INSTITUTO POLITÉCNICO DE LISBOA

ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

Internship - Exposure assessment of microbial contamination in different indoor environments

MARGARIDA RODRIGUEZ SOUSA

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Mestrado em Tecnologias Clinico-Laboratoriais

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JÚRI:
PRESIDENTE: DOUTORA EDNA SORAIA RIBEIRO, ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA
ARGUENTE: DOUTORA RAQUEL FILIPA PINHEIRO SABINO, FACULDADE DE FARMÁCIA DA UNIVERSIDADE DE LISBOA

Mestrado em Tecnologias Clinico-Laboratoriais

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Abstract

This internship report is part of the Project/Thesis/Internship course in the 2nd year of the Master's program in Clinical Laboratory Technologies at the Lisbon School of Health Technology (ESTeSL). The professional internship took place at ESTeSL's Environmental Health Laboratory from September 2023 to May 2024, focusing on Public Health and Occupational Exposure.

During the internship, sampling campaigns were conducted in primary schools and sawmills to evaluate microbiological contamination (bacteria and fungi) by collecting bioaerosols, using active and passive methods. In the laboratory, activities encompassed classical microbiology (culture~~e~~omics) and molecular techniques such as qPCR and DNA extraction. Additionally, an SOP was developed for screening azoles in *Aspergillus* section *Fumigati* isolates to define the resistance profile, a systematic review on WWTP was authored, alongside participation in a congress.

These tasks facilitated the consolidation of theoretical knowledge from the 1st year of the Master's program and the acquisition of competencies in Occupational Health, Microbiology, and Molecular Techniques, achieving the programmed objectives. This enriching experience also provided valuable skills in research and the daily life of a researcher, contributing significantly to future professional development.

Key Words: Occupational Exposure, Microbiology, *Aspergillus* section *Fumigati* and Bioaerosols

Resumo

Este relatório de estágio faz parte do curso Projeto/Tese/Estágio do 2º ano do Mestrado em Tecnologias Clínico-Laboratoriais na Escola Superior de Tecnologia da Saúde de Lisboa (ESTeSL). O estágio profissional ocorreu no Laboratório de Saúde Ambiental da ESTeSL de setembro de 2023 a maio de 2024, com foco em Saúde Pública e Exposição Ocupacional.

Durante o estágio, foram realizadas campanhas de amostragem em escolas primárias e carpintarias para avaliar a contaminação microbiológica (bactérias e fungos) através da colheita bioaressóis, através de métodos ativos e passivos. No laboratório, foram realizadas atividades de microbiologia clássica (culturoômica) e técnicas moleculares como qPCR e extração de DNA. Além disso, foi desenvolvida uma SOP para o screening de azóis em isolados de *Aspergillus* seção *Fumigati* para definir o perfil de resistência, e foi escrita uma revisão sistemática sobre WWTP, além da participação em um congresso.

Essas atividades permitiram a consolidação dos conhecimentos teóricos adquiridos no 1º ano do programa de mestrado e a aquisição de competências nas áreas de Saúde Ocupacional, Microbiologia e Técnicas Moleculares, cumprindo os objetivos estabelecidos. Esta experiência enriquecedora também proporcionou adquirir capacidades valiosas em investigação e no cotidiano de um investigador, contribuindo significativamente para o desenvolvimento profissional futuro.

Palavras-Chave: Exposição Ocupacional, Microbiologia, *Aspergillus* secção *Fumigati*, Bioaressóis

List of contents

List of Tables

List of Figures

List of Abbreviations

ABPA	Allergenic Bronchopulmonary Aspergillosis
AFST	Antifungal Susceptibility Testing
AMB	Amphotericin B
CFU	Colony-Forming Unit
CLSI	Clinical and Laboratory Standards Institute
DG18	Dichloran (18%) Glycerol Agar Base
EA	Essential Agreement
EDC	Electrostatic Dust Cloths
EDCT	Electrostatic Dust Cloths from T-shirt
ESTeSL	Escola Superior de Tecnologia da Saúde de Lisboa
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FRPD	Filtering Respiratory Protecting Devices
H&TRC	Health & Technology Research Center
ITZ	Itraconazole
MEA	Malt Extract Agar
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistance *Staphylococcus aureus*
OD	Optical Density
PBS	Phosphate Buffered Saline

PCZ	Posaconazole
qPCR	Quantitative Polymerase Chain Reaction
RPMI	Roswell Park Memorial Institute
SDA	Sabouraud Dextrose Agar
SOP	Standard Operating Procedure
TSA	Tryptic Soy Agar
VCZ	Voriconazole
VRBA	Violet Red Bile Agar
WGS	Whole Genome Sequencing
WHO	World Health Organization

1. Introduction

This report is incorporated into the second-year curriculum of the master's degree in Clinical-Laboratory Technologies at the Escola Superior de Tecnologia da Saúde de Lisboa (ESTeSL), part of the Instituto Politécnico de Lisboa.

1.1. Internship Objectives and Importance

As part of the academic curriculum, undertaking a professional internship is essential for acquiring practical and theoretical skills in environmental and public health. This internship, which spanned from September 2023 to May 2024 (Table 12.1, Appendix 1), totalled 600 hours and took place in an environmental/public health laboratory specializing in environmental and occupational microbiology at the Escola Superior de Tecnologia da Saúde de Lisboa (ESTeSL). The internship was supervised by Professor Carla Viegas. After the 600 hours completed, I continued to go to the Laboratory on a voluntary basis to help the bench work of the ongoing projects. In Appendix 2 – Figure 12.1 some activities done throughout the internship time.

The objectives of this internship were as follows:

- Conduct studies in various occupational environments, such as sawmills, primary schools, and poultry farms, to characterize the microbial contamination distribution (bacteria and fungi) and microbial resistance in different indoor environments (namely, *Aspergillus* section *Fumigati*).
- Integrate practical experience with the theoretical knowledge acquired in the master's courses, particularly in microbiology, by using culture methods applied to public health. This included inoculating samples collected from primary schools, poultry farms, and sawmills (such as settled dust, filters, and swabs) in different culture media.
- Apply molecular biology techniques, such as quantitative Polymerase Chain Reaction (qPCR), to target specific indicators of harmful fungal contamination.
- Develop writing skills by writing articles and communication skills by presenting posters at conferences.
- Create a Standard Operating Procedure (SOP) for antifungal suceptibilty testing of *Aspergillus* section *Fumigati* using an Etest method to determine minimum inhibitory concentration for ITZ, VCZ, PCZ and AMB.

1.2. Characterization of the internship location

The professional internship was conducted in the Environmental and Occupational Microbiology Laboratory at the Health & Technology Research Center (H&TRC), integrated within ESTeSL. During the internship, I followed three research projects carried out by four doctoral students (Bianca Gomes, Marta Dias, Pedro Pena, and Renata Cervantes). All doctoral students were supervised by Professor Carla Viegas.

2. Background

2.1. Exposure assessment to biological agents

Exposure assessment involves the qualitative or quantitative determination or estimation of the magnitude, frequency, duration, and rate of exposure. It is a crucial component of any quantitative risk assessment, being one of the four primary steps in the risk assessment framework established by the National Academy of Sciences in the United States(1).

Exposure to biological agents in various occupational environments is often neglected, resulting in adverse health impacts for workers. Unlike the well-documented impacts of chemical agents, the effects of biological agents are less recognized(2). However, biological agents can lead to diverse health issues in humans, acting as infectious, allergenic, toxic, and carcinogenic agents(2,3). When discussing biological risk, the focus is primarily on bacteria, fungi, viruses, and parasites(4).

Bioaerosols are the key responsible for the movement of microorganisms by air(4). Inhalation is considered the principal cause of exposure linked to adverse effects on workers' health, being relevant in occupational exposure(5). Bioaerosol sampling includes the collection of particles of biological origin from the air, make it possible to evaluate microbial contamination in environments (6,7).

2.2. Active and Passive Sampling

Occupational exposure to culturable bioburden is assessed using both active and passive sampling techniques (Figure 2.1.), influenced by environmental factors such as ventilation, seasonal variation, occupant quantity and cleanliness, building layout, and cleaning procedures(8–13).

2.2.1. Active Sampling

Active sampling techniques, including impaction (Andersen six-stage and MAS-100), impinger (Coriolis µ), and filtration (button air samplers), involve using an air sampling pump to draw air through a collection device, measuring

microorganisms in Colony-Forming Unit/m³ (CFU/m³) of air (14). In Table - 2.1. are described advantages and disadvantages of active air sampling methods.

2.2.2. Passive Sampling

Passive sampling techniques, which don't require air pumps, offer greater assay variety and easier handling, collecting contamination over longer periods (days to months) using devices like Filtering Respiratory Protecting Devices(FRPD), settled dust, Electrostatic Dust Cloths (EDC), and swabs (14,15). These methods reflect longer-term contamination levels, unlike the short-term load captured by active techniques(14).

To ensure accurate bioburden assessment, combining both active and passive sampling is recommended, enabling industrial hygienists to better characterize risks and improve occupational safety (10,11,14,16).

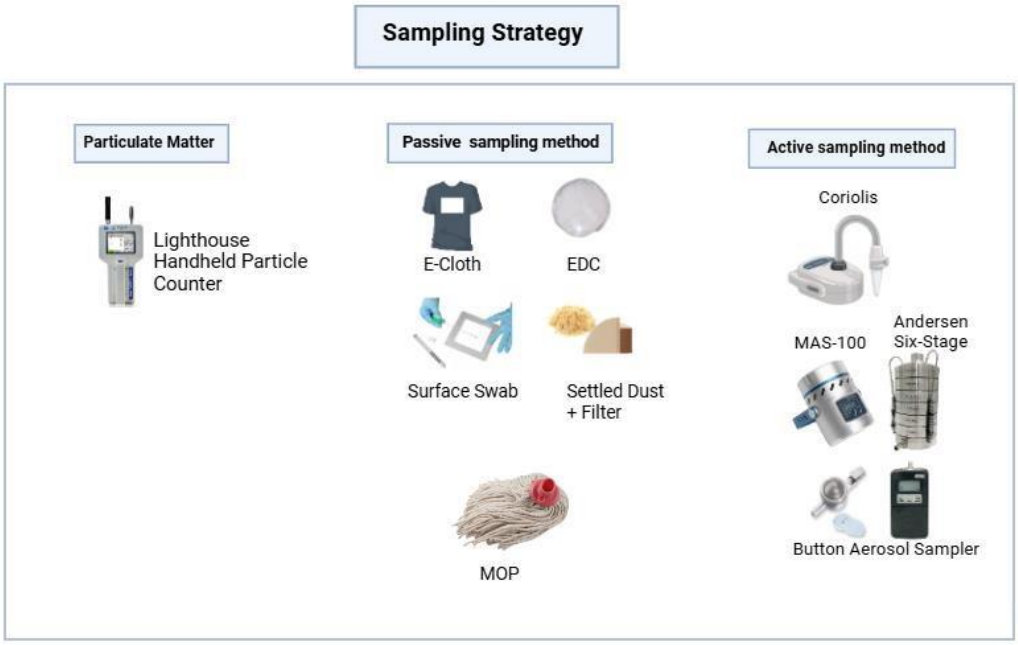

Figure 2.1 - Sampling strategies applied for occupational exposure assessment. Made with biorender, Scientific Image and Illustration Software | BioRender.

Table 2.1 - Advantages and Disadvantages of active air sampling methods, adapted from Whitby et al., 2022.

Air Sampling Method	Advantages	Disadvantages	References
Impaction (Andersen six-stage and MAS-100)	Suitable as particle size classifiers. Direct collection of microorganisms; Total sample to be examined.	Cell viability may be lost due to impact stress. Possibility of microbial overload on plates.	(14,17)
Impinger (Coriolis µ)	Liquid collection matrix distributed for different analysis. Airflow rates are higher, permitting shorter sampling periods compared to filtration.	Cell viability to be lost due to impact stress. Less efficient for culture- based methods.	
Filtration (Button Sampler)	Personal samplers provide human exposure assessment.	Personal samplers have low flow rates so longer sampling times are needed.	

2.3. Culture-based methods and molecular tools

2.3.1. Culture-based Methods

Culture-based methods have distinct advantages and limitations. Since the viable component of the bioburden impacts biological mechanisms like inflammatory and cytotoxic responses, it's essential to evaluate it using culture-based approaches(18–20). However, culture-based methods face challenges such as the varying growth requirements and rates of different fungal species, which can lead to overcrowding and chemical competition, thus biasing results(14,21). Moreover, these methods are limited by selective incubation temperatures and the culture media used (14,21).

2.3.2. Molecular Tools

Molecular tools offer benefits like speed, accuracy, high analytical sensitivity, and the capability to detect dormant or dead microorganisms and toxigenic fungal strains(14,22–24). Despite these advantages, molecular methods cannot assess the viable portion of the bioburden, crucial for health risk estimation(18). Additionally, qPCR assays may yield false negatives due to PCR inhibitors, ineffective DNA release, or poor DNA recovery. Airborne particles can also introduce inhibitors(25,26).

Therefore, integrating culture-based techniques with molecular technologies is recommended for comprehensive analysis (14).

2.4. Aspergillus section *Fumigati*

Aspergillus species are filamentous fungus that are typically found in soil, where they grow as saprophytes. Several *Aspergillus* species are thought to be opportunistic human infections, and the majority of the species are found in a variety of indoor settings(15,27,28).

Aspergillus section *Fumigati* is one of the most found in different environments among *Aspergillus* sections and one of the genus's sections that is frequently linked to respiratory problems such as asthma, allergic sinusitis, cough, and bronchial hyperresponsiveness because of the tiny conidia(15,29–32).

Moreover, *Aspergillus* section *Fumigati* is the causative agent of invasive aspergillosis, a disease that can be fatal for immunocompromised people at high rates(15,33). The development of resistance to antifungal drugs in *Aspergillus* spp., especially in section *Fumigati*, is an emerging public health problem(15).

Aspergillus fumigatus (A. fumigatus) is the most common and clinically relevant of the 63 species of this section (34) and it is included in the fungal pathogens list as a critical priority by World Health Organization (WHO) (WHO,2022). *A. fumigatus* are acquiring resistance to antifungals because of environmental selective pressure(34). Most azole-resistant illness cases are caused by resistant A. *fumigatus* (35).

2.5. Treatment with Antifungals

In the clinical treatment of invasive fungal infections, three distinct classes of antifungals are currently used: polyenes, echinocandins, and azoles (36–38).

Triazoles have a broader spectrum of applications, higher efficacy against various fungal species, and lower toxicity compared to Amphotericin B (AMB) (31). These antifungals interfere with ergosterol synthesis, a fundamental component of the fungal plasma membrane, by inhibiting the cytochrome P450 (CYP) enzyme, 14-α-demethylase (CYP51)(37,39–42), encoded by the CYP51A and CYP51B genes in Aspergillus spp. species(42). There has been an increase in the number of resistance cases in different fungal species to one or more azole antifungals, being the most common resistance mechanism mutations in the genes encoding the 14-α-demethylase enzyme, CYP51A and CYP51B (39–41), with CYP51A gene mutations being more frequent in *Aspergillus fumigatus*(42). In Portugal, has already been reported the TR34/L98H mutation, most frequent mutation described in CYP51A gene, in environmental isolates from occupational environments(29).

2.5.1. Amphotericin B

Amphotericin B is classified as the antifungal drug with the highest efficacy rate in treating invasive aspergillosis, among other invasive fungal infections. It also shows significant activity against various fungal species.(39,41,43,44). However, with the development of new antifungals, such as azoles and echinocandins, AMB is now administered only in specific cases of invasive fungal infections(45).

2.5.2. Itraconazole

Itraconazole (ITZ), a first-generation triazole, was the first clinically effective triazole in treating invasive aspergillosis (39,40). This antifungal is commonly used in treating chronic pulmonary aspergillosis and Allergenic Bronchopulmonary Aspergillosis (ABPA)(41).

2.5.3. Voriconazole

Voriconazole (VCZ), a second-generation triazole used in the treatment of various fungal infections, notably invasive fungal infections caused by *Aspergillus* spp.(39,46). Is considered the drug of choice for treating most cases of invasive aspergillosis (37,39–41,43).

2.5.4. Posaconazole

Posaconazole (PCZ), also a second-generation triazole, has a broad spectrum of antifungal activity (43,46). It is administered to immunocompromised patients in the treatment of invasive aspergillosis (46). It shows significant activity against *Aspergillus* spp., including *Aspergillus fumigatus* (41).

3. Projects ongoing

There were three research projects ongoing during the internship period. Although all projects shared the same objective, quantifying microbial contamination and characterizing patterns of microbial resistance, each focused on a different setting - sawmills, primary schools, and poultry farms.

3.1. Guidance for Microbial Occupational Exposure Assessment in Sawmills

This project aimed to assess and characterize the exposure of sawmill workers to microorganisms such as fungi and bacteria, as well as their metabolites (endotoxins, cytotoxins, and mycotoxins), and particulate matter. This is the project I will focus on to present results and to discuss them.

3.2. Identifying Determinants for Indoor Air Quality and their Health Impact in Environments for Children: Measures to Improve Indoor Air Quality and Reduce Disease Burdens

The InChildHealth project is included in HORIZON-HLTH-2021-ENVHLTH-02-02, dedicated to studying indoor air quality (IAQ) and its implications for health. The project seeks to identify factors that affect IAQ and their effects on the health of school-age children, covering chemicals, particle concentrations, microbes, and physical characteristics.

3.3. The Impact of Animals Bedding Material on the Sustainability of an Industrial Portuguese Poultry Farm through a One Health Perspective

This project aims to evaluate the potential health risks linked to microbial exposure among poultry farm employees, assess its impact on animal productivity, and investigate the indirect environmental effects of Portuguese poultry pavilions, focusing on the materials used in animal bedding.

Participation was limited to conducting post-sampling procedures for samples collected via passive methods, as sampling took place outside mainland Portugal.

4. Description of activities

The assessment of microbiological occupational exposure can be conducted using both active and passive sampling methods, along with laboratory procedures that enable microbial characterization. Figure 4.1. illustrates the workflow from fieldwork to laboratory analysis, beginning with the preparation of the sampling campaign and concluding with the various assays employed.

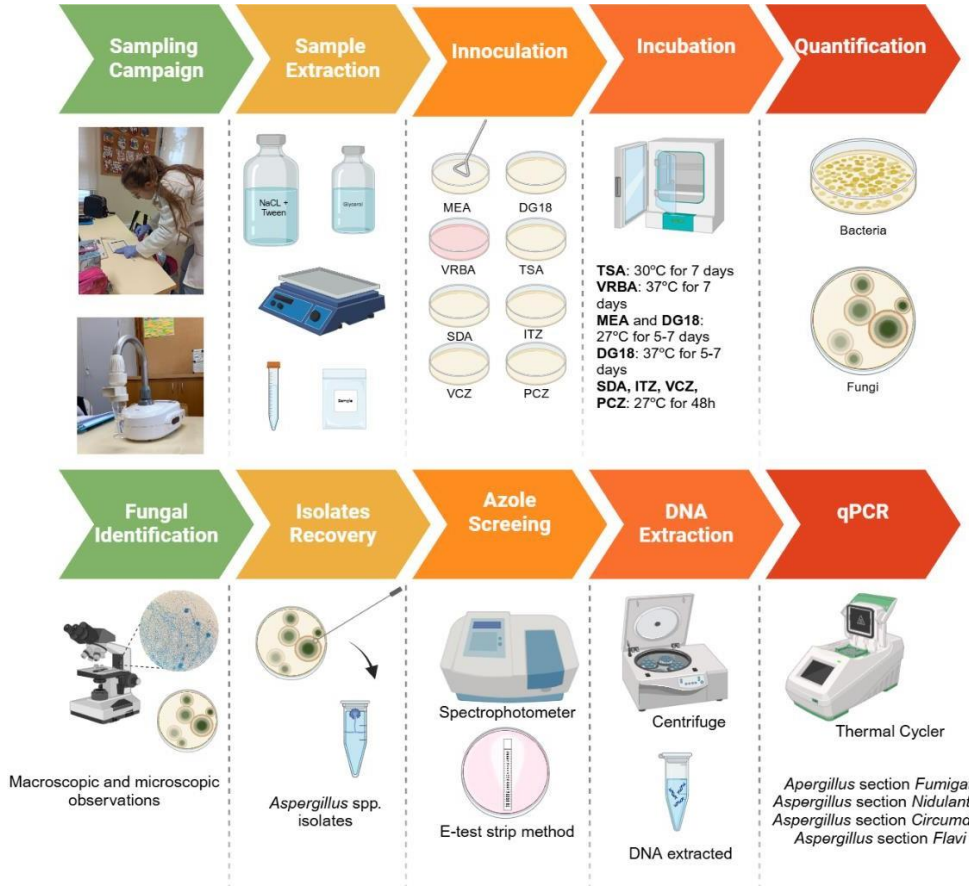

Figure 4.1 - Schematic representation of workflow from field work to laboratory work. Made with biorender, Scientific Image and Illustration Software | BioRender. Note: DNA extraction and qPCR are assays applied in all samples and not only in azole-resistant isolates.

4.1. Preparation of Sampling Campaigns

A checklist was created to ensure nothing was overlooked, including the number of swabs, EDCs, plastic bags, filters, equipment's, media plates, gloves, solutions like Phosphate Buffered Saline (PBS), distilled water and 70% alcohol, a permanent pen, and a lab coat. It was essential to ensure that all equipment had fully charged batteries.

4.2. Sampling Campaigns

4.2.1. Active Sampling

The active methods used in the sampling campaigns were as follows: particulate matter as Lighthouse Handheld Particle Counter measurement; impaction methods including Andersen six-stage impactor and MAS-100 air sampler; impinger methods such as Coriolis µ air sampler collection, and filtration methods like Button Sampler air sampler (personal air sampling). In sawmills and primary schools were variations on the sampling sites, these differences are described below.

Sampling in sawmills:

- Sampling sites: bench zone (BZ), machine zone (MZ), office (O), warehouse (W), and exterior (E).

- Sampling strategy : Lighthouse Handheld Particle Counter; MAS-100 using four different culture media (TSA, VRBA, MEA, and DG18); Ander six-stage using three different culture media (TSA, VRBA and DG18 – DG18 is collected in duplicate because the plates are incubated at 27ºC and 37ºC - to evaluate the pathogenic potential); Button Sampler placed in breathing area of two workers, one in BZ and other in MZ.

Sampling in primary schools:

- Sampling sites: library (L), classrooms (C), canteen (Ca), bathrooms (B), gymnasium (G), and exterior surroundings (E).

- Sampling Strategy: In each location, MAS-100 using four different culture media (TSA, VRBA, MEA, and DG18 media), Lighthouse Handheld Particle Counter, and Coriolis µ. Andersen Six-Stage sampling using three different culture media (TSA, VRBA, and DG18 - DG18 is collected in duplicate because the plates are incubated at 27ºC and 37ºC - to evaluate the pathogenic potential) in L, C, Ca and G. Button sampler placed in

breathing zone in a teacher in a sampled classroom and another in a school auxiliary.

In Table 4.1. are described the sampling strategies and sampling sites by sampling method.

4.2.2. Passive Sampling

Passive methods collected during the sampling campaigns included surface swabs, Electrostatic Dust Cloths (EDCs) (10 cm^2), EDC from t-shirt (EDCT), settled dust (collected with a sterile coffee filter into a vacuum cleaner tube) and mops. In Table 4.1. are described the sampling strategies and sampling sites by sampling method.

Sampling in sawmills:

- Sampling sites: BZ, MZ, O and W.

- Sampling Strategy: Surface Swabs on floor using a 10 × 10 cm stencil disinfected with 70% alcohol between samples, settled dust, placement of EDCS in hard-to-access locations for 15 days positioned 1.5 meters above the floor. EDCT were attached to sawmill workers, one in BZ and other in MZ, near the breathing area (in the same workers that carried the button sampler).

Sampling in primary schools:

- Sampling sites: L, C, Ca and G.

- Sampling Strategy: Surface swabs on floors, doors, and tables using a 10 × 10 cm stencil disinfected with 70% alcohol between samples, settled dust, placement of EDC booklet strategically at heights of 1.5-2.5 meters in each location, left for 30 days, attachment of EDCT ate one student per classroom (collected at the end of each class session), mops from C and B whenever its possible. Additionally, handle and table swabs were done for Methicillin-Resistance *Staphylococcus aureus* (MRSA) assessment, Due to their nature, the samples are transported in Stuart medium for further analysis.

In Table 4.1. are described the sampling strategies and sampling sites by sampling method.

Table 4.1 - Sampling strategy and sites for each sampling method.

Sampling Method	Sampling Strategy	Sampling site: Sawmills	Sampling site: Schools
Coriolis µ	2 min at flow rate 300L/min	MZ, BZ, O and W	C
MAS – 100	2 min at flow rate 100L/min	MZ, BZ, O, W and E	C, Ca, L, G, B, O
Andersen six-stage	9 min at a flow rate 28L/min	MZ, BZ, O, W	C, Ca, L
Lighthouse Handheld Particle Counter	7 min	MZ, BZ, O, W and E	C, Ca, L, G. O
Button Sampler (BS)	2 hour at a flow rate of 4L/min	Workers in BZ and MZ	Teacher and school auxiliary
EDC	Placed at 1.5-2.5 meters for 15-30 days	BZ, MZ, O and W	C, Ca, L, G
EDCT	Placed in t-shirt for a defined period	Workers in BZ and MZ (Same ones as BS)	Student
Surface Swabs	10 × 10 cm stencil disinfected with 70% alcohol between samples	Floor: MZ, BZ, O and W	Table, door and floor: C and Ca; Door and floor: B; Floor: L and G

4.3. Laboratory Work

4.3.1. Samples Extraction

Upon arrival at the laboratory, passive and active samples were processed differently according to their collection methods. Active samples collected through impaction methods were incubated for 5-7 days at specific temperatures

(MEA and DG18 - 27°C and DG18 - 37°C for fungi, TSA - 30°C and VRBA - 37°C). Fungal plates were incubated upside down to prevent condensation from dripping onto the medium, while bacterial plates were incubated right-side up to maintain dry conditions favourable for bacterial growth(47).

Samples Extraction from Coriolis µ, Filters from Button Sampler and samples collected from passive methods:

NaCl 0.9% + Tween 80 0.05% solution preparation: for 100 mL of distilled water, it was weighed 0.9 g of NaCl and measured 0.05 mL of Tween 80, then autoclaved at 120°C for 15 minutes.

- Coriolis µ (liquid samples): The liquid was divided into two Eppendorf tubes for DNA extraction and cytotoxic assays, while the remaining liquid was split into two Falcon tubes for mycotoxin and endotoxin assays.
- Samples from EDCs, EDCTs, Mops, and filters (from vacuumed dust): EDCS, EDCTS and Filters cut into two pieces. One quarter of each sample was placed in two sterile bags for mycotoxin and endotoxin assays. The remaining half was added to a Falcon tube containing a solution of 0.9% NaCl and 0.05% Tween 80, then agitated agitated in the orbital shaker (*Light Duty Orbital Shaker*) at 250 rpm for 30 minutes. After, the EDC, EDCT, and filter samples were squeezed, the liquid was divided into two tubes: one for cytotoxic assays and the other with added glycerol, stored until inoculation.
- Filters (Button sampler): The filter was placed in a Falcon tube with NaCl 0.9% + Tween 80 0.05% and agitated at 250 rpm for 60 minutes. The filter was squeezed, glycerol was added, and the sample was stored until inoculation.
- Surface Swabs: swab was placed in an Eppendorf tube containing NaCl 0.9% + Tween 80 0.05%, agitated at 250 rpm for 30 minutes. Swab was removed, and the sample was frozen until DNA extraction.
- Settled dust: Dust samples were collected and, where feasible, divided into two tubes for mycotoxin and endotoxin assays. When less than 2 grams of dust were available, which was often the case in schools, a composite sample was created from all sampled locations. The collected dust was then mixed with a solution of 0.9% NaCl and 0.05% Tween 80, agitated at 250 rpm for 30 minutes. Half of this mixture was set aside for

cytotoxicity assays, while glycerol was added to the other half, which was then stored until inoculation.

Glycerol was added to the samples in a proportion of 250 microliters per milliliter of solution.

Samples extracted for mycotoxin assays and DNA extraction were stored at -20°C, whereas samples for inoculation, endotoxic, and cytotoxic assays were kept at -80°C. It is important to note that cytotoxic, endotoxic, and mycotoxin assays were not conducted in our current laboratory and are therefore not described here.

4.3.2. Microbial contamination assessment

Culture media preparation was done by manufacturer instructions and prior to inoculation.

4.3.2.1. Bacteria contamination assessment

The extracted samples reserved for inoculation were introduced into different media: Tryptic Soy Agar (TSA) and Violet Red Bile Agar (VRBA) to assess bacterial presence. A volume of 150 µL from each liquid sample was spread across the media using a spreader. The plates were then incubated for 7 days at specific temperatures: TSA at 30°C for total bacterial counts, and VRBA at 37°C for gram-negative bacteria. Only bacterial colonies were quantified, expressed in colony-forming units (CFU).

4.3.2.2. Fungi contamination assessment

For fungi, samples were inoculated onto Malt Extract Agar (MEA) and Dichloran Glycerol Agar (DG18), with DG18 being done in duplicate. A volume of 150 µL from each liquid sample was spread across all media using a spreader. The plates were incubated for 5-7 days at specific temperatures (MEA and DG18 - 27°C and DG18 - 37°C) Figure 4.2. shows plates after incubation. Additionally, samples were inoculated onto azole-supplemented media, including Sabouraud Dextrose Agar (SDA), and SDA supplemented with 4mg/L Itraconazole (ITZ),

1mg/L Voriconazole (VCZ), and 0.5 mg/L Posaconazole (PCZ), to assess fungal resistance(48). The plates were incubated for 48h at 27ºC.

For fungi grown on MEA, DG18, SDA, ITZ, VCZ and PCZ different colonies were counted and identified macroscopically. A small piece of each fungus was cut and sliced with a sterilized scalpel (the scalpel was sterilized between cuttings) and placed on a slide with a drop of lactophenol cotton blue, then covered with a coverslip. The slides were observed under a microscope while the respective fungi were visualized macroscopically on the plates.

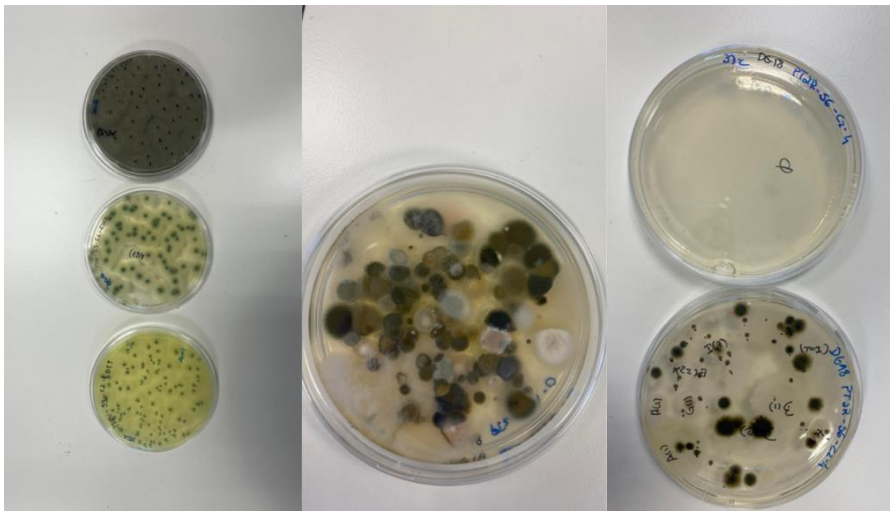

Figure 4.2. - Plates of MEA and DG18 after incubation

4.3.3. Isolates recovery

After identifying the fungal colonies, each was isolated into 1.5 mL Eppendorf tubes containing PBS solution. Using an inoculation loop, a piece of each fungus was collected and transferred to the Eppendorf tube (49). Isolates from *Aspergillus* spp. and *Mucor* spp. were recovered, with all isolates of *Aspergillus* section *Fumigati* and *Aspergillus* section *Flavi* being collected in duplicate. Glycerol was added to the samples, which were then stored at -80°C until further analyses. Glycerol was added to the samples in a proportion of 250 microliters per milliliter of solution.

PBS solution preparation: 10 mL of PBS are measured for 90 mL of distilled water, then autoclaved at 120°C for 15 minutes.

4.3.4. Antifungal susceptibility testing of *Aspergillus* Section *Fumigati*

During my internship, I concentrated on developing a protocol for antifungal susceptibility testing in isolates of *Aspergillus* section *Fumigati*. For internal use at environmental/public health laboratory in Health & Technology Research Centre (H&TRC), I developed a standard operating procedure (SOP) for this protocol. This is an Antifungal Susceptibility Testing (AFST) by gradient strips Etest Method(50). The objective is to evaluate the susceptibility of isolates to antifungals such as ITZ, VCZ, PCZ, and AMB. In brief, the process begins with creating a pure colony from each isolate, then incubating it and verifying that it has grown without contamination. If the colony is confirmed to be pure, the screening protocol can proceed. The turbidity must be adjusted to 0.5 on the McFarland scale, corresponding to an Optical Density (OD) of 0.06 (51). Then, inoculate the solution into Roswell Park Memorial Institute (RPMI) 1640 Medium, place the four azole strips on the plate, and incubate at 35ºC. The minimum inhibitory concentrations (MIC) are measured at 24 and 48 hours. The detailed protocol can be found in the SOP in Appendix 3.

4.3.5. DNA Extraction

After the inoculation, the remaining sample was subjected to DNA extraction. The extraction procedure was adapted from the Quick-DNA™ Fungal/Bacterial Microprep Kit. The protocol can be consulted in Attachment 1.

4.3.6. qPCR for target specific harmful fungal sections

After DNA extraction, quantitative Real-Time Polymerase Chain Reaction (qPCR) was done to detect specific toxigenic sections of *Aspergillus*, namely the sections *Circumdati, Flavi, Nidulantes,* and *Fumigati* (52) using the CFX-Connect PCR System (Bio-Rad). Reactions included 1× iQ Supermix (Bio-Rad, Portugal), 0.5 µM of each primer, and 0.375 µM of TaqMan probe in a total volume of 20 µL (Table 4.2). Amplification was carried out using a three-step PCR protocol: 40 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds (53). For each gene amplified, a non-template control and a positive control consisting of DNA obtained from reference

strains (kindly provided by the Mycology Laboratory of the National Institute of Health Dr. Ricardo Jorge) were included.

Table 4.2 - Sequence of Primers and TaqMan probes used for Real Time PCR

Fungal species/sections targeted	Sequences	Reference
***Flavi* (Toxigenic Strains)**		(54)
Forward Primer	5'-GTCCAAGCAACAGGCCAAGT-3'	
Reverse Primer	5'-TCGTGCATGTTGGTGATGGT-3'	
Probe	5'-TGTCTTGATCGGCGCCCG-3'	
Fumigati		
Forward Primer	5'-CGCGTCCGGTCCTCG-3'	(55)
Reverse Primer	5'-TTAGAAAAATAAAGTTGGGTGTCGG -3'	
Probe	5'-TGTCACCTGCTCTGTAGGCCCG -3'	
Circumdati		
Forward Primer	5'-CGGGTCTAATGCAGCTCCAA-3'	(56)
Reverse Primer	5'-CGGGCACCAATCCTTTCA-3'	
Probe	5'-CGTCAATAAGCGCTTTT-3'	
Nidulantes		
Forward Primer	5' – CGGCGGGGAGCCCT-3'	(57)
Reverse Primer	5' – CCATTGTTGAAAGTTTTGACTGATcTTA-3'	
Probe	5' –AGACTGCATCACTCTCAGGCATGAAGTTCAG-3'	

4.4. Citizen and Science

Citizen science was included into InChildHealth, one of the projects I participated on. By strengthening the connection between the general people and the scientific community, citizen science promotes a greater respect and knowledge of science. It has an educational component designed to improve the general public's understanding of scientific ideas and procedures. In this specific project, elementary school students worked together to complete scientific tasks that mimicked laboratory work utilising matrix that were used in the sampling campaign in schools.

Activities:

1 – EDC extraction: This exercise replicated the methods used in laboratories to extract EDCs. The children were familiar with the matrix used because it had been explained to them that they should protect it from being touched, fostering a sense of care and responsibility toward the EDC in their classroom.

2 – Inoculation of Swab: Identifying and swabbing the dirtiest areas. After collecting the samples, the children inoculated the swabs into petri dishes containing culture media. The plates were then given to the teacher, who monitored them for a week to observe the microorganisms that developed. Promoting the consolidation of their knowledge of hygiene and cleanliness.

3 - Microscopic Observation of Fungi**:** This activity involved observing fungi under a microscope. It produced curiosity and numerous questions from the children, demonstrating their interest in science. (Figure 4.3)

4 - Questionnaire**:** The children answered a questionnaire designed to have knowledge of their perceptions of factors affecting air quality.

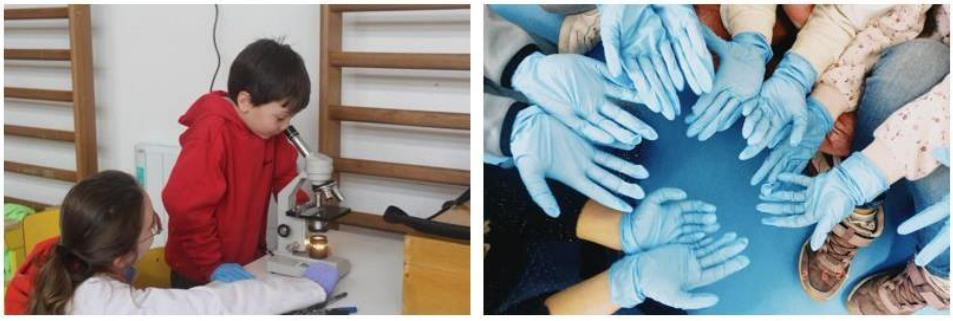

Figure 4.3 – Activities done during citizen and science.

5. Timeline of Activities

Table 5.1 - Timeline of activities during internship, in each project involved.

Project	Task	2023			2024				
		Sep	Nov	Dec	Jan	Feb	Mar	Apr	May
Primary Schools	Sampling								
	Assays								
	Citizen and Science								
Sawmills	Sampling								
	Assays								
Poultries Farms	Assays								

6. Results and Discussion

For results and discussion, I will focus solely on the results obtained in the Sawmills project, namely from the azole screening of isolates of *Aspergillus* section *Fumigati.*

6.1 *Aspergillus* section *Fumigati* recovered isolates

A total of 1,153 isolates of *Aspergillus* spp. were recovered from two woodworking environments: 656 isolates from DIY stores and 497 isolates from sawmills. Of these isolates, 42.94% from DIY stores were identified as *Aspergillus* section *Fumigati*, while 20.56% of isolates recovered from sawmills belonged to *Aspergillus* section *Fumigati.* In total, there are 383 isolates of *Aspergillus* section *Fumigati* across the two environments: 280 isolates from DIY stores and 103 isolates from sawmills. *Aspergillus* sections are found in various occupational environments like sawmills and waste sorting, in clinical and in the environment, being *Aspergillus* section *Fumigati* one of the most common (29,30). A. *fumigatus* is included in fungal pathogens list as a critical priority by World Health Organization (WHO) (WHO,2022), emphasising the necessity of performing further analysis on all obtained isolates to find mutations and possible resistance mechanisms.

6.2. Azole-resistant *Aspergillus* section *Fumigati* isolates

In terms of azole screening in isolates of *Aspergillus* section *Fumigati*, only the isolates recovered from DG18 incubated at 37°C were considered. In DIY stores, 196 isolates were recovered from DG18 37°C, while in sawmills, 74 isolates were recovered. In total, 270 isolates were tested for screening. Of these, 54 isolates are resistant, specifically, 16 are resistant to ITZ, 0 are resistant to VCZ, 35 are resistant to PCZ, and 2 are resistant to AMB. Only one isolate exhibits resistance to both ITZ and PCZ simultaneously.

The isolates that grew in DG18 at 37ºC were chosen because they allow the evaluation of the pathogenic potential and thus focused on the isolates that may present a greater risk to the health of workers.

ITZ, VCZ, PCZ and AMB are antifungals used with high frequency and are the most effective to treat invasive aspergillosis and another invasive infections caused by *Aspergillus* sp. (39,44). Obtaining 54 isolates of *Aspergillus* section *Fumigati* that show resistance to one or two of these antifungals is a concerning health risk to the workers in this environment.

Aspergillus section *Fumigati* cryptic species are known to possess inherent resistance to most antifungals, however, A. *fumigatus* sensu stricto lacks this ability, yet their acquisition of resistance is appearing because of environmental selective pressure due to the similarity of antifungals used in industries and agriculture to the ones used in clinical practice (Brauer et al., 2019; Van Der Torre et al., 2020). Most azole-resistant illness cases are caused by resistant A. *fumigatus* that reaches the body from the environment (35) . Antifungal susceptibility testing makes early discovery of these resistances possible, enabling the selection of the most suitable and successful therapy for a given fungal species (36).

The next step should pass by using the reference method to preform susceptibility testing - European Committee on Antimicrobial Susceptibility Testing (EUCAST) 9.4 method (microdilutions), in resistant isolates to confirm these results(60).

6.3. SOP of Azole screening in isolates of *Aspergillus* section *Fumigati*

Antifungal susceptibility testing (AFST) is essential for managing invasive fungal infections and tracking resistance epidemiology (60–63). Broth microdilution techniques, standardized by Clinical and Laboratory Standards Institute (CLSI) and EUCAST, are the reference method, are precise but time-consuming, suitable for large-scale studies and reference labs(60). Commercial, ready-to-use methods, like the gradient concentration Etest strip, are practical alternatives for routine clinical use. Etest determines the minimum inhibitory concentration (MIC) using an agar-based dilution method and is the one used in the SOP developed for azole screening in *Aspergillus* section *Fumigati* isolates (60).

Dannaoui e Espinel-Ingroff, 2019, wrote a review that compares results of Etest and microdilutions, and these are the conclusions:

- Amphotericin B: 80-100% essential agreement (EA) between Etest and microdilution, with Etest yielding higher MICs.
- Itraconazole, Voriconazole, Posaconazole: EA over 90%. Etest gives higher MICs for itraconazole and lower for voriconazole and posaconazole.

Etest is an alternative to reference methods for AFST in A. *Fumigatus* versus the triazoles. Despite the minor differences microdilutions are the reference method and should be preferred when possible. (60)

7. Scientific Production

During the internship, I had the opportunity to engage in various activities, these included conducting a scope review on Waste Water Treatment Plants (WWTPs), and presenting a poster at an international symposium.

7.1. Filling the knowledge gap regarding microbial occupational exposure assessment in Waste Water treatment plants – A scoping review (64)

- Riesenberger, B.; Rodriguez, M.; Marques, L.; Cervantes, R.; Gomes, B.; Dias, M.; Pena, P.; Ribeiro, E.; Viegas, C. Filling the Knowledge Gap Regarding Microbial Occupational Exposure Assessment in Waste Water Treatment Plants: A Scoping Review. Microorganisms 2024, 12, 1144. https://doi.org/ 10.3390/microorganisms12061144

This review followed the Preferred Reporting Items for Systematic Reviews (PRISMA) checklist. It aimed to provide an overview of the assays and sampling techniques used in Waste Water Treatment Plants (WWTPs) to assess worker exposure to microbiological substances. The collected data may help identify gaps in knowledge regarding microbial exposure in the workstation and potentially inform future criteria and recommendations to ensure reliable microbiological characterization. The paper is in Appendix 4.

7.2. International Symposium Occupational Safety and Hygiene (SHO 2024)

7.2.1. Oral Presentations:

- Dias, M.; Gomes, B.; Pena, P.; Cervantes, R.; **Rodriguez, M**.; Riesenberger, B.; Marques, L.; Ribeiro, E.; Viegas, C. (2024) Sampling protocol to assess Aspergillus section Fumigati in woodworking environments. International conference of Occupational safety and Hygiene (SHO 2024). https://www.sposho.pt/wp-content/uploads/2024/07/SHO2024-Program.pdf

- Gomes, B.; Dias, M.; Pena, P.; Cervantes, R.; **Rodriguez, M.**; Marques, L.; Riesenberger B.; Viegas, C. (2024) Levels of fungi in the air of poultry

farms following different stages of birds´ growth cycle. International conference of Occupational safety and Hygiene (SHO 2024). https://www.sposho.pt/wp-content/uploads/2024/07/SHO2024-Program.pdf

- Cervantes, R.; Pena, P.; Gomes, B.; Dias, M.; Riesenberger, B.; **Margarida, R.**; Marques, L.; Viegas, C. (2024) Understanding and Addressing Fungal Exposure Risks in Primary Schools: Implications for Children's Health and Wellbeing. International conference of Occupational safety and Hygiene (SHO 2024). https://www.sposho.pt/wp-content/uploads/2024/07/SHO2024-Program.pdf Awarded as the best proceeding paper.

- Pena, P.; Cervantes, R.; Dias, M.; Gomes, B.; Riesenberger, B.; Marques, L.; **Rodriguez, M**.; Viegas, C. (2024). Preliminary results concerning school staff personal exposure to microbial load – Worry to be considered? International conference of Occupational safety and Hygiene (SHO 2024). https://www.sposho.pt/wp-content/uploads/2024/07/SHO2024-Program.pdf

7.2.2. Posters

- Dias, M.; **Rodriguez, M**.; Vasques, C.; Riesenberger, B.; Marques, L.; Gomes, B.; Pena, P.; Cervantes, R.; Viegas, S.; Viegas, C. (2024) Budget-friendly protocol for TR34/L98H and TR46/Y121F/T289A mutation detection in Aspergillus section Fumigati isolates. International conference of Occupational safety and Hygiene (SHO 2024). https://www.sposho.pt/wp-content/uploads/2024/07/SHO2024-Program.pdf Awarded the honourable mention for the abstract. This poster was presented by me in SHO 2024. Poster in Appendix 5.

- Gomes, B.; Dias, M.; Pena, P.; Cervantes, R.; **Rodriguez, M**.; Marques, L.; Riesenberger, B.; Viegas, C. (2024) A multi-approach sampling strategy to assess exposure to microbiologic agents in poultries. International conference of Occupational safety and Hygiene (SHO 2024). https://www.sposho.pt/wp-content/uploads/2024/07/SHO2024-Program.pdf Poster in Appendix 6.

- Cervantes, R.; Pena, P.; Riesenberger, B.; Marques, L.; **Rodriguez, M.**; Gomes, B.; Dias, M.; Viegas, C. (2024) Fungal Contamination in Lisbon's Primary Schools - Sampling Insights and Analytical Approaches. International conference of Occupational safety and Hygiene (SHO 2024). https://www.sposho.pt/wp-content/uploads/2024/07/SHO2024-Program.pdf Poster in Appendix 7.

7.3. Epidemiology in Occupational Health (EPICOH) 2024

Submitted work by Renata Cervantes with title “Mitigating Health Risks in Wastewater Treatment Plants: Identifying Key Microbial Contamination and Protocol Needs”. Appendix 8.

7.4. Bootcamp H&TRC 2024

- Submitted abstract by Renata Cervantes with title “The Power of Citizen Science: Insights and Achievements from the InChildHealth Project”. Appendix 9.

- Submitted abstract by Bianca Gomes with title “Indoor microbial levels in poultry pavilions”. Appendix 10.

- Submitted abstract by Marta Dias with title “Protocol to detect CYP51A mutations in *Aspergillus* section *Fumigati* isolates”. Appendix 11.

- Submitted abstract by Bruna Reisenberger with title “Microbial Exposure Assessment in Waste Water Treatment Plants”. Appendix 12.

8. Project of Investigation: Detection of CYP51A Gene Mutations in Azole-resistant Isolates of *Aspergillus fumigatus*

Based on the activities conducted during the internship period, a potential research project has been designed, titled "Detection of CYP51A Gene Mutations in Azole-resistant Isolates of Aspergillus *fumigatus"*.

8.1. Introduction

Aspergillus section *Fumigati* is one of the most prevalent *Aspergillus* sections in clinical, environmental and different occupational environments (29). A. *Fumigatus* is the most clinically relevant specie of this section (34). Cryptic species within the *Fumigati* section exhibit intrinsic resistance to several antifungals, however, in *A. fumigatus* sensu stricto resistance is increasingly emerging due to selective pressure from prolonged azole treatment or selective pressure from environmental(29).

Azole resistance mechanisms are frequently linked to mutations in genes involved in the ergosterol pathway of A. *fumigatus*, particularly the CYP51A gene. This gene encodes cytochrome P450 14-α-lanosterol demethylase, the primary target of azole antifungals(59,65). The most common mutation conferring pan-azole resistance is the TR34/L98H mutation, which consists of a 34-base pair tandem repeat in the promoter region combined with a leucine-to-histidine substitution at codon 98 (59,66).

Infection of individuals with these resistant strains can lead to treatment failure with triazole therapy, consequently, the increased morbidity and mortality rates associated with azole resistance are likely to become a significant public health concern(29). In Portugal, has already been reported the TR34/L98H mutation in environmental isolates from occupational environments(29).

8.2. Objectives

The goals of these study are:

- Identifying *Aspergillus* section *Fumigati* cryptic species by thermotolerance.
- Identifying *Aspergillus fumigatus* by sequencing beta tubulin gene.

- Characterize azole-resistant *Apergillus fumigatus* by performing susceptibility testing by EUCAST 9.4 microdilutions.
- Identify TR34/L98H mutation in CYP51A gene in these isolates using PCR.
- Apply Whole Genome Sequencing (WGS) in isolates that are azole-resistant but not positive for mutation in CYP51A gene.

8.3. Methodologies

8.3.1. Identifying *Aspergillus* section *Fumigati* cryptic species by thermotolerance and sequencing

Re-inoculate the isolates of *Aspergillus* section *Fumigati* that were storage at - 80ºC in Malt Extract Agar medium, work in laminar flow chamber. Then incubate the plates at 50ºC for 7 days, so that only cryptic species of *Aspergillus* section *Fumigati* grow, as *Aspergillus fumigatus*. After 7 days, inspect for grow and check for pure colonies and absence of contamination. If this verifies, the protocol can be proceed. (29,34). Then, sequencing the beta tubulin gene is necessary to identify specifically *Aspergillus fumigatus*.

8.3.2. Susceptibility testing by microdilution

The EUCAST 9.4 method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds was applied for determining MIC for ITZ, VCZ and PCZ, using the microdilution approach (performance of broth dilution in microdilution plates(29). An internal control strain with known susceptibility should be included in each test as a positive control of antifungals. ITZ, VCZ and PCZ MICs for CYP51A mutants are typically: ≥ 4mg/L, >1 mg/L and ≥ 0.25 mg/L, respectively (67,68).

8.3.3. Detection of TR34/L98H mutation in CYP51A gene

Fungal DNA of azole-resistant isolates was extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, USA) according to the manufacturer's instructions(53). After DNA extraction, isolates were tested with AsperGenius multiplex real-time PCR assay following the manufactures instructions. This multiplex real-time PCR tests for TR34/L98H and TR46/Y121F/T289A mutations found in CYP51A gene (29,69).

8.3.4. Whole Genome Sequencing

If there is no relationship between the isolates identified as azole-resistant by microdilution method and the identification of mutation by PCR. The next step will be to perform whole genome sequencing of the isolates no correlation between the two tests(34). Genomic DNA was prepared as described (70) and sequencing was conducted in a MiSeq system, according to the manufacturer's protocols (Illumina)(70). Then the sequences are analysed as described(70).

Figure 8.1. shows a schematic representation of the protocol used.

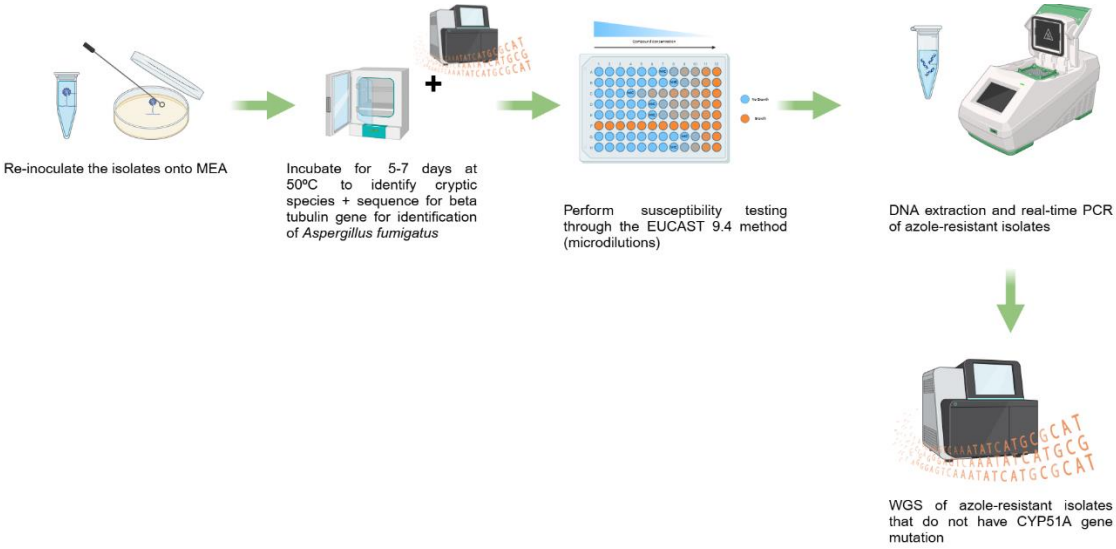

Figure 8.1 - Schematic protocol for the investigation line. Made with biorender, [Scientific Image and Illustration Software | BioRender]

8.4. Timeline

Table 8.1 - Timeline of activities for Detection of CYP51A gene mutations in azole-resistant Aspergillus Fumigatus isolates in one year.

Tasks	Months											
	1	2	3	4	5	6	7	8	9	10	11	12
Identifying cryptic species by thermotolerance												
Susceptibility testing by microdilution												
DNA extraction												
Mutation detetction												
WGS												
Analysis of the sequences												

8.5. Expected Results

With this methodology, it is expected to identify the most common mutation in the CYP51A gene in *Aspergillus fumigatus* isolates. In addition to identifying this mutation, by using whole genome sequencing, it is possible to identify other azole-resistance mechanisms already described but not so common, as well as the possibility of identifying new resistance mechanisms(34,70).

8.6. Conclusions

This study aims to provide critical insights into the genetic basis of azole resistance in *Aspergillus fumigatus*. By elucidating specific CYP51A mutations and their impact on antifungal susceptibility, this research will contribute to the development of targeted therapeutic strategies. By using this approach, a better understanding of the mechanisms of resistance is possible, and in consequence, improving patient outcomes(34,44).

This methodology using thermotolerance over sequencing to identify *Aspergillus* section *Fumigati* cryptic species, is less expensive, making this a budget-friendly protocol for mutation detection in *Aspergillus fumigatus*(34).

9. Conclusion and Reflexion of Internship

The field of public health and occupational exposure assessment are important for disease prevention and control, environmental health, worker´s health and regulations and compliance. Given its significant relevance, this was the chosen field for the professional internship of the Project/Thesis/Internship course unit in the 2nd year of the Master's in Clinical-Laboratory Technologies at ESTeSL. This internship was carried out at environmental/public health laboratory at ESTeSL , from September 2023 to May 2024, in the areas of environmental and occupational microbiology, with the objective of characterize the microbial contamination and microbial resistance in different indoor environments, using active and passive sampling methods and apply culture-based methods and molecular tools. As an internship in a research laboratory, writing articles and prepare posters for conferences were goals too.

The completion of this internship was an enriching experience, which enabled the consolidation of theoretical knowledge acquired during the 1st year of the Master's and the acquisition of theoretical and practical skills in the occupational microbiology area, thus fulfilling the established objectives. Integration into the research team from environmental/public health laboratory allowed for an understanding how to maintain a laboratory, how to interpret some results and how to adapt protocols to the methods practiced in laboratories that we have a partnership. In terms of me as a researcher in formation, this internship permitted me to improve my skills of questioning the why of things, a fundamental characteristic in the world of research. The encouragement to participate in the process of writing papers and to present posters at conferences gave me important baggage for the future.

The laboratory provided always all the necessary conditions to complete the defined objectives, the protocols used were adequate to the proposed tasks. Whenever necessary, there were adjustments of the protocols to obtain the best results. The research team was always ready to help and clarify all the doubts that could appear. Participating in this internship was an advantage for my academic career and for my professional life.

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11. Attachments

Attachment 1

Protocol for DNA Extraction

1. The samples were defrosted.
2. Samples in Falcon tubes were centrifuged at 3500 rpm for 30 minutes using a Thermo Heraeus Labofuge 400, and samples in Eppendorf tubes were microcentrifuged at speed 10 for 5 minutes using a VWR® Micro Star 21/21R Microcentrifuge. Most of the supernatant was discarded, and the pellet was retained.
3. To lyse the fungal cells, the pellet was resuspended, and 200 µL were placed into a ZR Bashing Bead Lysis tube containing 750 µL of Bashing Bead Buffer. The tube was vortexed for 10 minutes at speed 4 and then centrifuged for 1 minute at speed 10.
4. To remove the larger fungal organelles, 600 µL of the supernatant was transferred to a Zymo-Spin III-F Filter in a collection tube and centrifuged at speed 8 for 1 minute.
5. The column was discarded, and 1200 µL of DNA Binding Buffer was added to the collection tube and mixed well.
6. 875 µL of this mixture was transferred to a Zymo-Spin IC Column in a new collection tube and centrifuged for 1 minute at speed 10 to facilitate DNA binding and recovery.
7. The liquid was discarded, and step 6 was repeated.
8. In a new collection tube, 200 µL of DNA Pre-wash Buffer was added to the same column and centrifuged for 1 minute at speed 10 to remove protein contaminants.
9. The filtrate was discarded. To remove salts and contaminants before DNA elution, 500 µL of DNA Wash Buffer was added to the same column and centrifuged at speed 10 for 1 minute.
10. The Zymo-Spin IC Column was then transferred to an Eppendorf tube. To purify the DNA, 200 µL of DNA Elution Buffer was added to the column. After 2-3 minutes, the Eppendorf tubes with the columns were centrifuged at speed 10 for 30 seconds.
11. The eluted DNA was transferred again into the same column and, after 2-3 minutes, centrifuged for 30 seconds at speed 10.

12. The column was discarded, and 50 µL of the eluted DNA was transferred to two different Eppendorf tubes: one for qPCR assays and the other for fungal biomass assays (dPCR). The remaining 100 µL in the Eppendorf tube was reserved for detection of toxigenic strains (RT-PCR).
13. All Eppendorf tubes were stored at -20°C until use.

12. Appendix

Appendix 1

Table 12.1 - Number of hours per day until complete 600 hours.

Day	Number of hours	Day	Number of hours	Day	Number of hours
11/09/2023	7	02/11/2023	6	16/01/2024	8
12/09/2023	6	03/11/2023	9	17/01/2024	7
13/09/2023	5	06/11/2023	7	18/01/2024	9
14/09/2023	6	07/11/2023	7	19/01/2024	6
18/09/2023	5	08/11/2023	7	22/01/2024	6
19/09/2023	5	09/11/2023	6	23/01/2024	8
20/09/2023	9	10/11/2023	6	24/01/2024	8
21/09/2023	6	13/11/2023	6	25/01/2024	6
22/09/2023	5	14/11/2023	5	29/01/2024	9
25/09/2023	3	15/11/2023	4	31/01/2024	7
26/09/2023	5	16/11/2023	6	01/02/2024	8
27/09/2023	5	17/11/2023	4	05/02/2024	7
28/09/2023	6	20/11/2023	6	06/02/2024	6
02/10/2023	8	21/11/2023	6	07/02/2024	6
03/10/2023	6	22/11/2023	7	08/02/2024	8
04/10/2023	7	23/11/2023	5	19/02/2024	8
06/10/2023	6	24/11/2023	5	20/02/2024	7
9/10/2023	5	27/11/2023	6	21/02/2024	6
10/10/2023	9	28/11/2023	6	26/02/2024	6
11/10/2023	9	29/11/2023	6	27/02/2024	6
12/10/2023	5	30/11/2023	5	28/02/2024	6
13/10/2023	3	04/12/2023	8	05/03/2024	8
16/10/2023	5	05/12/2023	6	06/03/2024	7
17/10/2023	7	07/12/2023	7	11/03/2024	6
18/10/2023	7	11/12/2023	5	12/03/2024	8
23/10/2023	5	03/01/2024	6	13/03/2024	7
24/10/2023	7	04/01/2024	7	14/03/2024	6

25/10/2023	7	08/01/2024	6	15/03/2024	6
26/10/2023	8	09/01/2024	8	18/03/2024	6
30/10/2023	8	11/01/2024	8	19/03/2024	8
31/10/2023	6	15/01/2024	6	20/03/2024	7
Total of hours:	191	Total of hours:	383	Total of hours:	600

Appendix 2

Figure 12.1 - Activities performed in field and laboratory during the internship.

Appendix 3

Standard Operating Procedure

Antifungal Susceptibility Testing of *Aspergillus* section *Fumigati*

Authors

Lead author:

This document has been developed by Margarida Rodriguez.

Scope

This Standard Operating Procedure (SOP) is partially based on internal SOPs of the H&TRC-Health & Technology Research Center from Polytechnic Institute of Lisbon after the recovery of *Aspergillus* section *Fumigati* isolates. This SOP pretend to perform an antifungal susceptibility testing of *Aspergillus* section *Fumigati* isolates to determine the MICs for ITZ, VCZ, PCZ and AMB.

After isolates recovery, the obtained isolates may be re-inoculated in SDA to obtaining pure colonies of *Aspergillus* section *Fumigati,* then proceed to next analysis:

- Antifungal susceptibility testing by gradient strips Etest Method;

Materials, consumables and equipment required for Azoles Screening in isolates

Below is a list with the different materials needed for performing azole screening in *Aspergillus* section *Fumigati* after isolates recovery.

Materials and consumables:

- Petri dish with culture media as follow:
  - SDA (Sabouraud Dextrose Agar)
  - RPMI 1640 (Roswell Park Memorial Institute) (14mm)
- Spreaders
- Loops
- 1 Column specific for visible spectrum per isolate + 1 column for blank
- 3 Eppendorfs of 1,5ml per isolate
- Micropipette 20 µl, 200µl and 1000µl
- Pipet tips
- NaCl (saline solution)
- Distilled water
- Azole stripes of ITZ, VOZ, POZ and AMB

Solution Preparation:

- Saline Solution – buy or prepare 900 ml of distilled water for 9.1 g of NaCl
- Medium – Manufacturer protocol

Equipment:

- 2 Laboratory ovens:
  - 1 x 27ºC (for pure colonies)
  - 1 x 35ºC (for MICs)
- Laminar Flow Chamber
- Spectrophotometer
- Vortex

Recommendations and precautions for azole screening

- Appropriate health and safety practices should be established to ensure compliance with regulatory requirements and avoid any possible cross contamination.
- Before inoculation, culture media preparation and the Petri dishes with culture media should be stored in sceptic conditions.
- Work in Lamine Flow Chamber when opening petri dishes with *Aspergillus* section *Fumigati* and when spores are likely to spread.
- Before measure the Optical Density (OD), set the wavelength of the spectrophotometer to 600 nm and calibrate the spectrophotometer with a blank solution consisting of 1 mL of saline solution.
- Once touching the media, the antifungal strips cannot be removed or moved.

Azole screening in *Aspergillus* section *Fumigati* procedure (pure colonies and azole screening in isolates)

Pure Colonies Procedure

- Re-inoculate each isolate into SAB medium in laminar flow chamber to maintain sterility.
- Incubate the petri dishes at 27°C for 5-7 days.
- After the incubation period, inspect the cultures for any signs of contamination.
- If no contamination is detected, proceed with the azole screening in *Aspergillus* section *Fumigati* isolates procedure (note: in doubt, colonies should be confirmed with microscopic observation or other preferred methods based on culture).

Azole Screening in isolates

- Add 250 µL of saline solution to an Eppendorf.
- Scrape off with a loop a pure colony and add it to Eppendorf.
- Vortex for 10 seconds.
- Allow Eppendorf to stand for 5 minutes to enable the medium and fungal particles to settle.
- Carefully transfer 200 µL of the supernatant to a new Eppendorf.
- Prepare the columns specific for the visible spectrum by adding 900 µL of saline solution and 100 µL of each solution.
- Set the wavelength of the spectrophotometer to 600 nm(1).

- Calibrate the spectrophotometer with a blank solution consisting of 1mL of saline solution.
- Measure the OD of each solution at 600 nm.
- Adjust the OD to 0.06 using the formula(2):

$$Adjusted\ volume = \frac{0{,}06\ \times 100}{Measured\ OD}\ \mu L$$

- Prepare a new Eppendorf with the solution adjusted to the desired OD.
- Inoculate 400 µL of the adjusted solution into a large RPMI petri dish.
- Place four antifungal strips (ITZ, VCZ, PCZ, AMB) on the RPMI plate. (note: once touching the media, the strips can not be removed or moved)(3)
- Incubate the petri dishes at 35°C.
- Observe and record the Minimum Inhibitory Concentrations (MICs) at 24 hours and 48 hours. Seeing the halo where you stop seeing growth.

Figure 1 – Shows the protocol for azole screening of *Aspergillus* section *Fumigati* isolates.

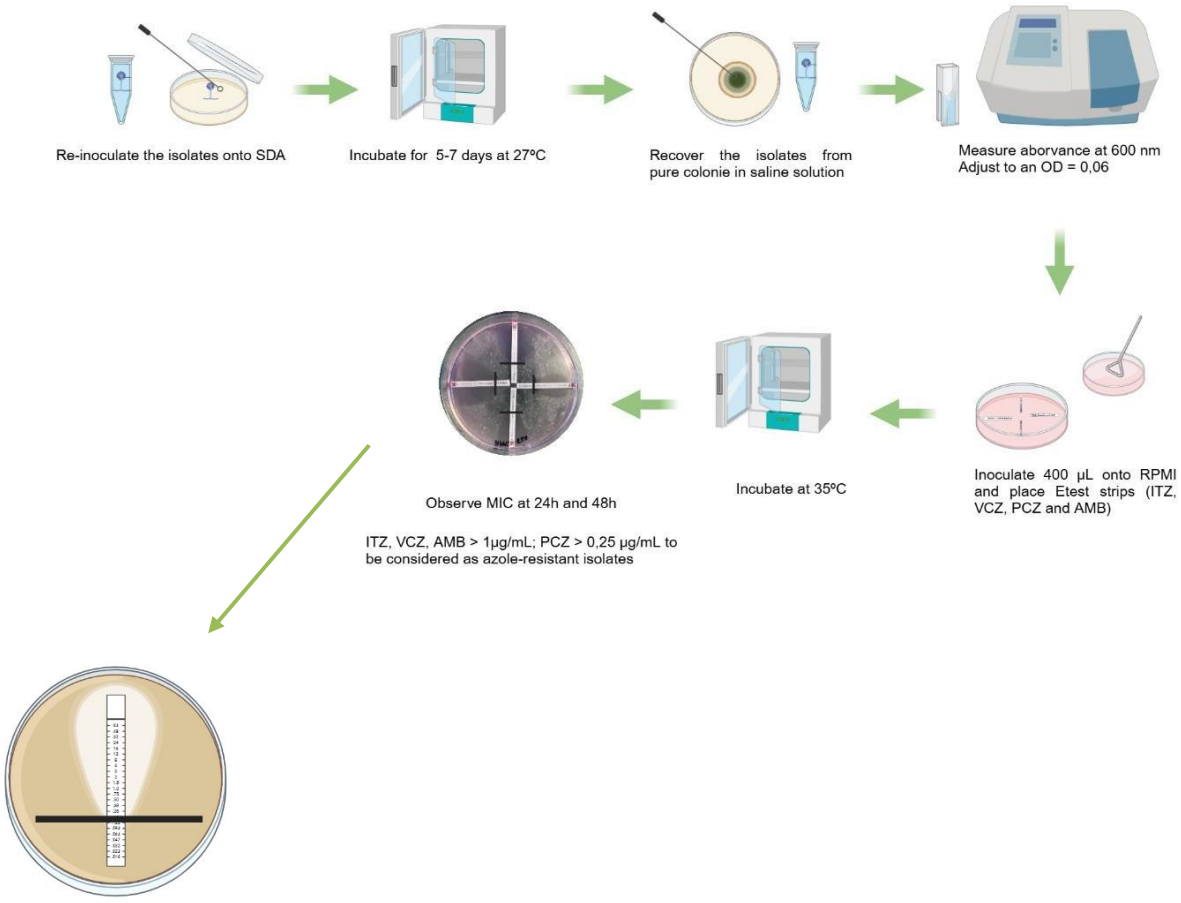

Figure 1 – Schematic protocol for azole screening of Aspergillus section *Fumigati isolates and how to identify the MIC. Made with biorender, Scientific Image and Illustration Software | BioRender.*

Sample traceability and contextual information

A standardized convention identification for the isolates should be mentioned in the petri dishes regarding pure colonies procedure and in the Eppendorfs used in azole screening of isolates.

Storage

After measurement of MICs at 48h, used petri dishes must be disposed as chemical waste and called for pick up by Chemical Waste. Isolates can be re-storage at -80 Celsius degrees.

Quality control

To check for possible contamination during the inoculation procedure, blanks should be used. This blank should be handled in the same way as the other samples during the incubation period. In doubt, pure colonies should be confirmed with microscopic observation or other preferred methods based on culture.

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Appendix 4 – Systematic Review

microorganisms

MDPI

Review

Filling the Knowledge Gap Regarding Microbial Occupational Exposure Assessment in Waste Water Treatment Plants: A Scoping Review

Bruna Riesenberger [1], Margarida Rodriguez [1], Liliana Marques [1], Renata Cervantes [1,2], Bianca Gomes [1], Marta Dias [1,2], Pedro Pena [1,2], Edna Ribeiro [1] and Carla Viegas [1,2,*]

[1] H&TRC—Health & Technology Research Center, ESTeSL—Escola Superior de Tecnologia e Saúde, Instituto Politécnico de Lisboa, 1990-096 Lisbon, Portugal
[2] NOVA National School of Public Health, Public Health Research Centre, Comprehensive Health Research Center, CHRC, REAL, CCAL, NOVA University Lisbon, 1099-085 Lisbon, Portugal
* Correspondence: carla.viegas@estesl.ipl.pt

Abstract: Background: Wastewater treatment plants (WWTPs) are crucial in the scope of European Commission circular economy implementation. However, bioaerosol production may be a hazard for occupational and public health. A scoping review regarding microbial contamination exposure assessment in WWTPs was performed. Methods: This study was performed through PRISMA methodology in PubMed, Scopus and Web of Science. Results: 28 papers were selected for data extraction. The WWTPs' most common sampled sites are the aeration tank (42.86%), sludge dewatering basin (21.43%) and grit chamber. Air sampling is the preferred sampling technique and culture-based methods were the most frequently employed assays. *Staphylococcus* sp. (21.43%), *Bacillus* sp. (7.14%), *Clostridium* sp. (3.57%), *Escherichia* sp. (7.14%) and *Legionella* sp. (3.57%) were the most isolated bacteria and *Aspergillus* sp. (17.86%), *Cladosporium* sp. (10.71%) and *Alternaria* sp. (10.71%) dominated the fungal presence. Conclusions: This study allowed the identification of the following needs: (a) common protocol from the field (sampling campaign) to the lab (assays to employ); (b) standardized contextual information to be retrieved allowing a proper risk control and management; (c) the selection of the most suitable microbial targets to serve as indicators of harmful microbial exposure. Filling these gaps with further studies will help to provide robust science to policy makers and stakeholders.

Keywords: wastewater treatment plants; sampling methods; assays; microbial contamination assessment; bacteria; fungi

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1. Introduction

The European Commission (EC) strongly recommends circular economy implementation aiming at a zero-waste strategy, by instigating water innovations technologies for water reuse and recycling [1]. In this scope, wastewater treatment plants (WWTPs) are designed to maximize energy and water recovery, becoming of pivotal importance for the achievement of the EC's goals [2].

On WWTPs, the wastewater of domestic, hospital and industrial uses undergoes preliminary, primary, secondary, and in some cases, tertiary biological treatments [3,4]. During these treatments, bioaerosol formation is higher throughout discharging, mixing and aerating processes, as well as during the spraying of sewage [3–6]. The bioaerosols contain microorganisms, such as fungi, viruses, bacteria, and their metabolites, including endotoxins and mycotoxins, which may be potentially pathogenic to humans. Infection can occur through ingestion, dermal contact, or inhalation, and it is highly possible that due to prolonged exposure, a decline in the health status of WWTPs workers may be

observed [5–7]. In fact, several negative health outcomes associated with bioaerosol occupational exposure have been reported, including respiratory and gastrointestinal effects or allergies [4,6]. In addition, WWTPs are recognized as key emission sources for the discharge of antimicrobial-resistant (AMR) bacteria and antibacterial resistance genes (ARGs) [8].

Although it is crucial to assess occupational exposure to bioaerosols in WWTPs, there is a lack of consensus regarding sampling approaches and analyses that should be performed, as well as the targets that can be used as surrogates to identify harmful microbial contamination, which is a common problem in settings where (micro)biologic agents need to be assessed. However, suggestions regarding the procedures to be employed from the field to lab have already been described in different occupational environments [9–11]. Thus, this study aims to perform a scoping review to provide a broad overview of the state-of-the-art methods (sampling and analyses) applied to perform microbial contamination assessments in WWTPs, as well as to identify the most suitable targets to be used as indicators of hazardous microbial contamination.

2. Materials and Methods

2.1. Search Strategy, Inclusion and Exclusion Criteria

This study adopted the PRISMA methodology and the Preferred Reporting Items for Systematic Review (PRISMA) checklist [12] (was completed (Supplementary Materials Table S1).

This study reports available data published between 1 January 2010 and 8 November 2023. The search aimed at selecting studies on microbiologic agents' assessment in WWTP and included the terms "Waste Water Treatment Plants", "bacteria", "fungi", "viruses", "exposure" and "sampling", with English as the chosen language. The databases chosen were PubMed, Scopus and Web of Science (WoS). Articles that did not meet the inclusion criteria were not subjected to additional review (Table 1).

Table 1. Inclusion and exclusion criteria on article selection.

Inclusion Criteria	Exclusion Criteria
Articles published from 1 January 2010 to 8 September 2023	
Articles published in English	Articles published prior to 2010
Articles summarising research results from any country	Articles published in other language
Original scientific articles on the subject	Abstracts of congress, reports, reviews/state-of-the-art articles
Articles focused to microbial occupational exposure	

2.2. Studies Selection and Information to Be Retrieved from the Papers

The articles were selected by using the Rayyan—Intelligent systematic review tool, a free online tool that significantly accelerates the process of screening and choosing papers for academics working on systematic reviews. Article selection followed three rounds: 1st: All titles were screened to identify and remove duplicated papers or those unrelated to the topic. The selected papers were uploaded to Rayyan for additional examination; 2nd: screening of all the abstracts; 3rd: Selected papers were reviewed considering the inclusion and exclusion criteria. Possible differences in the study's selection were discussed by three investigators (BR, MR and LM), and eventually decided by the remaining investigators (BG, MD, PP, RC). Data extraction was then performed by two investigators (BR, and LM), while another (MR) examined the results. The data that follows were manually extracted: Database, Title, Country, Type of WWTP, Sampling Strategies and Methods, Assays applied, Main Findings, and References.

2.3. Quality Assessment

The assessment of the risk of bias was performed by 4 investigators (BR, MR, LM, and CV). Within each research article, an evaluation of the risk of bias was performed across two parameters divided as key criteria ("Sampling methods" and "Assays"). Each parameter's risk of bias was rated as "low" "medium" "high", or "not applicable". The studies for which all the key criteria and most of the other criteria were characterized as "high" were removed.

3. Results

The workflow illustrated in Figure 1 was used for selecting studies. Initially, 191 studies were found in the database search, from which 105 abstracts were analyzed, and 40 complete texts were deemed eligible for further examination. A total of 12 papers were rejected for not satisfying the inclusion and exclusion criteria, mostly because they did not have any information regarding microbial occupational exposure in WWTPs. Overall, the selection process yielded 28 studies on microbiologic contamination occupational exposure assessment.

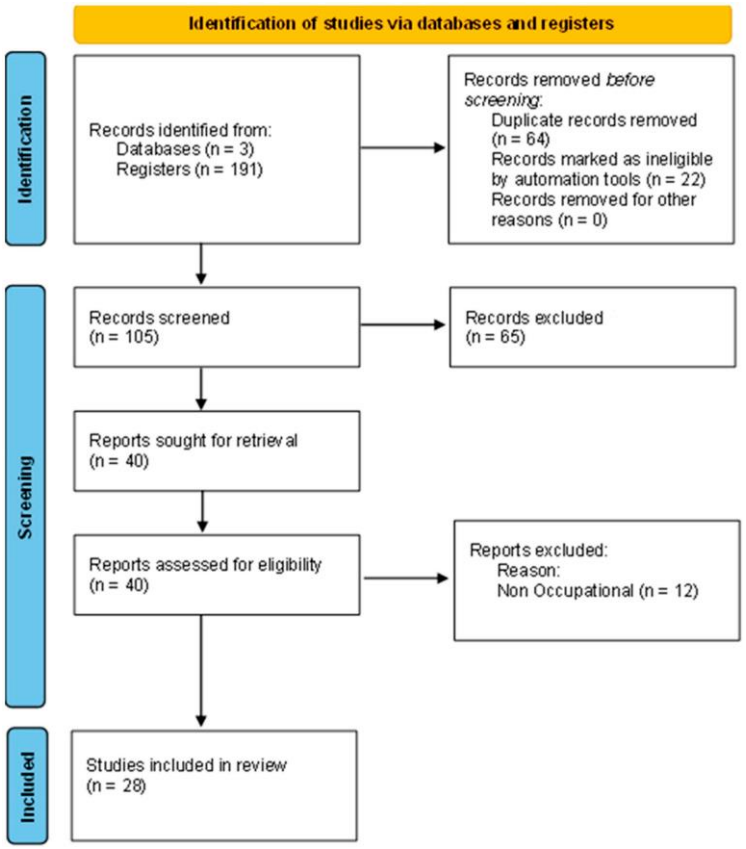

Figure 1. PRISMA methodology of selection of papers [12].

Extracted Data

After the selection of the 28 studies on microbiologic contamination occupational exposure assessment, the relevant data were extracted; the key findings are summarized in Table 2.

Among the 28 chosen studies, 9 were conducted in Europe (3 in Portugal [13–15], 2 in Poland [16,17], 2 in Denmark [18,19], 1 in Switzerland [20], and 1 in Austria [21]), 9 in Asia (specifically in China [4,5,22–28]), 6 in the Middle East (Iran [6,29–33]), and 4 in North America (3 in the USA [34–36] and 1 in Canada [37]).

Table 2. Data extracted from the chosen papers.

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
Exposure to Airborne Noroviruses and Other Bioaerosol Components at a Wastewater Treatment Plant in Denmark	no data	Noroviruses (NoVs), Adenoviruses, Endotoxin, Bacteria and Molds	Air samples: Active methods—Filtration (Personal Dust Sampling-Inhable GSP samplers with teflon filters or polycarbonate filters, average sampling period 242 min), Stationary measurements of "total dust"	no data	Personal dust sampling was carried out on 16 workers, on different wastewater processes; stationary sampling was carried out in the aeration basin at 1.5 m above the ground level	Culture-based methods (DG18 agar for cultivable moulds, nutrient agar for cultivable bacteria)	NoVs and endotoxin were detected at concentrations that could pose an occupational health risk. Positive correlations between exposure to endotoxin, bacteria, moulds and NoVs were found and indicate that the exposure to bioaerosols may be related to work tasks.	[18]
ADMS simulation and influencing factors of bioaerosol diffusion from BRT under different aeration modes in six wastewater treatment plants	Municipal WWTP	Bacteria and Intestinal Bacteria	Air samples: Active methods—Impaction (Andersen six-stage cascade impactor, flow rate = 28.3 L/min; TH-150 medium flow sampler)	Seasonal (spring)	1.5 m above aeration tanks of 6 Municipal Wastewater Treatment Plant (MWWT), 6 samples were taken at each sampling site, (n = 36)	Culture-based methods (LB medium for bacteria, and for intestinal bacteria, MAC); Ion chromatography and Illumina MiSeq high-throughtput sequencing	The concentrations of bacteria and, specifically, intestinal bacteria in the bioaerosols ranged from 389 CFU/m^3 to 1536 CFU/m^3 and 30 CFU/m^3 to 152 CFU/m^3, respectively, and the proportion of the intestinal bacteria was 8.85%. The proportion of intestinal bacteria (75.79%) produced via surface aeration by Biological Reaction Tanks (BRT) attached to large-sized bioaerosol particles was higher than that of a BRT undergoing the bottom aeration process (37.28%). The main microorganisms found in the bioaerosols included Moraxellaceae, Escherichia–Shigella, Psychrobacter, and Cyanobacteria.	[22]
Spatio-temporal variations in airborne bacteria from the municipal wastewater treatment plant: a case study in Ahvaz, Iran	Municipal WWTP	Airborne Bacteria	Air Samples: Passive methods (microbiological sampling index of microbial air contamination (1/1/1 standard))	Seasonal (warm and cold)	Grit chamber (GCh), primary sludge dewatering basin (PSDB) and at the aeration tank (AT); (n = 180)	Culture-based methods (Trwith nystatin (250 mg/L) to inhibit fungal growth); PCR-RFLP	The dominant bacterial genus included *Bacillus pumilus* (26.7%), *Staphylococcus arlettae* (23.2%), *Kocuria turfanensis* (13.6%), and *Alicycliphilus* (9.2%), and they increased with high temperatures and wind speed, and decreased with high humidity.	[6]

Table 2. *Cont.*

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
Emission level, particle size and exposure risks of airborne bacteria from theoxidation ditch for seven months observation	WWTP with orbal oxidation ditch process	Airbone Bacteria	Air samples: Active—Impaction (Andersen six-stage cascade impactor, flow rate = 28.3 L/min); Material collection (raw water in the oxidation ditch)	Seasonal (spring and summer)	ConS: Control site was set 300 m upwind from the oxidation ditch; AWS (above water surface): above water surface; AWS-0.5: above water surface 1 m; AWS-3: above water surface 3 m; ARB (above rotating brushes)-25: after the rotating brushes 25 m; ARB-55: after the rotating brushes 55 m; ARB-210: after the rotating brushes 210 m; (n = 168)	Culture-based methods (with nutrient agar for mesophilic bacteria) for air samples; Gradient gel electrophoresis for 16S rDNA; PCR	Spatial and seasonal variations in the concentrations of airborne bacteria emissions were detected. The highest concentration was observed near the rotor disc aerators (RDAs) (835 ± 91 CFU/m^3 to 8916 ± 155 CFU/m^3) during each sampling process, with the concentration decreasing by 76.70% and 79.91% as sampling distance and height increased, respectively. Most of the airborne bacteria were coarse particles that exceeded 4.7 µm in size. The dominant bacteria were *Bacillus* sp., *Lysinibacillus* sp., and *Sphingomonas* sp.	[23]
Aerosol partitioning potential of bacteria presenting antimicrobial resistance from different stages of a small decentralized septic treatment system	On-site/ decentralized WWTP	Antibiotic-Resistant Bacteria (ARB)	Air samples: Active method—Impinger (Wetted wall cyclone collectors (WWC)); Material collection (stainless steel porTable 600 mL water dipper (Grainger Industrial Supply))	Seasonal (summer and winter)	Aerosol and water samples were collected at the four tanks; 600 mL of water and 1500 L of air at each tank	Kirby–Bauer testing for antibiotic resistance, quantitative Polymerase Chain Reaction (qPCR); 16SrRNA-based Illumina sequencing	As expected, the higher concentration of bacteria was found when the lids were open; in the summer, *Legionella* was found in the water tanks 1 and 3, and in the water tank 1 *Pseudomonas* was present; in the winter, *Legionella* was also present in the water tank 1; bioaerossol samples showed a higher antimicrobial resistance of 50% (at four of the eight antbiotics tested), and the higher antimicrobial resistance of the water samples was 87.5% (resistance in the 7 of the 8 antibiotics).	[36]

Table 2. *Cont.*

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
Identification of airborne fungi's concentrations in indoor and outdoor air of municipal wastewater treatment plant	Municipal WWTP with conventional activated sludge treatment process	Fungi	Air Samples: Passive methods (microbiological sampling index of microbial air contamination (1/1/1 standard))	Seasonal (warm and cold)	Grit chamber tank, primary sludge dewatering basin, aeration tank, upstream and downstream of dominant wind blowing at the site and at the administrative building (n = 240)	Culture-based methods (SDA with chloramphenicol antibiotic (100 mg/L) to inhibit bacterial growth)	The greatest release of fungal aerosols occurred in the cold season while the minimum release occurred in the warm season; the highest concentrations of fungi were observed in the grit chamber unit; *Cladosporium* (39.23%) and *Alternaria* (19.87%) were the airborne fungal genera most common.	[29]
Aspergillus spp. prevalence in different Portuguese occupational environments: What is the real scenario in high load settings?	no data	*Aspergillus* spp.	Air samples: Active methods—Impaction (Millipore air Tester, flow rate = 140 L/min) and Impinger (Coriolis µ air sampler, flow rate = 300 L/min); Passive methods: surface samples (swabs)	1 year longitudinal study	Sampling occured at 2 Watewater Treatment Plant (WWTP); 26 air sample and 15 surface samples	Culture-based methods (MEA); Real Time PCR (RT-PCR)	At both WWTPs were found 33 different species of *Aspergillus* spp. (18 at WWTP1 and 15 at WWTP2), 7 species were only isolated in surfaces (5 in the WWTP1 and 2 at WWTP2), and 12 different Aspergillus sections were identified (6 in both WWTP).	[14]
Wastewater treatment plant workers' exposure and methods for risk evaluation of their exposure	WWTP with anaerobic–anoxic–oxic process	Airborne Bacteria, Enteric Bacteria, Endotoxins	Air Samples: Active methods—Filtration (personal and stationary GSP samplers with polycarbonate filters or with Teflon filter, flow rate = 3.5 L/min) and Impaction (Andersen six-stage cascade impactor, flow-rate 28.3 L/min)	1 year longitudinal study	Stationary samples were taken in the grid chamber house and in the aeration tank (106 personal GSP samples, 12 stationary GSP samples), and 141.5 L to 843 L of air by ASCI were taken over the year	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)	A total of 22.36% of the bacteria potentially inhaled by WWTP workers seem to be from the air around the aeration tank and 22.40% from the grid house; *Staphylococcus* (13.2%) and *Aeromonas* (11.7%) were the dominant genera at the aeration tank, while *Acinetobacter* (25.6%) was the dominant in grid house.	[19]

Table 2. *Cont.*

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
Anaerobic bacteria in wastewater treatment plant	Mechanical–biological WWTP	Anaerobic Bacteria	Air samples: Active method—Impaction (Andersen six-stage cascade impactor, flow-rate = 28.3 L/min); Material collection (sewage and sludge samples were taken directly into 50 mL sterile screwed-off Falcon tubes)	Seasonal (summer and winter)	Bar screens, containers with solids in the screens' hall, primary settling tank, sewage sludge pumping station, aeration basins incineration plant, sludge-thickening building, and at the background of WWTP (n = 22)	Culture-based methods (Schaedler agar with 5% additive of sheep blood for bacterial growth), PCR (for confirmation of *Clostridium* isolates), Biochemical API 20A test (bioMérieux)	Some of the anaerobic bacteria identified belongs to the risk group 2 (according to the EU Directive 2000/54/EC); *Actinomyces*, *Bifidobacterium*, *Clostridium* and *Propionibacterium* genera were identified in wastewater and in the air	[16]
Bioaerosols emission characteristics from wastewater treatment aeration tanks and associated health risk exposure assessment during autumn and winter	Municipal WWTP with rotating disc aeration tank, adopted with DE oxidation ditch treatment process, and microporous aeration tank and adopted with Anaerobic–anoxic–oxic process	*Escherichia coli* and *Staphylococcus aureus*	Air samples: Active—Impaction (Andersen six-stage cascade impactor, flow-rate = 28.3 L/min); Material collection (500 mL wastewater samples were taken by a sterility water sampling bottle)	Seasonal (autumn and winter)	The sampling was carried out at 3 WWTPs, and they were located in the middle of the center corridor of the second microporous aeration tank and the first rotating disc aeration tank from north to south	Culture-based methods (for *S. aureus* MYP was used, and MAC for *E. coli*)	*Staphylococcus aureus* was about 2 times higher in winter than in autumn, while *Escherichia coli* in autumn was about 9 times higher than in winter.	[24]

Table 2. *Cont.*

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
Influence of seasons and sites on bioaerosols in indoor wastewater treatment plants and proposal for air quality indicators	Municipal WWTP with pre-, primary and secondary treatments	Bacteria and Endotoxins	Air samples: Active—Impaction (Andersen six-stage cascade impactor, flow rate = 28.3 L/min) and Filtration (37 mm cassettes (SKC) loaded with binder-free glass fiber filters, flow-rate = 2 L/min)	Seasonal (warm and cold)	Screening, degreasing/grit removal, settling tanks and biofiltration	Culture-based methods (TSA to collect total culturable aerobic bacteria and Gram-negative selective agar (GNSA) for culturable Gram-negative bacteria). In addition to total bacteria (bacteria 16S rDNA), specific qPCR was used to monitor bacteria from human flora: *E. coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and fresh water environment: *Aeromonas hydrophila*.	The average concentration of culturable Gram-negative bacteria was approximately 100 CFU/m^3 for both seasons. Only two WWTPs showed concentrations of culturable Gram-negative bacteria higher than the recommended exposure limit (1000 CFU/m^3 according to Institut Robert Sauvé en Santé et en Sécurité du Travail (IRSST). Several values were close to the limit.	[37]
Assessment of bioaerosol contamination (bacteria and fungi) in the largest urban wastewater treatment plant in the Middle East	Municipal WWTP with air diffusion by fine bubble diffusers	Airborne Bacteria and Fungi	Air samples: Active method—Impaction (QuickTake 30 sample pump equipped with the Bio Stage single-stage cascade impactor, flow rate = 28.3 L/min)	1 year longitudinal study	Area adjacent to the aeration tank and secondary sedimentation units, near the tricking filter, near the sludge storage tank and sludge dewatering unit, adjacent the screening, grit chamber, and primary sedimentation unit and outside of the WWTP were the locations of the sampling; (n = 240)	Culture-based methods (TSA for airborne bacteria growth, and SDA for fungal growth)	Maximum bacterial concentration was found in the aeration tank in the summer, and the minimum was in the sludge dewatering unit during the winter; maximum and minimum fungal concentrations were in primary treatment and sludge dewatering unit in winter and summer, respectively. *Micrococcus* spp. and *Staphylococcus* spp. had the highest emission of bacteria in the winter and summer, respectively. *Cladosporium* spp., Penicillium spp., *Aspergillus* spp. and *Alternaria* spp. were the dominant fungi.	[30]

Table 2. *Cont.*

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
Characterization of the airborne bacteria community at different distances from the rotating brushes in a wastewater treatment plant by 16S rRNA gene clone libraries	Municipal WWTP with orbal oxidation ditch treatment process	Airborne Bacteria	Air samples: Active methods—Impaction (six-stage Impacting Airborne Microorganism Sampler—FA-1, 28.3 L/min) and Impinger (SKC BioSampler, flow rate = 12.5 L/min)	no data	Aerosol samples were collected at different distances from the rotating brushes in the oxidation ditch; 1.5 L for each sample	Culture-based methods; PCR; Sequencing	The majority of bacteria in the bioaerosols were *Proteobacteria* and *Bacteroidetes* around the oxidation ditch. The study concluded that the rotating brush aeration was the main source of bioaerosols in the oxidation ditch.	[25]
Genomic insight into transmission mechanisms of carbapenem-producing Citrobacter spp. isolates between the WWTP and connecting rivers	WWTP with anaerobic–anoxic–oxic treatment process	Carbapenem-Resistant *Citrobacter* spp. (CRCS)	Material collection (wastewater and sludge mixtures samples with a total volume of 1 L)	Seasonal (spring, summer, autumn and winter)	Water inlet, anaerobic tank, aerobic tank, sludge thickening tank, activated sludge tank, mud cake storage area, and water outlet; In total, 136 river water and 51 river sediment samples were collected and 189 samples were gathered from the WWTP.	Culture-based methods; PCR; 16s RNA sequencing; MALDI-TOF MS	In total, 14 CRCS were detected in 376 environmental samples, including those from the inlet (n = 7), anaerobic tank (n = 2), and rivers (n = 5). *Citrobacter braakii* (n = 6) was the dominant subtype among 14 CRCS isolates, followed by *Citrobacter freundii* (n = 5), *Citrobacter sedlakii* (n = 2), and *Citrobacter werkmanii* (n = 1). All CRCS showed resistance to the studied antibiotics.	[26]
Aspergillus flavus contamination in two Portuguese wastewater treatment plants	WWTP with primary, secondary and tertiary treatment processes	*Aspergillus* spp.	Air samples: Active methods—Impaction (Millipore, flow rate = 140 L/min) and Impinger (Coriolis μ air sampler, flow rate = 300 L/min); Passive methods: surface samples (swabs)	Seasonal (winter)	Ten sampling locations were established at the two WWTP for assessing indoor air contamination: lift station, flotation sludge, sludge dewatering, screening, co-generation, aerobic digestion (secondary treatment), canteen, operation room, grit removal, and administration room. An outdoor reference sampling was also included; air samples: 26 indoor and 2 outdoor; surface samples: 17 indoor	Culture-based methods (MEA); RT-PCR	In both WWTPs, *Aspergillus versicolor* (38%), *Aspergillus candidus* (29.1%), and *Aspergillus sydowii* (12.7%) were the most common. In the surfaces were *Aspergillus flavus* (47.3%), *Aspergillus fumigatus* (34.4%), and *Aspergillus sydowii* (10.8%). The isolates of *Aspergillus flavus* that were inoculated in coconut agar medium were not identified as toxigenic, and were not detected by RT-PCR.	[13]

Table 2. *Cont.*

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
Bioaerosol emissions and detection of airbone antibiotic resistance genes from a wastewater treatment plant	Municipal WWTP with activated sludge treatment process	Culturable Bacteria and Fungi; Fluorescent Bioaerosols	Air Samples: Active method—Impaction (Reuter Centrifugal Sampler High Flow, flow-rate = 100 L/min) and Impinger (SKC Biosampler, flow-rate = 12.5 L/min; Particulate matter (Ultraviolet aerodynamic 190 particle sizer (UV-APS)	Seasonal (spring, summer, autumn, and winter)	Sludge thickening basin, biological reaction basin, screen room	Culture-based methods (with TSA and MEA for bacterial and fungal growth, respectively); PCR	Highest concentrations in sludge thickening basin (bacteria: 1697 CFU/m^3, fungi: 930 CFU/m^3). Bacterial concentrations met Chinese standards, but fungal levels exceeded World Health Organization (WHO) recommendations in some areas.	[4]
Occupational Exposure to *Staphylococcus aureus* in the Wastewater Treatment Plants Environment	Municipal WWTPs with mechanical, chemical and biological treatments processes	*Staphylococcus aureus*	Air samples: Active methods—Impaction (1-step portable air sampler made by Burkard, flow rate = 20 L/min) and Filtration (GilAir-5 pump and an open-faced aerosol sampler with a gelatin filter of a 37 mm in diameter and 3 µm pores at a flow rate of 3 L/min); Material collection (raw wastewater discharged into the wwtp and treated wastewater)	Seasonal (spring and summer)	The study was conducted in 16 WWTPs in Poland, representing different treatment technologies; a total of 286 samples were collected, including 253 air samples and 33 wastewater samples	Culture-based methods (chromogenic substrate CHROMID® S. aureus Elite agar); MALDI-TOF, and an automatic method for antibiotic resistence na alysis (WalkAway system)	The study identified *Staphylococcus aureus*, including antibiotic-resistant strains, in wastewater and air samples from WWTP. Workers engaged in mechanical treatment faced the highest health risk.	[17]

Table 2. *Cont.*

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
COVID-19 infection risk from exposure to aerosols of wastewater treatment plants	Municipal with activated sludge treatment process	SARS-CoV-2	Air samples: Active method—Impinger (Portable pumps; flow rate = 7.5–8.5 L/min); Material collection (Grab samples—raw wastewater was colected in 250 mL in sterile glasses)	1 year longitudinal study	Pumping station and activated sludge plants; a total of 24 raw wastewater samples were collected, with 12 samples from each of the two wastewater treatment plants (WWTPs) and 15 air samples.	RT-qPCR	SARS-CoV-2 RNA was found in 37.5% of wastewater samples. Detected in 5 of 12 samples from WWTP A and 4 of 12 samples from WWTP B. The highest concentration was observed at the pumping station.	[31]
Dispersion and Risk Assessment of Bacterial Aerosols Emitted from Rotating-Brush Aerator during Summer in a Wastewater Treatment Plant of Xi'an, China	WWTP with oxidation ditch process	Bacteria	Air samples: Active method—Impaction (Andersen six-stage cascade impactor, flow rate = 28.3 L/min)	Seasonal (summer)	Directly Downwind Sites: 2 m downwind 5 m downwind 10 m downwind 30 m downwind 50 m downwind 100 m downwind Lateral Sites: G1 (5 m laterally from the aerator) G2 (5 m laterally from the aerator)	Culture-based methods	Higher airborne bacteria concentrations were observed closer to the aerator.	[5]

Table 2. *Cont.*

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
Airborne bacteria in a wastewater treatment plant: Emission characterization, source analysis and health risk assessment	WWTP with anaerobic–anoxic–oxic process	Bacteria	Air samples: Active method—Impaction (Quartz membranes (90 mm, Whatman QM-A), flow-rate = 100 L/min and TH-150)	Seasonal (spring, summer and winter)	The WWTP has various treatment stages, including CS (possibly activated sludge), AGC (grit chamber), PST (primary settling tank), AnT (possibly anoxic tank), AeT (aeration tank), and SST (secondary settling tank). Indoor facilities like CS and SDH (sludge dewatering with decanter centrifuges) were compared with outdoor facilities like AGC, PST, and AeT.	High-throughput sequencing techniques	Concentrations varied by site and season. Treatment stages were significant emission sources.	[27]
Quantifying the Relationship between SARS-CoV-2 Wastewater Concentrations and Building-Level COVID-19 Prevalence at an Isolation Residence: A Passive Sampling Approach	no data	SARS-CoV-2	Passive method (tampons made from rayon with a polyester string)	Seasonal (spring)	Approximately 190 feet from the isolation residence, and the wastewater influent at this location was restricted to the isolation building	RT-qPCR	The virus was detected over 16 weeks, demonstrating its feasibility for identifying residential halls with infected individuals. The daily viral wastewater load showed a positive association with the building's COVID-19 prevalence.	[35]

Table 2. *Cont.*

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
Assessment of airborne virus contamination in wastewater treatment plants	no data	Adenovirus (AdV); Norovirus (NoV); Hepatitis E Virus (HEV)	Air samples: Active method—Impaction (3 µm pore size, 25 mm gelatine filters embedded in standard cassettes using MSA Escort Elf or SKC pocket pump 210–1002, flow rate = 4 L/min)	Seasonal (summer and winter)	Inside (Enclosed Area): One sample was collected in the enclosed area, specifically near the water inlet. The sampling point inside was close to the rake that removes large particles from incoming water. Outside (Unenclosed Area): Another sample was collected in the unenclosed area, specifically above the bubbling aeration basin; 123 air samples from 31 WWTPs.	qPCR	AdV-F was present in all WWTPs during summer and 97% during winter. Concentrations were higher in summer, reaching a maximum of 2.27×10^6 genome equivalent/m^3. AdV-E/D were detected in winter, only in a few samples. NoV was detected in only 3 out of 123 air samples, with concentrations below quantification limits. HEV was not detected in any of the samples.	[20]
Airborne bacteria and fungi in a wastewater treatment plant: type and characterization of bio-aerosols, emission characterization and mapping	no data	Bacteria and Fungi	Air samples: Active method—Impaction (One-Stage Andersen cascade impactor, flow rate = 28.3 L/min)	Seasonal (spring, summer and winter)	ETP (Entrance of Treatment Plant), Gch (Grit Chamber), SDB (Sludge Drying Bed), Aea tank (Aeration tank), and Lab (Laboratory Building). Within the mentioned areas, specific points were chosen for sampling, such as the pumping station, additional points in SDB, Gch, Aea tank, and the laboratory.	PCR; biochemical tests: urease, oxidative fermentative (OF), oxidase, catalase, triple sugar iron (TSI), eosin methylene blue (EMB), and Indole-Methyl red-Voges-Proskauer-Citrate (IMViC) test	Various bacteria were identified (some with pathogenic potential), and fungi were present in the air of the WWTP. Bacterial concentrations exceeded the standards, as is the case of *Staphylococcus* and *Enterobacteriaceae*. Fungal concentrations varied seasonally and by location. The relationship between meteorological parameters and bio-aerosols was explored, with temperature showing significance. Particulate matter, especially PM10, correlated significantly with fungal concentrations.	[33]

Table 2. *Cont.*

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
Exposure to Bioaerosol from Sewage Systems	no data	Mesophilic Bacteria; Coliform Bacteria; *Aspergillus fumigatus*	Air samples: Active methods—Impaction (MAS-100, flow rate = 100 L/min) and Impinger (SKC Biosampler, flow rate = 12.5 ± 0.1 L/min)	Seasonal (summer and winter)	At hospital sewage (K1), relief chamber of a combined sewage overflow (K2) and in the area of a city treatment plant (K3); 30 air samples	Culture-based methods (Blood agar was used for mesophilic bacteria and *Aspergillus fumigatus*, Endoagar for coliform bacteria, Coli-ID agar for *Escheriachia coli*, Hektoenagar for *Salmonella* sp., and *Camplylobacter* agar with selective supplement for *Camplylobacter* sp.)	Mesophilic Bacteria Concentrations: Location K1 had concentrations ten times higher than ambient air, attributed to the small chamber size. Location K2 exhibited concentrations comparable to ambient air, possibly due to the large size and good ventilation of the relief chamber. In the encased grit chamber (K3), mesophilic bacteria concentrations were significantly higher than in K1, K2, and ambient air. Coliform bacteria concentrations were generally low, with the highest load found in the encased grit chamber (K3). Coliform bacteria were infrequently found in aerosols of wastewater plants. *Aspergillus fumigatus* was detected at all sampling sites both indoors and outdoors.	[21]
Methicillin-Resistant *Staphylococcus aureus* (MRSA) Detected at Four U.S. Wastewater Treatment Plants	WWTP with primary, secondary and tertiary treatment processes	Methicillin-resistant *Staphylococcus aureus* (MRSA)	Material collection (Grab Samples—Samples were collected in 1-L sterile polyethylene Nalgene® Wide Mouth Environmental Sample Bottles)	1 year longitudinal study	Mid-Atlantic WWTP1 Mid-Atlantic WWTP2 Midwest WWTP1 Midwest WWTP2; 44 grab samples were collected	Gram stain; coagulase and catalase tests; PCR	MRSA was detected in 50% of wastewater samples, at all WWTPs studied. MSSA (Methicillin-Susceptible *Staphylococcus aureus*) was also detected in 55% of wastewater samples, at all WWTPs. The occurrence of MRSA and MSSA varied across WWTPs, sampling dates, and sampling locations. MRSA isolates showed resistance to multiple antibiotics, including those approved for treating MRSA infections. MSSA isolates also exhibited antibiotic resistance patterns that varied by WWTP. In total, 93% of MRSA isolates were multidrug-resistant (MDR), while 29% of MSSA isolates were MDR.	[34]

Table 2. *Cont.*

Title	Type of WWTP	Microbe Assessed	Sampling Methods	Season of Sampling	Sampling Sites and Number of Samples	Assays	Main Findings	Reference
Characterization and source analysis of indoor/outdoor culturable airborne bacteria in a municipal wastewater treatment plant	Municipal WWTP with anaerobic–anoxic–oxic treatment process	Airborne Bacteria, Enterobacteriaceae and Opportunistic Pathogens	Air Sample: Active method—Impaction (Andersen six-stage cascade impactor, flow rate = 28.3 L/min)	Seasonal (spring, summer, autumn and winter)	Four specific sampling sites were selected within the plant: fine screens room (FS), aeration tank (AT), sludge dewatering house (SDH), and an external upwind control site; 48 air samples	Culture-based methods; Illumina MiSeq sequencing	FS had over ten times higher concentrations of culturable airborne bacteria compared to the outdoor aeration tank. Particle size distribution of culturable airborne bacteria varied between sampling sites. Enterobacteriaceae and opportunistic pathogens were detected indoors, primarily sourced from wastewater and sludge (were not detected outdoors).	[28]
Assessment of indoor airborne contamination in a wastewater treatment plant	Municipal WWTP with preliminary, primary, secondary, tertiary and sludge treatments, and deodor-ization processes	Bacteria and Fungi	Air Sample: Active method—Impaction (MAS 100, flow rate = 100 L/min)	Seasonal (summer, autumn and winter)	Bar Rack Chamber SEDIPAC 3D (Degritting/Degreasing/ Primary Sedimentation Facility) Secondary Sedimentation Tanks (Two Locations) Sludge Thickener Sludge Dehydration Chamber Sludge Disposal Area Outdoor Control Sampling Point	Culture-based methods (TSA for total bacteria, Mannitol salt agar and MAC for Gram-positive and Gram-negative bacteria, respectively, and DG18 for total fungi)	Out of 3 sampling campaigns, in the first one (with the highest ambient temperature) the total airborne bacteria and fungi concentrations were the highest. Gram-positive bacteria were the most dominant, and *Aspergillus*, *Penicillium*, *Cladosporium*, and *Alternaria* were the most common fungi.	[15]
Estimation of health risks caused by exposure to enteroviruses from agricultural application of wastewater effluents	WWTPs with conven-tional activated sludge processes	Fecal Coliforms and Enteric Viruses	Material collection (effluent samples were collected in 1-L sterile glasses)	Seasonal (spring, summer, autumn and winter)	30 effluent samples (15 from each WWTP)	Culture-based methods	A high fecal coliform concentration was observed in the WWTPs. Enteric viruses were also detected, peaking in summer/autumn. There was a high risk for farmers (EV infection and disease burden) and risk for lettuce consumers, exceeding WHO guidelines.	[32]

Among the chosen studies, 12 (42.86%) were conducted within Municipal WWTP [4,6,15,17,22,24,25,28–31,37]. However, information regarding the type of WWTP was not explicit in 16 studies (57.14%) [5,13,14,16,18–21,23,26,27,32–36].

The most common sampled sites were the aeration tank (42.86%) [6,16,18,19,22,24,26–30,33], sludge dewatering basin (21.43%) [6,13,27–30] and grit chamber (17.86%) [6,19,27,29,30,33]. Some authors choose to perform the sampling at 1.5 m up on the aeration tanks (7.14%) [18,22]. Only one study (3.57%) [23] focused on sampling at different distances from the rotation brushes.

In terms of sampling strategy, seven papers opted to conduct sampling in two seasons (25%) [16,17,20,21,23,24,36]. Four studies (14.29%) were carried out in a single season [5,13,22,35] while another four studies covered all four seasons (14.29%) [4,26,28,32]. Furthermore, three authors conducted sampling activities across three seasons (10.71%) [15,27,33]. Five studies (17.86%) focused on a one-year longitudinal study [14,19,30,31,34]. Additionally, three studies (10.71%) differentiated sampling procedures between warm and cold seasons [6,29,37], whereas two studies did not specify the timing of their sampling activities (7.14%).

Air sampling emerged as the most employed technique, utilized in 24 out of 28 studies (85.71%) [4–6,13–25,27–31,33,36,37]. Active air sampling was carried out in 22 papers (78.57%) [4,5,13–25,27,28,30,31,33,36,37], and among these, the impaction method was predominant, with 19 studies (67.86%) [4,5,13–17,19–25,27,28,30,33,37] using different sampling devices such as the six-stage (32.14%) [5,16,19,22–25,28,37] and single-stage impaction (25%) [13–15,17,21,30,33]. The impingement method was employed in seven studies (25%) [4,13,14,21,25,31,36], while only five studies (17.86%) [4,13,14,21,25] utilized both impaction and impingement methods, simultaneously. Four studies used the filtration method (14.29%) [17–19,37]. Regarding passive sampling, it was employed in five studies (14.29%) [6,13,14,29,35], the 1/1/1 standard was used in two studies (in accordance with the microbiological sampling index of the air, a plate is placed at 1 m height, at 1 m distance to the (possible) source of contamination, and it is performed for a period of 1 h) (7.14%) [6,29], and surface samples were used in two papers (7.14%) [13,14]. Active and passive sampling strategies were carried out simultaneously in 2 out of the 28 studies (7.14%) [13,14]. Regarding the type of microbial contamination assessed, the majority of the studies (50%) [5,6,16,17,19,22–25,27,28,34,36,37] focused only on bacteria, while three studies (10.71%) [13,14,29] focused solely on fungi, and another three (10.71%) [20,31,35] evaluated only virus exposure. Six studies (21.43%) [4,15,18,21,30,33] included both fungi and bacteria, while one (3.57%) [18] examined bacteria, fungi, and viruses collectively, and another (3.57%) [32] assessed bacteria and viruses together.

Culture-based methods were the most frequently employed assays, utilized in 20 out of 28 studies (71.43%) [4–6,13–18,21–26,28–30,32,37]. Among the most used culture media, for fungal growth, three studies used MEA (Malt Extract Agar) (10.71%) [4,13,14], two studies Saboraud dextrose agar (SDA) (7.14%) [29,30], and one used Dichloran Glycerol agar (DG18) (3.57%) [18]. For bacteria, four studies used Tryptic Soy Agar (TSA) (14.19%) [4,15,30,37], three studies MacConkey Agar Medium (MAC) (10.71%) [15,22,24], and only one used Mannitol Egg Yolk (MYP) (3.57%) [24]. Nine of these studies only used one culture media for bacteria and/or fungi growth [4,6,13,14,16,17,23,29,30], and four used more than one culture media for bacteria growth [15,21,24,37]. In total, five studies did not mention the culture media used (17.86%) [5,25,26,28,32].

Molecular techniques were applied in 19 papers (67.86%): 13 employed Polymerase Chain Reaction (PCR) (46.43%) [4,6,13,14,20,23,25,26,31,33–35,37], and 6 used sequencing (21.43%) [22,25–28,36]. In PCR assays, to target bacterial strains, 5 out of 28 studies amplified bacterial 16S rRNA using universal primers (17.86%) [6,22,23,25,33], one amplified *Eschecrichia coli* MG1655 (3.57%) [36], and another used Chis150f and Clostlr primers for *Clostridium* sp. (3.57%) [16]. To detect bacterial pathogenic species, for *Staphylococcus aureus*, the primers used were NUC1 and NUC2 to target the NUC gene (3.57%) [34]. Another study targeted bacterial populations from human flora, such as *Escherichia coli*,

Klebsiella pneumoniae, and *Pseudomonas aeruginosa*, and bacterial populations from freshwater environments such as *Aeromonas hydrophila* (3.57%) [37]. Three out of twenty-eight studies focused on antibiotic resistance profiling, one for MRSA using ECA1 and MECA2 primers for the amplification of mecA gene (3.57%) [34], one study (3.57%) used PCR coupled with gel electrophoresis to detect antibiotic resistance genes, such as sul1, sul2, sul3 for sulfonamide, tetA, tetC, tetO, tet W for tetracycline and integrons (intl1, intl2 and intl3) [4], and other amplified blaNDM, blaKPC, blaOXA-48, blaIMP, and blaVIM genes for Carbapenem-Resistant Citrobacter spp. (CRCS) (3.57%) [26]. For targeting viruses, two papers focused on SARS-CoV-2 (7.14%), one on the N1 and N2 unique genes [35] and one on RdRp, ORF-1ab, and N [31]. In one study (3.57%), three duplex qPCR were performed to target NoV180 GGII/RYMV and HEV/RYMV for RNA viruses, and AdV-40/AdV-E/D for DNA viruses [20]. For fungi, PCR was used to target *Aspergillus* sections such as *Flavi* (toxigenic strains), *Fumigati* and *Circumdati* in one paper [14], and only *Aspergillus* section *Flavi* in another [13]. Regarding sequencing methodologies, three out of six studies targeted the identification of airborne bacteria [22,27,28]; one targeted 16 rRNA to delineate the composition and similarities of microbiomes in water and air samples [36], one targeted taxonomic species of CRS [26], and one used sequencing to evaluate the positive clones of *Eschecrichia coli* [25]. In total, 11 out of 28 studies (39.29%), applied both molecular techniques and culture-based methods [4,6,13,14,16,22,23,25,26,28,37].

Among the species identified, the most prevalent Gram-positive bacteria were *Staphylococcus* sp. (21.43%) [17,19,24,30,33,34], *Bacillus* sp. (7.14%) [6,23] and *Clostridium* sp. (3.57%) [16], and the most prevalent Gram-negative were *Escherichia* sp. (7.14%) [22,24] and *Legionella* sp. (3.57%) [36]. *Aspergillus* sp. (17.86%) [13–15,21,30], *Cladosporium* sp. (10.71%) [15,29,30] and *Alternaria* sp. (10.71%) [15,29,30] dominated the fungal presence. One study focused on the dissemination of Methicillin-resistant *Staphylococcus aureus* (MRSA) [34], while another investigated the occupational exposure to *Staphylococcus aureus* in wastewater treatment plants, particularly focusing on antibiotic resistance [17].

4. Discussion

WWTPs are crucial for the implementation of the zero-waste strategy which is in the scope of the EC's circular economy management. Interestingly, the geographical distribution of the analyzed studies corroborated the urge for tackling WWTPs' pollution threat and to answer to the determined environmental goals worldwide. In agreement with previous reviews held in different settings, such as poultries [9] and sawmills [10], no standardization was observed in the sampling campaigns performed, as well as in the assays employed. Furthermore, the lack of standardized contextual information retrieved through the developed studies hinders the possibility to identify the environmental variables that contribute effectively to the occupational exposure assessment, as well as to propose suitable recommendations to avoid microbial exposure and dissemination [38]. In fact, the contextual information (e.g., implemented occupational health measures, training on safety issues related to the working tasks, cleaning practices, ventilation conditions, number of workers in each workstation, protection devices used by workers), when retrieved, should allow the identification of the most critical scenario and, thus, the selection of proper sampling sites following the "worst case scenario" approach as a first step for exposure assessment. In those sampling sites considered as the most critical, besides the environmental sampling campaign, nasopharyngeal swabs should be collected from the workers' nose to obtain additional information regarding workers' exposure. In previous studies, nasopharyngeal swabs were also taken to assess MRSA prevalence in workers from different occupational settings [39] or to corroborate the predominant fungi present in the Portuguese cork industry and, more specifically, exposure to *Penicillium* section *Aspergilloides* [40]. In addition, this approach can help occupational health services to prioritize multiple interventions in workers' education or even in personal protection device (e.g., gloves, respiratory protection devices) selection and replacement frequency.

The assessment of microbial dynamics in WWTPs is critical for ensuring public health and environmental safety. Seasonal evaluation plays a crucial role in this assessment, particularly given the influence of global warming and human activities, such as intensive agriculture, on microbial ecology [41,42]. In fact, recent studies [43,44] suggest that these factors contribute to the emergence of new fungal species, underscoring the need for comprehensive monitoring strategies. Recognizing the prevalence of research in specific regions and climatic periods is vital for contextualizing findings and understanding their implications for human health. Moreover, linking environmental exposure to health outcomes emphasizes the importance of establishing regulatory limits based on health considerations. This underscores the interconnectedness of the environment, exposure, and health outcomes, necessitating comprehensive regulatory frameworks.

Most of the selected papers (78.57%) exclusively applied active sampling methods, with impaction being the most frequently used method (67.86%). This sampling strategy is based on culture-based methods, which only allows the evaluation of culturable microorganisms, and thus microorganisms' cells that are potentially damaged due to the high velocity of the airflow are not isolated [10,37,45]. Furthermore, it is critical to emphasize that air is not uniform in place or time and that it is always subject to change based on the kind and intensity of the activities occurring there or other environmental variables (e.g., climate conditions) [36,46]. Thus, the sampling period must match the setting of the research and the work being developed in that specific environment. Passive sampling methods were applied in only a few of the analyzed studies as a stand-alone method (14.29%). However, passive sampling methods are expected to be more reliable than active sampling methods since they can collect contamination over longer periods, allowing to cover all the changes that may happen in the environment [47] such as the ventilation, environmental features [48], water infiltrations and damage [49], as well as the type of task being developed in that workplace [10,50,51]. Additionally, passive sampling methods allow the combination of different assays such as culture-based methods and molecular tools increasing the accuracy of obtained results [52]. Although only two papers (7.14%) used active and passive sampling methods together, this should be the trend to follow, since this allows each sampling methods' drawbacks to be overcome [10].

The fact that culture-based methods are primarily used for microbial characterization as standard methods for microbial assessment [53,54] might justify its frequent use among the selected papers (71.43%). This methodology is crucial to estimate health risks, since microorganisms' viability can limit microorganisms' inflammatory and/or cytotoxic potential [10,54,55]. Despite the advantages, conventional approaches may underestimate results since incubation temperatures and culture conditions may favor specific species. Plus, typical procedures may not always be effective in cultivating certain common microorganisms [53]. Furthermore, a recent study [53] highlights the importance of culture media selection and its significant impact on fungal counts and species diversity. Although some studies (17.86%) did not mention what culture media were employed, accurate culture media selection is critical for exposure assessment in different environments, particularly when targeting *Aspergillus* sp. [53]. Overall, three cultural media were employed for fungal assessment (MEA, DG18, and SDA). MEA and SDA are the most used non-selective media for fungi and yeasts, whereas DG18 is a fast-growing fungi inhibitor, allowing more diversity in the growth of fungal strains [56]. MEA and DG18 have both been used alongside and have proven to be useful in the growth of *Aspergillus* species according to the matrix, sampling method employed, and indoor environment assessed [57]. For bacterial assessment, TSA was the most non-selective media related to the growth of fastidious bacteria, while MAC was the most used selective and differential media related to the growth of Gram-negative bacteria, useful for the identification of enteric bacteria [58]. MYP allows the identification of Gram-positive bacteria as *Bacillus cereus* [59]. The use of multiple culture media is fundamental for the isolation and identification of a wider spectrum of microorganisms. Also, the integration of multiple culture media and different incubation temperatures in culturomics methods (such as MALDI-TOF) permits a more

precise identification of unknown isolates [60,61]. This approach allows accurate microbial characterization, particularly the rapid identification of potential pathogens. In fact, culturomics methods bridge the gap between culture-based methods and molecular techniques, providing a comprehensive assessment of bioaerosols [38].

Recently, culture-independent techniques such as PCR and genome sequencing have been demonstrated to be useful for various bioaerosol measurements [52]. Indeed, PCR and sequencing were frequently performed by the authors in the selected papers. These techniques enable the detection of non-viable microorganisms as well as their potentially allergenic components [52,62], providing more information regarding microbial diversity in the evaluated environment [9]. Molecular techniques along with culture-based methods were applied by some papers (39.29%). This strategy is highly supported, since both viable and non-viable microorganisms are considered, providing a wider microbial characterization [9,10,52], and a more accurate characterization of the exposure scenario [14].

Furthermore, molecular techniques development has also enabled the assessment of Antibiotic Multidrug Resistance (AMD), including resistance genes associated with bacteria contamination. Recently, the World Health Organization (WHO) released an updated Bacterial Priority Pathogens List (BPPL) 2024, in which 15 families of antibiotic-resistant bacteria were grouped into critical, high and medium categories in order to allow an effective prioritization [63]. Additionally, the European Food Safety Authority (EFSA) panel on Biological Hazards recently emitted a Scientific Opinion in which the highest priority antimicrobial-resistant bacteria (ARB) and antibiotic resistance genes (ARG) were identified in different sources, including water. Among the most relevant ARB, the panel indicated carbapenem or extended-spectrum cephalosporin and/or fluoroquinolone-resistant *Enterobacterales*, fluoroquinolone-resistant *Campylobacter* sp., Methicillin-resistant *Staphylococcus aureus* and glycopeptide-resistant *Enterococcus faecium* and *E. faecalis*. Regarding the highest priority ARGs, the panel reported *blaCTX-M*, *blaVIM*, *blaNDM*, *blaOXA-48-like*, *blaOXA-23*, *mcr*, *armA*, *vanA*, *cfr* and *optrA* genes. The EFSA report also evidenced the existence of several data gaps regarding sources and the relevance of transmission routes and diversity of ARB and ARGs [64]. The data analyzed in this review demonstrate that antibiotic resistance profiling, including MRSA, *mecA gene* [31], sulfonamide, *sul1*, *sul2*, *sul3*, tetracycline, *tetA*, *tetC*, *tetO*, *tet W*, integrons, *intl1*, *intl2 and intl3* [4], and Carbapenem-Resistant *blaNDM*, *blaKPC*, *blaOXA-48*, *blaIMP*, and *blaVIM* genes [26] is already a reality. Moreover, despite the fact that the quantitative microbial risk assessment (QMRA) of WWTPs has been classically focused on risk-based monitoring targets, it is accepted that the expansion of QMRA methodologies, to include ARG, may be key for the assessment of the relative risk of these contaminants [65]. The assessment of ARG units is crucial for the identification of relevant/high-priority sources and natural reservoirs of AMR, allowing the establishment of effective mitigation strategies in a One Health approach. Despite the fact that microbial assessment in water samples and sewage treatment plants has been carried out, the development of official monitoring strategies and effective risk assessment in sewage treatment plants is crucial. In agreement with the newly updated WHO-BPPL, which demonstrates the highly dynamic nature of AMR, increasing evidence and expert reports clearly highlight the urge to promote a comprehensive public health approach and international coordination to engage innovation and mitigation strategies [63].

On the other hand, it is important to note that ARGs identification may be influenced by the different methods employed and divergences in the measuring process from sampling to wet-lab differences, among others [66]. In addition to the multi-criteria decision analysis (MCDA) method developed by the WHO in the 2017 WHO BPPL, which is still currently applied in the 2024 WHO BPPL [63] and EFSA Panel on Biological Hazards (BIOHAZ) risk assessment monitoring (https://www.efsa.europa.eu/en/topics/topic/biological-hazards), other international multi-disciplinary networks, such as NEREUS COST Action ES1403 [67], created to access the current challenges related to wastewater reuse and high-priority concerns regarding public health and environmental protection, concluded that scientific research and environmental management should follow system-

atic, quantitative, and comparable ARG datasets, and reported that the research community should adopt "ARG copy per cell" [66]. Thus, the development of effective mitigation measures including new monitoring technologies, such as on-line sensors that are able to detect and quantify bacterial pathogens, ARB and ARG, is crucial, as is the implementation and improvement of links between research and policy [65].

The identification of the most suitable fungal indicators in WWTPs is also critical for assessing treatment efficacy, environmental impacts, and public and occupational health risks [68]. Commonly used fungal species such as *Aspergillus* sp. and *Penicillium* sp. serve as markers for organic matter removal and microbial contamination [69]. Monitoring fungal indicators enables the identification of seasonal variations, climate influences, and anthropogenic impacts on wastewater quality, essential for tailoring treatment strategies. Additionally, their presence aids in the early detection of potential health hazards, such as opportunistic pathogens or allergenic molds, ensuring the safety of both workers and the public [70]. *Aspergillus* sp. was recurrent and also the most prevalent in the selected papers; the prevalence of this genera in waste management industries has already been recognized, highlighting the need for further research regarding occupational exposure [14]. In fact, *Aspergillus* section *Fumigati* was already suggested as an indicator of harmful fungal exposure in the waste management industry [71–74] and listed by the WHO as a critical priority, considering specific criteria such as antifungal resistance, mortality, evidence-based treatment, access to diagnostics, annual incidence and complications and sequelae [75]. However, the WHO list did not consider the toxicologic potential from fungal species, neglecting the possible occupational exposure to mycotoxins, as was already reported in different occupational environments [76].

In agreement with bacteria contamination analysis, fungal assessment should also cover the resistance profile. Indeed, antifungal drug resistance is a growing global concern in both space and time. This includes newly emerging species that are resistant to multiple antifungal drugs (like the yeast *Candida auris*), as well as novel resistant variants of previously susceptible pathogens (such as the ubiquitous mold *Aspergillus fumigatus*) [77]. Because of the selection of resistant strains triggered by the growing use of triazole drugs, azole resistance in *Aspergillus fumigatus* is currently seen as an emerging hazard to global public health [78,79]. In *Aspergillus fumigatus*, azole resistance can evolve through two different pathways. First, in the setting of chronic pulmonary aspergillosis, as in individuals with cystic fibrosis, resistant strains may be chosen during or following a long-term azole therapy [79,80]. Second, the prolonged use of azole antifungals in agriculture may be connected to azole resistance [79,81–84]. Relevantly, it is reported that several antifungals cause inherent resistance in *Fumigati* cryptic species. However, selected pressure brought on by the prolonged azole therapy of patients with chronic aspergillosis or environmental selection pressures are the reasons behind the emergence of resistance acquisition in *Aspergillus fumigatus* sensu stricto. Mutations in genes engaged in the *Aspergillus fumigatus* ergosterol pathway are frequently linked to the mechanisms of azole resistance, especially in the cyp51A gene that encodes cytochrome P450 14-lanosterol demethylase, the primary target of azole antifungals [79,85,86], highlighting the relevance of using these mutations as an indicator for fungal resistance.

Considering the above, further research should be performed to select the most suitable indicators of harmful microbial contamination for this occupational setting. The lists provided by the WHO regarding fungi [86] and bacteria [87] should be considered for this endeavor, but the resistance and toxicological potential from fungi and bacteria should not be neglected.

5. Conclusions

Overall, this scope review concluded what is needed to provide robust science for the guidance of occupational exposure assessments: (a) common protocol from the field (sampling campaign) to the lab (assays to employ) when aiming to perform exposure assessment in WWTPs; (b) standardized contextual information to be retrieved, allowing a

proper risk control and management; (c) the selection of the most suitable microbial targets to serve as indicators of harmful microbial exposure. Filling these gaps with further studies will allow robust science to be provided to policy makers and stakeholders.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms12061144/s1, Table S1: PRISMA Checklist.

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Appendix 5

Budget-friendly protocol for TR34/L98H and TR46/Y121FT289A mutation detection in *Aspergillus* section *Fumigati* isolates

M. Dias[a,b], M. Rodriguez[b], C. Vasques[b], B. Riesenberger[b], L. Marques[b], B. Gomes[b,c], P. Pena[a,b], R. Cervantes[a,b], S. Viegas[a,b], C. Viegas[a,b]

[a] NOVA National School of Public Health, Public Health Research Centre, Comprehensive Health Research Center, CHRC, REAL, CCAL, NOVA University Lisbon, Lisbon, Portugal (msf.dias@ensp.unl.pt)
[b] H&TRC- Health & Technology Research Center, ESTeSL- Escola Superior de Tecnologia da Saúde, Instituto Politécnico de Lisboa, Portugal (marta.dias@estesl.ipl.ptl)
[c] CE3C—Center for Ecology, Evolution and Environmental Change, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisbon, Portugal

Introduction

Aspergillus section *Fumigati* is one of the **most common sections**, in the environment [1].

It has been found in **different occupational environments, such as sawmills and waste sorting** [1,2].

Its **cryptic species show intrinsic resistance** to several antifungals [3].

Resistance in *A. fumigatus* is emerging due to selective pressure caused by the prolonged use of azoles.

It is often associated **with mutations in the Cyp51A gene** [3].

The **fungal priority pathogens list (WHO)**, includes ***A. fumigatus* with critical priority** [4].

Further analysis to identify potential resistance mechanisms and mutations is needed.

Objective

This evaluation aims to offer a protocol for mutation detection in *Aspergillus* section *Fumigati* isolates,

It will contribute for the development of guidance that can support future occupational exposure assessments.

Methodology

Hypotheses were determined based on the advantages and disadvantages of each suggested method, including its cost.

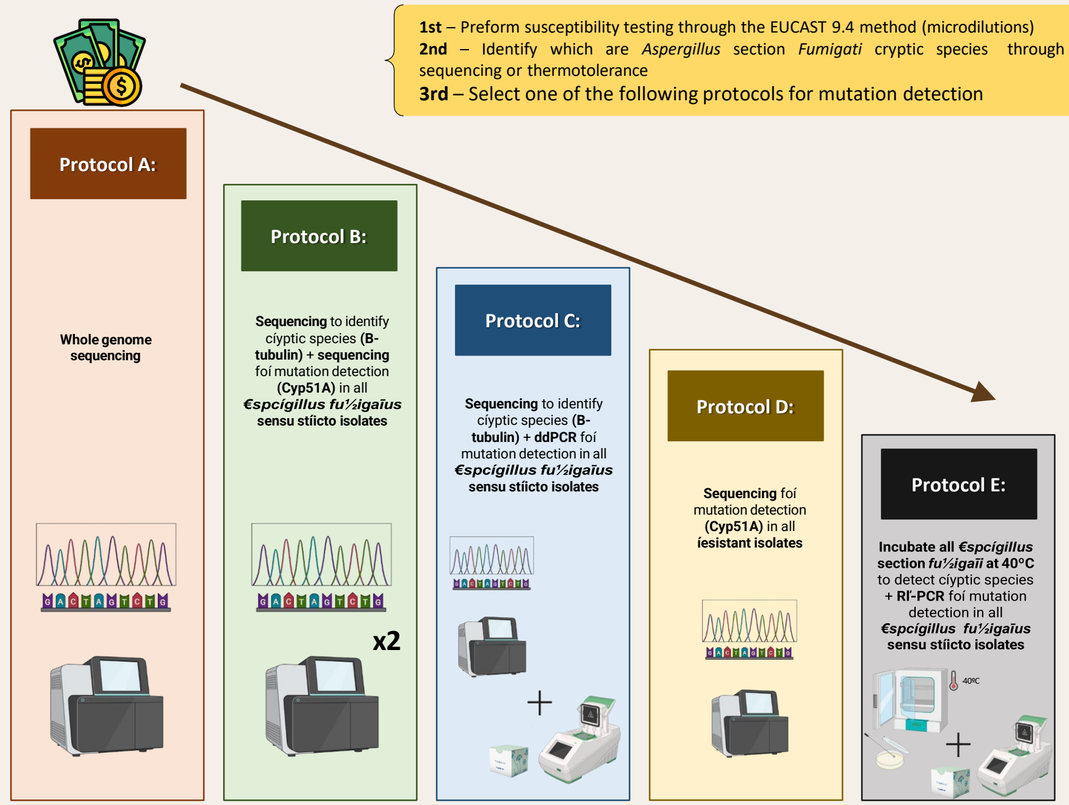
	Whole genome sequencing:	Sequencing (x2) of all isolates:	Sequencing (x2) of resistant isolates:	Sequencing + ddPCR:	Incubation + RT-PCR:
👍	All information with one analysis	All the necessary information	Information regarding the section and the presence of TR and point mutations	Information regarding the presence of TR and point mutations	All the necessary information
👎	Additional unnecessary information for this analysis		Loss of information regarding point mutations in the CYP51A	Cryptic species not identified	Loss of information regarding point mutations in the Cyp51A

*Tandem repeat

Results and discussion

- Azole resistance is mostly caused by **particular mutations in CYP51A** [5].
- **Wild-type CYP51A-resistant** isolates question the effectiveness of the available methods [5].
- **Whole-genome sequencing is** becoming increasingly common to address these issues [5].

1st – Preform susceptibility testing through the EUCAST 9.4 method (microdilutions)
2nd – Identify which are *Aspergillus* section *Fumigati* cryptic species through sequencing or thermotolerance
3rd – Select one of the following protocols for mutation detection



Conclusions

This study allowed determining several ways to detect mutation in *Aspergillus* section *Fumigati* isolates. It **provided the necessary tools to perform an accurate occupational exposure assessment to *Aspergillus* section *Fumigati*** and allowed a **more detailed risk assessment** while overcoming cost issues at the same time.

References

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Appendix 6

International Symposium

Occupational Safety and Hygiene

A multi-approach sampling strategy to assess exposure to microbiologic agents in poultries

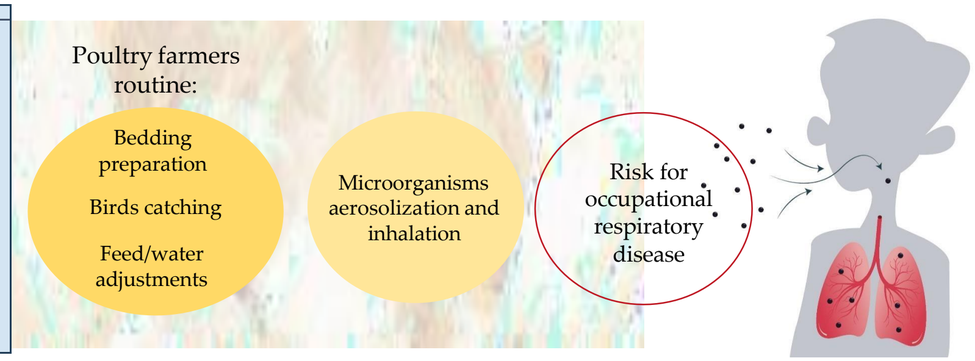
Bianca Gomes [1,2,*], Marta Dias [2,3], Pedro Pena [2,3], Renata Cervantes [2,3],Margarida Rodriguez[2], Liliana Marques, [2] Bruna Riesenberger[2], Carla Viegas[2,3]

[1] Center for Ecology, Evolution and Environmental Change, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisbon, Portugal, bianca.gomes@estesl.ipl.pt
[2] H&TRC – Health & Technology Research Center, ESTeSL – Escola Superior de Tecnologia e Saúde, Lisboa; pedro_migpena@hotmail.com; renata.cervantes@estesl.ipl.pt;
[3] NOVA National School of Public Health, Public Health Research Centre, Comprehensive Health Research Center, CHRC, REAL, CCAL, NOVA University Lisbon, Lisbon, Portugal; martasfd@gmail.com; carla.viegas@estesl.ipl.pt

Introduction

A reasonable number of studies focusing on **microbiological contamination** associated with the **poultry industry** evidence **various health concerns** [1,2]

In occupational studies focusing on microbiological contamination in poultry farms, **air sampling is typically the only sampling method used** [3]

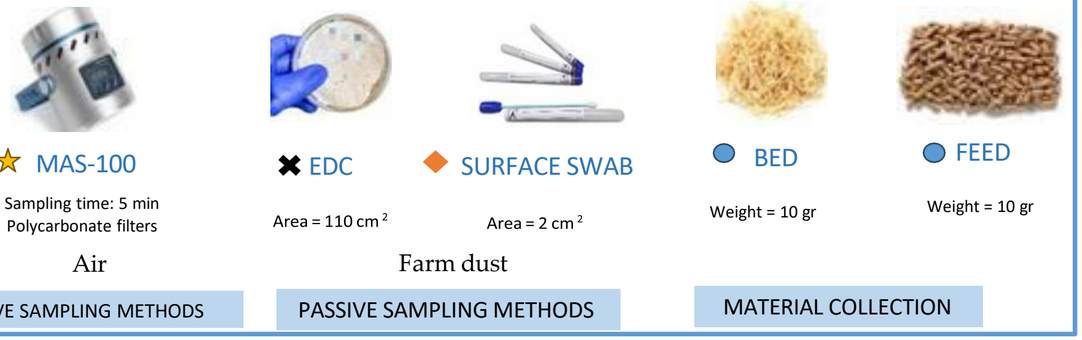


To better understand the relationship between factors influencing microbial contamination and adverse health effects, **data regarding the amount, composition, and risk category of the common microorganisms are needed** [4].

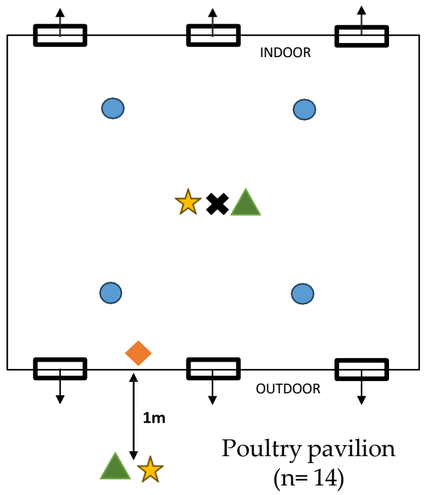
This study intends to apply a multi-approach sampling protocol and corroborate the importance of its application for a wider microbial characterization

Methodology

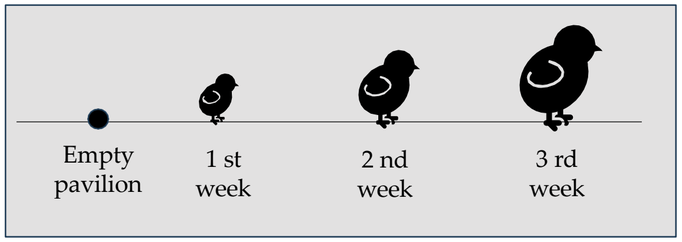
1. Sampling methods



2. Sampling strategy



3. Sampling Frequency



2 Seasons:

Summer

Winter

Results and discussion

Culture based-methods: Fungal prevalence in indoor air samples was highest during: the 3rd week (35%), followed by 2nd week (33%) and 1st week (10%).

Molecular analysis: Fungal detection in indoor air samples was highest during: the 3rd week (69%), followed by 2nd week (64%) and 1st week (43%).

Culture based-methods offer the advantage of enabling **identification and quantification of viable microorganisms** which is **essential to estimate health risks** since microorganisms' **viability** can restrain **microorganisms' pathogenic potential.** Culture-based methods, on the other hand, may understimate the results since incubation temperature and culture conditions may favor specific species [3].

PCRbased techniques have been widely used in detection of microorganisms from environmental samples to determine **accurately and quantitatively, the composition of microbial communities** [3]. These methods allow the **detection of non-viable microorganisms** which, may justify the differences between in the obtained results from conventional and molecular methods.

Conclusions

- Both **methods have advantages and limitations** when applied to characterize occupational exposure to biological agents in different settings. The results highlight the importance of using a multi-approach sampling strategy and laboratory assays including culture-based methods along with molecular tools [3].
- The multi-approach sampling strategy and assays will **enhance data findings**, **enabling a more accurate intervention** in order to propose strategies to improve poultry environment, enhance workers and animal safety while reducing environmental impact.

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Appendix 7

Fungal Contamination in Lisbon's Primary Schools - Sampling Insights and Analytical Approaches

Renata Cervantes [1,2], Pedro Pena [1,2], Bianca Gomes[1,3], Marta Dias[1,2], Bruna Riesenberger[1], Margarida Rodriguez[1], Liliana Marques[1], Carla Viegas [1,2]

[1] H&TRC—Health & Technology Research Center, ESTeSL—Escola Superior de Tecnologia e Saúde, Instituto Politécnico de Lisboa, 1990-096 Lisbon, Portugal;
[2] Public Health Research Centre, Comprehensive Health Research Center, NOVA National School of Public Health, CHRC, REAL, CCAL, NOVA University Lisbon, 1099-085 Lisbon, Portugal
[3] CE3C—Center for Ecology, Evolution and Environmental Change, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisbon, Portugal

Introduction

Climate change is posing challenges for Portugal due to intense weather changes, affecting public health and causing pathogens to adapt and spread, increasing the global risk of infectious diseases [1,2].

Azole fungicides are less effective against resistant fungi, raising concerns for children [1,2].

Warm and humid conditions promote the growth of pathogenic fungi and the production of mycotoxins, impacting health by causing gastrointestinal problems, organ damage and chronic diseases. Even after fungi removal, mycotoxins continue to pose risks [3,4,5].

Objectives

- Identifying fungal species present in indoor environments.
- Assessing spatial distribution and concentration levels within classrooms and other areas.
- Investigating factors influencing fungal proliferation, such as building characteristics and seasonal variations.
- Evaluating the effectiveness of existing cleaning protocols and providing insights into proactive management strategies to protect students' and staff members' health and well-being.

Methodology

Sampling Methods

Active

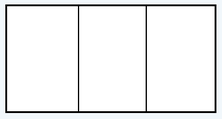
Andersen six-stages

MAS-100

Button Sampler

Passive

Surface swabs



Electrostatic dust cloth

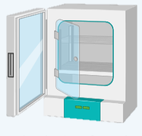
Settled Dust

Mop

Particulate Matter

Lighthouse Handheld
Airborne Particle Counters

Analytical Methods



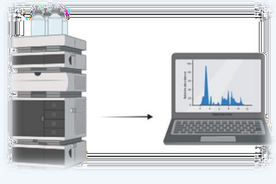
Incubation of samples
at 27°C

Fungal resistant profile

Microbial quantification (Fungi and bacteria)

Fungal identification

Metabolites Assessment



Mycotoxins detection
and
Cytotoxicity assay

Results and discussion

The expected results are that seasonal variations in fungal load show complex environmental interactions[1,2].

Examining fungal load distribution in DG18 media at 27°C and 37°C helps assess growth preferences at different temperatures[6].

Methods used to assess azole resistance and mycotoxin provide essential insights into the resilience and potential harm of fungal species under varying environmental conditions[3,4,5].

Addressing fungal exposure risks requires a comprehensive approach for an accurate risk assessment and to target mitigation strategies on educational environments [6].

Conclusions

- **Standardized protocols** need to be defined and implemented for **effective risk assessment.**
- It is essential to consider **climate changes and seasonal influences into health policies** to mitigate the risks associated with fungal exposure.

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Appendix 8

Epidemiology in Occupational Health (EPICOH) 2024

Mitigating Health Risks in Wastewater Treatment Plants: Identifying Key Microbial Contaminants and Protocol Needs

Renata Cervantes[1,2]; Bruna Riesenberger [1], Margarida Rodriguez [1], Liliana Marques [1], Bianca Gomes [1,3], Marta Dias [1,2], Pedro Pena [1,2], Edna Ribeiro [1] and Carla Viegas[1,2]

Appendix 9

The Power of Citizen Science: Insights and Achievements from the InChildHealth Project

Renata Cervantes* [1,2], Pedro Pena [1,2], Bruna Riesenberger [1], Margarida Rodriguez [1], Liliana Marques [1], Bianca Gomes [1,3], Marta Dias [1,2], Carla Viegas [1,2]

Appendix 10

Indoor microbial levels in poultry pavilions

Bianca Gomes[1,2*], Marta Dias [2,3], Renata Cervantes [2,3], Pedro Pena [2,3], Margarida Rodriguez [1], Liliana Marques [1], Bruna Riesenberger [1], Carla Viegas [2,3]

Appendix 11

PROTOCOL TO DETECT CYP51A MUTATIONS IN *ASPERGILLUS* SECTION *FUMIGATI* ISOLATES

Marta Dias[1,2], Margarida Rodriguez[2], Catarina Vasques[2], Bruna Riesenberger[2], Liliana Marques[2], Bianca Gomes[2,3], Pedro Pena[1,2], Renata Cervantes[1,2], Liliana Aranha Caetano[2,4], Susana Viegas[1,2], Carla Viegas[1,2]

Appendix 12

Microbial Exposure Assessment in Waste Water Treatment Plants

Bruna Riesenberger [1], Margarida Rodriguez [1], Liliana Marques [1], Renata Cervantes* [1,2], Pedro Pena [1,2], Bianca Gomes [1,3], Marta Dias [1,2], Carla Viegas [1,2]