibited MIC values of 1 mg/L against Amphotericin B, which is

considered non-wildtype according to ECOFFs, but “Susceptible” according to the breakpoint. The prevalence of resistance was lower among *A. fumigatus* isolates with 8 of 171 isolates tested with broth microdilution exhibiting resistance to one or more antifungal drugs and only 8/317 if including the triazole-resistance screening, equivalent to 2.5 %.

Notably, isolates of *A. niger* frequently exhibited paradoxical effects against Itraconazole. The paradoxical effects were observed as inhibition of growth at low drug concentrations, followed by growth at higher concentrations. Isolates with paradoxical growth were not classified as resistant according to EUCAST guidelines, and the MIC value was recorded as the lowest concentration to inhibit growth.

Of the 34 resistant isolates, 20 exhibited resistance towards one antifungal. Three exhibited resistance towards Amphotericin B alone, 13 isolates exhibited resistance towards Itraconazole alone, and four isolates exhibited resistance towards Voriconazole alone. Mono-resistance towards Posaconazole was not observed. Eleven isolates exhibited multi-resistance towards either Itraconazole and Voriconazole (5 isolates), or Itraconazole, Voriconazole and Posaconazole (4 isolates), or Amphotericin B and Voriconazole (2 isolates). Three isolates of *A. versicolor* exhibited MIC values >8 mg/L for itraconazole, deviating notably from the normal distribution of the published EUCAST MIC distributions for this species. One of these isolates, exhibited an MIC value of 2 mg/L against Posaconazole, appearing to fall outside the expected distribution. However, the deviation of this MIC from the normal distribution was less clear, and thus not characterized as resistance.

For species without defined breakpoints and ECOFFs or MIC distributions, characterizations as resistant or susceptible can be difficult, due to the lack of data for reference. In these cases, we use the term “tolerance”. High antifungal tolerance was observed in isolates of several species. One (1/4) isolate of *Rhizomucor pusillus*, had an MIC value of >8 mg/L against Itraconazole (**Figure 4**). Only a single isolate of *A. ochraceus* was tested, which showed a high MIC value against Amphotericin B (>8 mg/L). Several species, including *Byssochlamys spectabilis*, *Paecilomyces variotii*, *Lichtheimia corymbifera*, *L. ramosa*, and *Rhizomucor pusillus* consistently had high MIC values against Voriconazole. Isolates of *Scopulariopsis brevicaulis* had high MIC values against all tested antifungal drugs.

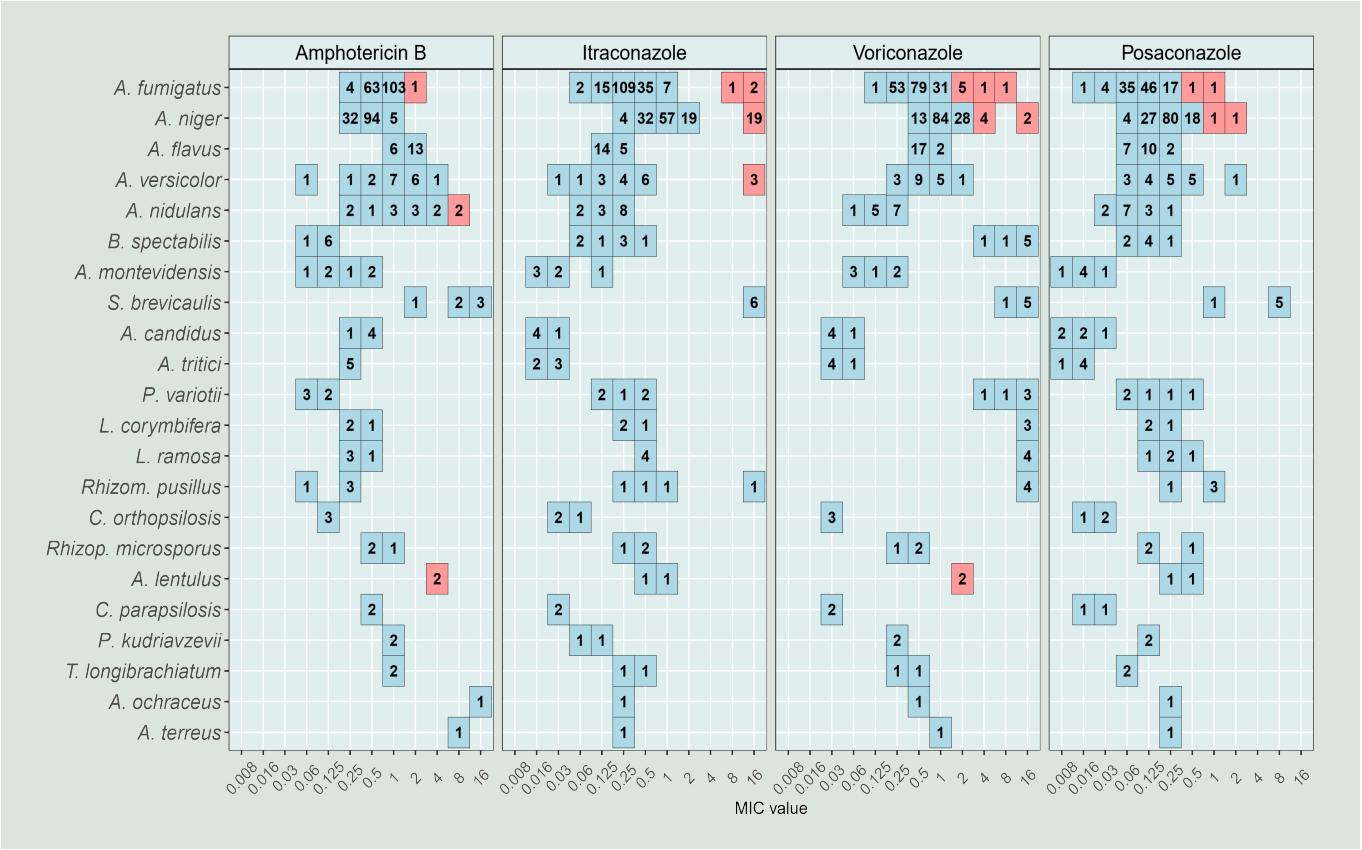

FIGURE 4. MIC value (mg/L) distributions of fungal species tested with EUCAST broth microdilution. The fungi were identified using MALDI-TOF MS. Numbers in tiles indicate the number of tested isolates with the respective MIC values. Red colored tiles, represent MIC values characterized as resistant, and blue tiles represent MIC values characterized as susceptible. Based on figure from Kofoed et al. (2024).

6.4 Identification of cryptic species by sequencing of *benA* and *camA*

Beta-tubulin and *calmodulin* (*benA* and *camA*) sequencing of fungal isolates (n = 40) were carried out for resistant isolates of *A. niger, A. fumigatus* (n = 4)*, A. nidulans*, and *A. lentulus* as well as 9 susceptible isolates of *A. niger*. The gene sequencing revealed a large proportion of cryptic species among the *A. niger* isolates. Out of 33 isolates that were initially identified as *A. niger* with MALDI-TOF MS, 25 were identified as *A. tubingensis*, 3 as *A. luchuensis*, 3 as *A. niger* and 1 was identified as *A. phoenicis* (**Table 4**). Interestingly, the three isolates identified as *A. niger* from sequencing shared similar susceptibility patterns, with an MIC value of 1 mg/L against Amphotericin B, which is considered non-wildtype according to ECOFFs. The nine non-resistant isolates included for sequencing, were all identified as *A. tubingensis*, indicating that this cryptic species was present throughout the *A. niger* isolates, regardless of resistance. For isolates of *A. fumigatus* and *A. nidulans*, identifications were identical between methods (MALDI-TOF MS and sequencing). Isolates identified as *A. lentulus* by MALDI-TOF MS were identified as *A. udagawe* by sequencing results; both species are cryptic species in the *A. fumigatus* complex.

TABLE 4. Species identity of resistant isolates as obtained by MALDI-TOF MS and by sequencing of benA and camA.

Isolat ID	MALDI-TOF id	Amplicon seq id	Resistance against[1)]	MIC-AMB	MIC-ITR	MIC-VOR	MIC-POS	Environment[2)]	Sampling Date	Sample type[3)]	Person/sample id.
biof-04_iso71	A. fumigatus	A. fumigatus	ITR, VOR, POS	1	16	2	1	Biofuel 2004	01-11-2004	P	1485
biow_iso25	A. fumigatus	A. fumigatus	ITR, VOR	1	16	2	0.5	Biowaste	04-05-2021	P	A3.P4
biof-04_iso111	A. fumigatus	A. fumigatus	VOR	0.5	0.25	4	0.06	Biofuel 2004	26-10-2004	P	167
wc22_iso54	A. fumigatus	A. fumigatus	VOR	0.5	0.25	8	0.06	Waste collection 2022	28-03-2022	P	V1.P3
biof-00_iso138	A. nidulans	A. nidulans	AMB	8	0.25	0.25	0.125	Biofuel 2000	03-10-2000	P	629
sosu_iso5	A. nidulans	A. nidulans	AMB	8	0.25	0.125	0.06	SOSU	18-06-2018	P	L4E1
biow_iso46	A. niger	A. luchuensis	ITR, VOR, POS	0.5	16	16	2	Biowaste	30-06-2021	P	D1.P1
biow_iso52	A. niger	A. tubingensis	ITR	0.5	16	2	0.5	Biowaste	30-06-2021	P	D1.P3
biow_iso62	A. niger	A. tubingensis	ITR	0.5	16	4	0.5	Biowaste	27-10-2021	P	F1.P1[5)]
biow_iso63	A. niger	A. tubingensis	ITR, VOR	0.5	16	4	0.5	Biowaste	27-10-2021	P	F1.P1[5)]
biow_iso73	A. niger	A. tubingensis	ITR	0.25	16	2	0.25	Biowaste	27-10-2021	S	F1.S2[5)]
biow_iso74	A. niger	Missing(6	ITR, VOR	0.5	16	4	0.5	Biowaste	27-10-2021	S	F1.S2[5)]
biow_iso94	A. niger	A. tubingensis	ITR	0.25	16	2	0.5	Biowaste	05-10-2021	P	E1.P1
biow_iso98	A. niger	A. tubingensis	ITR	0.25	16	2	0.25	Biowaste	05-10-2021	P	E1.P2[5)]
biow_iso100	A. niger	A. tubingensis	ITR	0.25	16	2	0.25	Biowaste	05-10-2021	P	E1.P2[5)]
biow_iso104	A. niger	A. tubingensis	ITR, VOR	0.5	16	4	0.5	Biowaste	05-10-2021	P	E1.P3
pbiow_iso4	A. niger	A. tubingensis	ITR	0.5	16	2	0.25	Biowaste-pilot	07-01-2020	S	A2.S2
pbiow_iso8	A. niger	A. phoenicis	ITR	0.25	16	2	0.25	Biowaste-pilot	03-02-2020	S	B1.S2
sosu_iso1	A. niger	A. tubingensis	ITR	0.5	16	2	0.5	SOSU	25-06-2018	P	L5E2

sosu_iso2	A. niger	A. tubingensis	ITR	0.5	16	2	0.5	SOSU	18-06-2018	P	L1E1
sosu_iso3	A. niger	A. tubingensis	ITR	0.5	16	2	0.25	SOSU	25-06-2018	P	L4E2
wc-22_iso234	A. niger	A. tubingensis	ITR	0.5	16	1	0.25	Waste collection 2022	04-01-2023	P	V5.P9
wc-18_iso243	A. niger	A. tubingensis	ITR	0.25	16	2	0.25	Waste collection 2018	01-03-2018	P	P8.B
wc-18_iso248	A. niger	A. tubingensis	ITR	0.5	16	2	0.5	Waste collection 2018	11-06-2018	P	P19.C[5)]
wc-18_iso249	A. niger	A. luchuensis	ITR, VOR, POS	0.5	16	16	1	Waste collection 2018	11-06-2018	P	P19.C[5)]
wwtp_iso1	A. lentulus	A. udagawe	AMB, VOR	4	1	2	0.5	WWTP	08-09-2021	P	C106P
wwtp_iso2	A. lentulus	A. udagawe	AMB, VOR	4	0.5	2	0.25	WWTP	08-09-2021	P	C109P
wc15_iso11	A. niger	A. luchuensis	AMB*4)	1	0.5	1	0.25	Waste collection 2015	26-01-2015	P	2A
biof-00_iso172	A. niger	A. niger	AMB*	1	0.5	0.5	0.25	Biofuel 2000	29-05-2001	P	846
biow_iso9	A. niger	A. tubingensis	AMB*	1	1	1	0.25	Biowaste	04-05-2021	P	A3.P3
sosu_iso131	A. niger	A. niger	AMB*	1	0.5	1	0.25	SOSU	22-01-2018	P	L1C1
pbiow_iso12	A. niger	A. niger	AMB*	1	1	0.5	0.125	Biowaste-pilot	03-02-2020	P	B1.P1
greenfw_iso9	A. versicolor	Not included	ITR	2	16	0.5	0.5	Greenhouse-flower	24-10-2012	P	P243
greento_iso18	A. versicolor	Not included	ITR	1	16	2	0.5	Greenhouse-tomato	12-11-2007	P	C643
greento_iso32	A. versicolor	Not included	ITR	2	16	1	2	Greenhouse-tomato	12-11-2007	P	C632
sosu_iso62	A. fumigatus	Not included	ITR, VOR	1	8	2	*	SOSU	18-06-2018	P	L3E1
sosu_iso17	A. fumigatus	Not included	AMB	2	0.5	0.5	*	SOSU	20-11-2017	P	L13B1
pbiow_iso5	A. fumigatus	Not included	VOR	1	1	2	*	Biowaste-pilot	07-01-2020	S	A2.S1
pbiow_iso6	A. fumigatus	Not included	VOR	1	1	2	*	Biowaste-pilot	07-01-2020	S	A1.E2

1) AMB = Amphotericin B, ITR = Itraconazole, VOR = Voriconaozle, POS = Posaconazole. 2) Environment in which the sample is collected, 3) P = Personal sample type, S = Stationary. 4)*: non-wild type according to ECOFF, but susceptible according to breakpoint. 5) Samples with identical IDs are from the same person. 6) re-cultivation of isolate was not possible after storage. Table from Kofoed et al. (2024).

6.5 Mutations in *cyp51A* gene and promoter region

The *cyp51A* gene (including promoter) of seven resistant *A. fumigatus* isolates (biof04_iso71, sosu_iso62, biow_iso25, biof04_iso111, pbiow_iso5, pbiow_iso6 and wc22_iso54) was sequenced, to determine the underlying mechanism conferring azole resistance. Sequencing revealed that three isolates (biof04_iso71, biow_iso25 and sosu_iso62) carried the TR_{34}/L98H mutation (**Figure 5A, Figure 5B**), all of which were multi-drug resistant towards two or three triazoles. In addition, the isolate biow_iso25 contained an additional mutation (T→G) in the non-coding region 67bp upstream of the start codon (not shown). Isolate, biof04_iso111, contained a base substitution (position 117, G→A). This point mutation leads to a missense mutation (M39I) in *cyp51A*. The isolate pbiow_iso5, contained a silent point mutation (C→T, positon 735), not causing any changes in the amino acid sequence (not shown). In the isolates, pbiow_iso6 and wc22_iso54, no changes were observed in the regions sequenced, indicating that the observed resistance was due to other mechanisms than changes in *cyp51A*.

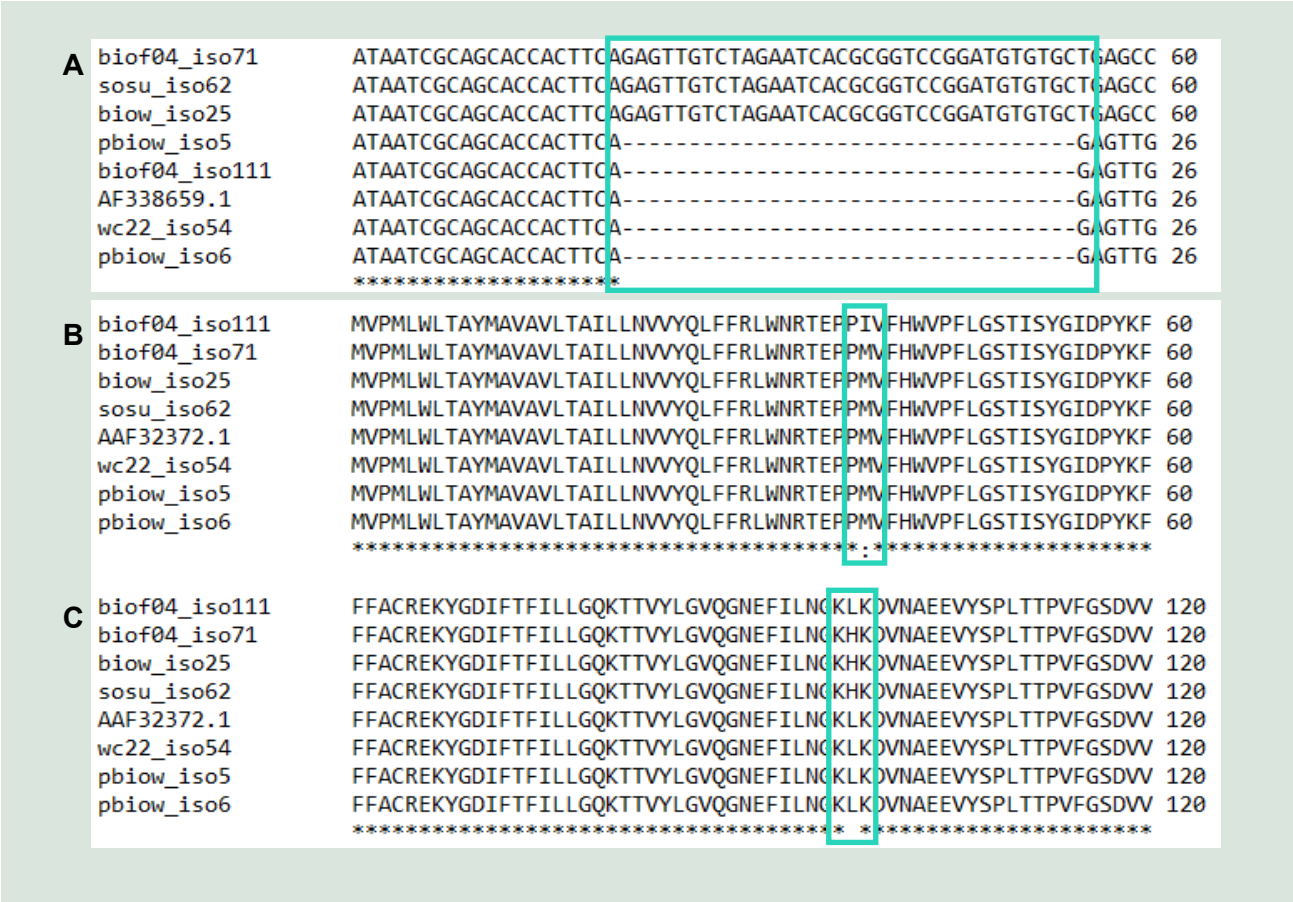

FIGURE 5. Multiple Sequence Alignment of the Cyp51A nucleotide and amino acid sequence of wild-type reference *Aspergillus fumigatus* and azole resistant isolates. The *cyp51A* DNA sequence (AF338659.1) and Cyp51A amino acid sequence (AF32372.1) was retrieved from NCBI. A) In focus is a section of the nucleotide sequence of the promoter regions, highlighting the well-characterized TR34 tandem repeat. B) Highlighted is the M39I mutation of isolate biof04_iso111 and C) the L98H mutation in the TR34/L98H isolates (bottom). Sequences were aligned using the CLUSTAL O(1.2.4) multiple sequence alignment tool.

6.6 Antifungal resistance prevalence and occupational exposure to resistant fungi across environments and time

We investigated the prevalence of antifungal resistance and the exposure to resistant *A. fumigatus*, *A. niger* (and sibling species), *A. flavus*, *A. nidulans*, *A. terreus*, *A. lentulus*/*A. udagawe*, *A. versicolor*, and *Candida parapsilosis* across working environments and time-periods (**Table 5**). When factoring in the exposure (thereby analyzing the exposure to resistant fungi), an effect of working environment was observed both for “All species” and for individual species (*A. fumigatus*, *A. niger* complex, *A. nidulans*, and *A. versicolor*). Biofuel plants had the highest occupational exposure to resistant fungi, for “All species”, *A. fumigatus*, and *A. nidulans*. The exposure to resistant fungi of the *A. niger* complex was highest in biowaste plants, while the exposure to resistant *A. versicolor* was highest in greenhouse facilities (**Table 5**). A significant effect of time was evident when considering “All species” together and specifically for *A. fumigatus*; the time-period 2000 to 2009 had the highest exposure.
The type of working environment had a significant effect on the prevalence of resistance (P = 0.024; **Table 5**) when considering all species together. However, the effect was not significant

when analyzing species individually. The number of resistant isolates for individual environments was as follows: Biowaste plants with 15 out of 145 isolates, nursing homes (6/110), waste collection (4/62), biofuel plants (3/124), and greenhouses (3/16). The effect of working environment was beyond what could be explained by time, which was also included in the model (likelihood ratio test).

TABLE 5. P-value for effect of environment or time period on exposures (GM of positive samples in CFU/m3) to resistant fungi and their prevalence.

	All species	A. fumigatus	A. niger	A. nidulans	A. versicolor
Environment					
P-value	**<0.0001 1)**	**<0.0001**	**<0.0001**	**<0.0001**	**0.008**
Exposure (CFU/m3):					
Biofuel	23.5a, 2)	30.0a	0.46 NR	248.0a	*
Cabbage	*3)	*	*	*	*
Grass seeds	0.30 NR	0.30 NR	*	*	*
Greenhouse	13.4ab	0.27 NR	*	*	48.9
Pig farm	*	*	*	*	*
Pigeons	0.33	0.33 NR	*	*	*
Biowaste	9.2b	13.8b	64.5a	0.95 NR	*
Waste collection	5.2b	2.17c	36.1a	*	0.85 NR
WWTP	0.43d	0.30 NR	0.23 NR	0.95 NR	0.85 NR
Homes	0.23 NR, 4)	0.23 NR	*	*	*
Hospital	0.23 NR	*	0.23	*	*
SOSU	1.23c	0.93c	1.43b	2.49b	*
Outdoor air	0.31	0.31	*	*	*
Time period for exposure					
P-value	**0.0013**	**0.0080**	**0.30**	**NA5)**	**0.82**
Exposure (CFU/m3):					
1) 2000-2009	26.09a	26.7a	0.95 NR	248.0a	37.6a
2) 2010-2018	1.73c	0.955c	2.5491b	2.49b	21.6a
3) 2019-2023	4.75b	3.67b	48.28a	0.95 NR	0.85 NR
Interaction for exposure					
Enviroment:year (P-value)	**0.69**	**0.55**	**NA**	**NA**	**NA**
Resistance prevalence					
Environment (P-value)	0.024	0.80	0.086	0.339	0.085
Time period (P-value)	0.46	0.22	0.40	NA	0.70
Interaction (P-value)	0.99	1	NA	NA	NA

1)P-values <0.05 are in bold. 2)Numbers in the same column followed by the same letter are not significant different. 3)NR = None resistant (None of the tested isolates were resistant), 4)* Below detection (Species were not found in samples, and exposure is thus below detection), 5) Not applicable. Table from Kofoed et al. (2024).

7. Discussion and perspectives

Based on exposure assessments during whole workdays combined with species identification and resistance testing, the occupational exposure to airborne resistant fungi in different working environments in Denmark has been estimated for 510 persons and 159 area samples. Data are analyzed as exposure to resistant fungi and as prevalence of resistance within a species or an environment. We show that workers are exposed to different species compositions, human pathogens, and azole resistant fungi in different working environments. Knowledge about exposure to azole resistant fungi in occupational settings is of particular interest as an ageing workforce coupled with higher rates of medical vulnerabilities constitute risk factors for serious fungal infections (Bongomin et al., 2017; Kauffman and Yoshikawa, 2001). Accordingly, investigating the exposure to resistant fungi in potential high-risk environments could contribute to risk assessment and prevention strategies. Moreover, the prevalence of emerging pathogens, including new and rare species, has increased in recent years (Geddes-McAlister and Shapiro, 2019). Insights as to the resistance and occurrence of less studied species have thus become progressively more important. This will be discussed in the following sections.

7.1 Overall fungal exposure and species composition

The overall fungal exposure varied significantly between working environments. Workers in grass seed production were exposed to extremely high concentrations of fungi. However, this was a special case in which workers had developed organic dust toxic syndrome (ODTS), and may not reflect the daily environment (Madsen et al., 2015). The development of infection is dose-dependent. At present, limited information is available on the infectious dose of fungi, which may be highly variable between species (Lass-Flörl et al., 2021). Infectious dose has been studied for inhalation of *A. fumigatus* in mouse models, but numbers may not translate to humans (Leleu et al., 2013). Nevertheless, it is safe to assume that higher exposure carries a higher risk of infection.

The working environments showed different species compositions. In biofuel- and biowaste plants, workers were highly exposed to pathogenic species of *Aspergillus*. Workers in biofuel plants had high exposure to *A. fumigatus*, only surpassed by workers in the grass seed plant, indicating that growth and aerosolization are facilitated in this environment. Workers in biowaste plants, were in addition to pathogenic *Aspergillus* spp. such as *A. fumigatus* and *A. niger*, exposed to a multitude of other fungal species, with this working environment showing the highest number of individual species. This may be explained by the highly diverse material (household organic waste), handled by biowaste workers, which could accommodate a range of different species. Waste collection workers were exposed to fewer *Aspergillus* species than the biowaste workers and had lower exposure in general. This may be related to the handled waste typically being “newer” at the time of collection leaving less time for fungal growth and that waste collection workers work outdoors.

Workers in greenhouse facilities were rarely exposed to pathogenic *Aspergillus* spp. despite comparable overall exposure levels to workers in biofuel plants and biowaste plants. Instead, workers in greenhouse facilities, were predominantly exposed to species of *Penicillium*, which are rarely associated with human infections (Perrone and Susca, 2017).

Fungal species were selected for susceptibility testing based on several criteria outlined in **Table 3**. First, we aimed to select all species, with extensive resistance data, in the form of clinical breakpoints, ECOFFs or MIC distributions including *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger, A. versicolor*, *A. terreus* and *Candida parapsilosis*. Furthermore, we aimed

to select additional species within the genus *Aspergillus* and the order of Mucorales, as these encompass several emergent pathogens (Geddes-McAlister and Shapiro, 2019). Certain species, including *Aspergillus ustus* and *A. scletorium* were not included due to insufficient sporulation, even after long incubation on a variety of agar media types. Lastly, additional species, for which human infections have also been described including, *Paecilomyces variotii* and *Scopulariopsis brevicaulis*, were frequently found in samples and were also selected for resistance testing.

7.2 Exposure to resistant fungi and prevalence of antifungal resistance across environments and time

The type of working environment had a significant effect on the overall prevalence of resistance while the sampling time-period did not show a significant effect. Resistant isolates were most numerous in samples from biowaste plants, while samples from greenhouse facilities showed the highest frequency of resistance among the isolates tested. Azole residues have previously been found in organic household waste as well as in plant material (Schoustra et al., 2019), which along with abundant substrate for growth, could facilitate selection for resistant phenotypes. The use of fungicides in horticulture is mentioned in **Table 1**.

The occupational exposure to resistant fungi varied between both sampling time-periods and environments. The exposure to resistant fungi (all species together) was highest in biofuel plants, and specifically for the species *A. fumigatus*. In line with this result, woodchips, often used for heat production in biofuel plants, have been associated with presence of high quantities of azole resistant *A. fumigatus* on the surface (Schoustra et al., 2019). Environmental hotspots for presence of resistant *A. fumigatus* on materials (such as green waste or woodchips) have previously been identified, and are generally linked to certain factors, including the presence of fungicide residues and favorable growth conditions (Schoustra et al., 2019; Verweij et al., 2020). However, presence of antifungal resistant fungi on a material such as wood chips is not an expression of how many resistant fungi are spread and if people are exposed to them. Thus, the number of fungi on surfaces of wood chips and straw do not correlate with how many are released to the air (Madsen et al., 2006). Previously we have found that 1 kg straw or woodchips in average released 3.5×10^5 CFU of *A. fumigatus* per kg (Madsen et al., 2004). To prevent exposure to and spread of resistant *A. fumigatus* during work in biofuel plants, preventive measures should be identified.

As mentioned in the introduction, serious invasive fungal infections more often develop in immunocompromised individuals compared to healthy people at work. One of the working environments causing high exposure to antifungal resistant *A. fumigatus* and *A. niger* is biowaste plants. In a previous study, we found that workers collecting biowaste in the end of their workday had up to 3×10^6 CFU *A. fumigatus* and 1×10^4 CFU *A. niger* per m^2 on their work t-shirt (Madsen et al., 2022a), and similarly the exposure level to airborne fungi has a significant impact on the accumulation of fungi on work t-shirts and the aerosolisation of fungi from the t-shirt (Møller et al., 2022). Therefore, exposure to airborne *A. fumigatus* and *A. niger* during work at biowaste plants may, without showering and change of clothes, be associated with para-occupational exposure (via hands and air) to antifungal resistant fungi. This may occur during post-work activities such as transportation from work, shopping, and in the home.

Regarding the effect of time, we observed the highest exposure in the earliest time-period (2000 – 2009), and the result was significant. Perhaps contrary to this result, the resistance prevalence has been suggested to increase over time for *A. fumigatus* in Denmark, as discussed in Risum et al. (2022). Here it is important to distinguish between the prevalence and the exposure, as high prevalence of resistance, does not in itself cause high exposure to resistant fungi. Thus, other factors, such as the overall fungal exposure could also contribute to a higher exposure to resistant fungi.

7.3 Resistance among individual fungal species

While the exposure to *A. fumigatus* was high in some environments, only a small portion of the tested *A. fumigatus* isolates showed resistance. The overall prevalence of 2.5 % resistance, is in line with recent, large scale data of environmental samples from Germany (Barber et al., 2020), while other studies have found higher prevalence (Mortensen et al., 2010; Sewell et al., 2019). Direct comparison is however problematic, as resistance may vary depending on location and sample type (e.g. air samples or samples of soil or material), with some environments regarded as hotspots for resistant *A. fumigatus* (Verweij et al., 2020). Despite *A. fumigatus* being recovered from most working environments in this study, conditions facilitating resistance development may not be present in all. In addition, 85 of 317 *A. fumigatus* isolates were recovered from biofuel plant workers' exposure between 2000 and 2005. Due to the proposed increase in resistance prevalence during recent years (Risum et al., 2022; Verweij et al., 2020), older samples could potentially harbor fewer resistant isolates, compared to more recent samples (we did not observe this for the overall prevalence of resistance). Despite the few azole resistant isolates of *A. fumigatus* observed, these should arguably hold more weight, due to the pathogenicity of the species (Latgé, 1999). In addition, due to the high observed exposure to *A. fumigatus* in particular for biofuel plant workers, the exposure to resistant *A. fumigatus*, could be high despite the relatively low prevalence of resistance for this species.

The prevalence of resistance for individual species was further nuanced by the identification of cryptic species within the *Aspergillus niger* complex. In this study, MALDI-TOF MS was used for species identification throughout, while sequencing of *benA* and *camA* was predominantly carried out for resistant isolates. Previous studies have shown that MALDI-TOF MS can identify cryptic species, but is limited by the quality and size of the database (Imbert et al., 2019; Vidal-Acuña et al., 2018). Thus, sequenced-based techniques remain the most reliable for identification of cryptic species (Bian et al., 2022). Using MALDI-TOF MS we were able to distinguish *A. lentulus/A. udagawe* from *A. fumigatus*, and we identified other cryptic species including *A. westerdijkiae* and *A. calidoustus*. In our study MALDI-TOF MS could not distinguish between species in the *Aspergillus niger* complex, likely as a result of them not being present in the database. The majority of resistant isolates, were identified as *A. tubingensis* after *benA* and *camA* sequencing. Cryptic *Aspergillus* species are often associated with resistant phenotypes (Gautier et al., 2016). However, the results of the sequencing, which also included susceptible isolates of the *A. niger* complex, suggest that *A. tubingensis*, was prevalent among susceptible isolates as well.

Some clinical laboratories are described to rely on morphological identification of clinical fungal isolates; while the identification to complex level appears to be accurate, morphology is often insufficient for species level identification due to similarity between cryptic species within the same complex (Takeda et al., 2022). Not accurately identifying to the species level could lead to misinterpretation of resistance data. As an example, if a cryptic species (e.g. *A. lentulus*) is not distinguished from a non-cryptic sibling species (e.g. *Aspergillus fumigatus*), a falsely higher rate of resistance may be attributed to the non-cryptic species, due to the often naturally occurring (intrinsic) resistance among the cryptic species.

A part of this study was to obtain knowledge about resistance in species, which are not commonly investigated. One of these species is *A. nidulans*, for which two out of thirteen isolates were resistant to Amphotericin B according to the ECOFF. Exposure to *A. nidulans* in different environments is not well described, and it may be because it is not the most commonly present fungus. Another species, *A. versicolor* is commonly found in indoor environments, especially in humid buildings (Knudsen et al., 2017; Pasanen et al., 1997). In this study, *A. versicolor* was mainly found in the greenhouse workers' exposure. Due to the high frequency of resistance among the tested *A. versicolor* isolates combined with a common presence of this fungus in environments with many people, it is relevant to put further light on this species in terms of resistance to antifungals.

Clinical breakpoints, ECOFFs and published MIC distributions allow for better interpretation of resistance, but these have only been determined for a few fungal species. The interpretation of resistance is thus limited by the scarcity of available data. In this study, a number of species

with limited resistance data underwent susceptibility testing. While these were not included for statistical analysis, obtained MIC values can contribute to species specific data for less common fungi. In accordance with previous studies, high MIC values against Voriconazole were observed for species of Mucorales (Espinel-Ingroff et al., 2015). One isolate of *R. pusilus*, had a high MIC value against Itraconazole, and appeared to deviate from the remaining Mucorales isolates. More information is needed to determine if this phenotype is associated with a non-wildtype isolate. In addition, the high MIC values against Voriconazole for isolates of *Paecilomyces variotii* (and *Byssochlamys spectabilis*), and the high MIC values against all tested antifungal drugs for *Scopulariopsis brevicaulis* isolates, correspond with the existing literature (Cuenca-Estrella et al., 2003; Houbraken et al., 2010).

7.4 Perspectives

Several factors are important to consider when interpreting the occurrence of resistance and assessing the risk of occupational exposure to resistant fungi. Certain species are more pathogenic and more frequently cause infections, and resistance among these is more concerning from a clinical and occupational perspective. Furthermore, while fungi can acquire resistance through mutations, some species appear to show higher rates of resistance. In this study, the observed prevalence of resistant fungi appears to be more associated with cryptic species, rather than susceptible species acquiring resistance. Cryptic species such as *A. tubingensis* and *A. lentulus* are known to cause serious invasive infections (Imbert et al., 2021), and resistance among these is thus important to consider from the perspective workers' health. A distinction between cryptic and non-cryptic species is however important in trying to gain a further understanding of how resistance develops.

We frequently observed isolates with resistance towards one or more of the tested triazole drugs. In addition to clinical usage, azole fungicides are used extensively in agriculture, with sales in Denmark estimated at 165 tons in 2018 (Burks et al., 2021). With a possible environmental route of resistance to medical triazoles, driven by exposure to azole fungicides, the widespread use of these agents is concerning.

With this study, we have gained knowledge about certain working environments, in which workers may have an increased risk of being exposed to antifungal resistant fungi. Resistance is a risk factor for individuals prone to infections and can severely worsen patient outcomes. It would be pertinent to monitor the occurrence of resistant fungi in working environments where the exposure to potentially pathogenic fungi is high. Moreover, with *A. fumigatus* being the primary cause of invasive aspergillosis, special attention should be given to environments where this species is prevalent. Yet, due to the increased occurrence of new and emerging pathogens, care should be taken not to disregard other potentially human infectious species. Based on the results of our study, biofuel plants could constitute an environment with increased risk of exposure to resistant fungi, and may benefit from further investigation.

Due to the potential selective pressure of fungicides, measuring the presence of fungicide residues in material handled by workers could be valuable in assessing this as a risk factor for increased occupational exposure to antifungal resistant fungi.

7.5 Conclusions

We sought to answer six research questions in order to highlight the risk of occupational and para-occupational exposure to resistant fungi.

First, we determined to which fungal species and at what concentrations workers are exposed in different environments. As expected, we observed that the fungal species composition and the total fungal exposure varied significantly between the working environments ($p < 0.0001$), with exposures varying between 2.6 and 6.7 $\times 10^6$ CFU/m^3. High exposure levels were measured in working environments including the grass seed plant, biofuel plants and biowaste plants. The lowest exposure levels were measured in the WWTPs (GM = 12 CFU/m^3). This low level is comparable to the level of reference outdoor air samples. In total 53 different fungal species were isolated and identified, across working environments, with the most prevalent fungal species being *A. fumigatus*, with an average exposure of 207

CFU/m^3 (GM of positive samples). The highest exposure of *A. fumigatus* was observed in grass seed plant workers' exposure at 2.60×10^5 CFU/m^3. *A. niger* was the second most frequently observed species. The most diverse working environment in regards to species diversity was the biowaste plants, followed by greenhouse facilities.

Our second research question explored the exposure levels to antifungal resistant fungi and the prevalence of antifungal resistance in the working environments. Our observations indicate that the type of working environment has a significant effect on the exposure to antifungal resistant fungi, both when analyzing all species together and for individual species. Work in biofuel plants was associated with the highest exposure levels to resistant fungi for all species, and for the species *A. fumigatus*, which is the most frequent cause of aspergillosis, and thus a species for which resistance is of particular concern. The type of working environment also had an effect on the overall prevalence of antifungal resistance, however the effect was not significant on the individual species level.

We also sought to explore, if a higher prevalence of antifungal resistance existed for certain fungal species. The species *A. niger*, had the most isolates that exhibited resistance towards antifungals. However, the majority of these isolates, previously identified as *A. niger*, were later re-identified as cryptic species of the *A. niger* complex, such as *A. tubingensis*. Moreover, the species *A. versicolor* showed a high frequency of resistance, but the number of isolates was low (n = 18), and possibly not sufficient to conclude on the resistance prevalence for the species. Despite *A. fumigatus* being the most dominant fungus detected across working environments, the species represented a small part of fungal isolates exhibiting antifungal resistance, with an overall resistance prevalence of 2.5% for the species. However, among these few resistant isolates, multidrug resistance was observed, including the TR_{34}/L98H resistance mechanism. In addition, the work-exposure to *A. fumigatus* was high in several working environments. Combined with the pathogenicity of *A. fumigatus*, these factors suggest that greater significance should be given to this species.

An important part of our research was to determine whether, we observed **an overall increase in antifungal resistant fungi in the working environment over time.** However, the data presented here did not reflect an overall increase in antifungal resistant fungi in the working environment over time, neither when analyzing the prevalence of resistance, or when factoring in the occupational exposure.

Finally, we wanted to determine whether, we would find more resistance towards certain types of antifungals. Of the four antifungals included in this study, Itraconazole was the drug, which most fungal isolates exhibited resistance towards. Of the 34 isolates described in this work as resistant, mono-resistance towards Itraconazole (13/34) was the most frequently observed phenotype, and Itraconazole resistance was also observed in several multiresistant isolates. Itraconazole (a second generation azole drug) has been available for a longer time compared to the other azole drugs in this study and this could in part explain the higher frequency of resistance towards the drug. Relatively few isolates exhibited resistance towards Amphotericin B, three isolates exhibited mono-resistance (3/34), and two isolates exhibited multi-resistance in combination with Voriconazole. No isolates exhibited mono-resistance towards Posaconazole, and resistance to this drug, was only observed in multiresistant isolates.

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Appendix 1.

TABLE S1. Geometric mean (GM) and geometric standard deviation (gsd) of expo-sure to fungi cultivated at 37℃ across environments. Only active air samplers (Personal GSP samples, n = 510, and stationary GSP, n = 137) included. Table from Kofoed et al. (2024).

	Environment	GM (CFU/m³)	gsd
	Agriculture and biofuel		
1	Biofuel plants	807	10.2
2	Cabbage	23.7	2.0
3	Grass seeds	365724	16.1
4	Greenhouse	311	6.4
	Animal handling		
5	Pig farm	149	3.2
6	Pigeons	64.9	2.3
	Waste management		
7	Biowaste plants	633	7.8
8	Waste collection	86.5	3.7
9	WWTP	12.4	2.8
	Home & Healthcare		
10	Homes	24.4	1.1
11	Hospitals	20.1	1.3
12	Nursing homes	15.8	2.7
	Outdoor air		
13	Outdoor references	17.4	4.0

WWTP: Wastewater treatment plants

Appendix 2.

TABLE S2. MIC values (mg/L) as GM (geometric mean), Modal MIC, MIC50, MIC90, min and max. n = number of tested isolates (EUCAST broth microdilution).

Species	Parameter	AMB	ITR	VOR	POS
Aspergillus candidus	GM	0.435	0.018	0.034	0.014
	Modal MIC	0.5	0.016	0.03	0.008
	MIC50	0.5	0.016	0.03	0.016
	MIC90	0.5	0.0244	0.048	0.0244

	Min	0.25	0.016	0.03	0.008
	Max	0.5	0.03	0.06	0.03
	n	5	5	5	5
Aspergillus flavus	GM	1.607	0.150	0.538	0.103
	Modal MIC	2	0.125	0.5	0.125
	MIC50	2	0.125	0.5	0.125
	MIC90	2	0.25	0.6	0.15
	Min	1	0.125	0.5	0.06
	Max	2	0.25	1	0.25
	n	19	19	19	19
Aspergillus fumigatus	GM	0.753	0.302	0.486	0.105
	Modal MIC	1	0.25	0.5	0.125
	MIC50	1	0.25	0.5	0.125
	MIC90	1	0.5	1	0.25
	Min	0.25	0.06	0.125	0.016
	Max	2	16	8	1
	n	171	171	171	105
Aspergillus lentulus	GM	4.000	0.707	2.000	0.354
	Modal MIC	4	0.5	2	0.25
	MIC50	4	0.75	2	0.375
	MIC90	4	0.95	2	0.475
	Min	4	0.5	2	0.25
	Max	4	1	2	0.5
	n	2	2	2	2
Aspergillus montevidensis	GM	0.197	0.028	0.109	0.016
	Modal MIC	0.125	0.016	0.06	0.016
	MIC50	0.1875	0.023	0.0925	0.016
	MIC90	0.5	0.0775	0.25	0.023
	Min	0.06	0.016	0.06	0.008
	Max	0.5	0.125	0.25	0.03
	n	6	6	6	6
Aspergillus nidulans	GM	1.532	0.171	0.172	0.071
	Modal MIC	1	0.25	0.25	0.06
	MIC50	2	0.25	0.25	0.06
	MIC90	7.2	0.25	0.25	0.125
	Min	0.25	0.06	0.06	0.03
	Max	8	0.25	0.25	0.25
	n	13	13	13	13
Aspergillus niger	GM	0.433	1.338	1.178	0.234
	Modal MIC	0.5	1	1	0.25
	MIC50	0.5	1	1	0.25
	MIC90	0.5	16	2	0.5
	Min	0.25	0.25	0.5	0.06
	Max	1	16	16	2
	n	131	131	131	131

Aspergillus ochraceus	GM	16.000	0.250	0.500	0.250
	Modal MIC	16	0.25	0.5	0.25
	MIC50	16	0.25	0.5	0.25
	MIC90	16	0.25	0.5	0.25
	Min	16	0.25	0.5	0.25
	Max	16	0.25	0.5	0.25
	n	1	1	1	1
Aspergillus terreus	GM	8.000	0.250	1.000	0.250
	Modal MIC	8	0.25	1	0.25
	MIC50	8	0.25	1	0.25
	MIC90	8	0.25	1	0.25
	Min	8	0.25	1	0.25
	Max	8	0.25	1	0.25
	n	1	1	1	1
Aspergillus tritici	GM	0.250	0.023	0.034	0.014
	Modal MIC	0.25	0.03	0.03	0.016
	MIC50	0.25	0.03	0.03	0.016
	MIC90	0.25	0.03	0.048	0.016
	Min	0.25	0.016	0.03	0.008
	Max	0.25	0.03	0.06	0.016
	n	5	5	5	5
Aspergillus versicolor	GM	0.998	0.461	0.583	0.230
	Modal MIC	1	0.5	0.5	0.25
	MIC50	1	0.375	0.5	0.25
	MIC90	2	16	1	0.5
	Min	0.06	0.03	0.25	0.06
	Max	4	16	2	2
	n	18	18	18	18
Byssochlamys spectabilis	GM	0.113	0.166	11.888	0.112
	Modal MIC	0.125	0.25	16	0.125
	MIC50	0.125	0.25	16	0.125
	MIC90	0.125	0.35	16	0.175
	Min	0.06	0.06	4	0.06
	Max	0.125	0.5	16	0.25
	n	7	7	7	7
Candida orthopsilosis	GM	0.125	0.038	0.030	0.024
	Modal MIC	0.125	0.03	0.03	0.03
	MIC50	0.125	0.03	0.03	0.03
	MIC90	0.125	0.054	0.03	0.03
	Min	0.125	0.03	0.03	0.016
	Max	0.125	0.06	0.03	0.03
	n	3	3	3	3
Candida parapsilosis	GM	0.500	0.030	0.030	0.022
	Modal MIC	0.5	0.03	0.03	0.016
	MIC50	0.5	0.03	0.03	0.023

	MIC90	0.5	0.03	0.03	0.0286
	Min	0.5	0.03	0.03	0.016
	Max	0.5	0.03	0.03	0.03
	n	2	2	2	2
Lichtheimia corymbifera	GM	0.315	0.315	16.000	0.157
	Modal MIC	0.25	0.25	16	0.125
	MIC50	0.25	0.25	16	0.125
	MIC90	0.45	0.45	16	0.225
	Min	0.25	0.25	16	0.125
	Max	0.5	0.5	16	0.25
	n	3	3	3	3
Lichtheimia ramosa	GM	0.297	0.500	16.000	0.250
	Modal MIC	0.25	0.5	16	0.25
	MIC50	0.25	0.5	16	0.25
	MIC90	0.425	0.5	16	0.425
	Min	0.25	0.5	16	0.125
	Max	0.5	0.5	16	0.5
	n	4	4	4	4
Paecilomyces variotii	GM	0.080	0.250	10.556	0.141
	Modal MIC	0.06	0.125	16	0.06
	MIC50	0.06	0.25	16	0.125
	MIC90	0.125	0.5	16	0.4
	Min	0.06	0.125	4	0.06
	Max	0.125	0.5	16	0.5
	n	5	5	5	5
Pichia kudriavzevii	GM	1.000	0.087	0.250	0.125
	Modal MIC	1	0.06	0.25	0.125
	MIC50	1	0.0925	0.25	0.125
	MIC90	1	0.1185	0.25	0.125
	Min	1	0.06	0.25	0.125
	Max	1	0.125	0.25	0.125
	n	2	2	2	2
Rhizomucor pusillus	GM	0.175	1.189	16.000	0.707
	Modal MIC	0.25	0.25	16	1
	MIC50	0.25	0.75	16	1
	MIC90	0.25	11.5	16	1
	Min	0.06	0.25	16	0.25
	Max	0.25	16	16	1
	n	4	4	4	4
Rhizopus microsporus	GM	0.630	0.397	0.397	0.198
	Modal MIC	0.5	0.5	0.5	0.125
	MIC50	0.5	0.5	0.5	0.125
	MIC90	0.9	0.5	0.5	0.425
	Min	0.5	0.25	0.25	0.125
	Max	1	0.5	0.5	0.5

	n	3	3	3	3
Scopulariopsis brevicaulis	GM	8.980	16.000	14.254	5.657
	Modal MIC	16	16	16	8
	MIC50	12	16	16	8
	MIC90	16	16	16	8
	Min	2	16	8	1
	Max	16	16	16	8
	n	6	6	6	6
Trichoderma longibrachiatum	GM	1.000	0.354	0.354	0.060
	Modal MIC	1	0.25	0.25	0.06
	MIC50	1	0.375	0.375	0.06
	MIC90	1	0.475	0.475	0.06
	Min	1	0.25	0.25	0.06
	Max	1	0.5	0.5	0.06
	n	2	2	2	2

Human exposure to antifungal resistant fungi across working environments and time

In recent years, resistance towards commonly used antifungal drugs has been observed for previously susceptible fungal species. Accumulative evidence suggests that the observed resistance can in part be attributed to the use of fungicides in agriculture, due to structural similarities between the agents used in the environment and in the clinic. While antifungal resistant fungi have repeatedly been found in environmental samples, the extent to which humans are exposed to these remains elusive. This study aimed to investigate the human exposure to resistant fungi across different working environments during a span of twenty years. To this end, more than 500 personal air samples taken from 12 different types of working environments were utilized and potentially pathogenic fungi present in the samples underwent susceptibility testing against medical triazoles, Itraconazole, Voriconazole and Posaconazole, as well as the polyne drug Amphotericin B. Working environments varied significantly in their overall fungal exposure and showed vastly different species compositions. Resistance was observed for 34 fungal isolates, most of which were found to be cryptic species of the *Aspergillus niger* complex. The results indicate that the working environment significantly contributes to the exposure to resistant fungi. Work in biofuel plants was associated with the highest exposure to resistant fungi overall and to resistant *Aspergillus fumigatus*, which is arguably the most concerning species from the perspective of workers health.

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