INSTITUTO POLITÉCNICO DE LISBOA
ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

CHARACTERIZATION OF THE AZOLE RESISTANT ISOLATES OF *ASPERGILLUS* SECTION *FUMIGATI* DISTRIBUTION - INTERNSHIP

STUDENT: Liliana Raquel Pais Marques

ADVISOR: Carla Sofia Viegas, PhD - Escola Superior de Tecnologia da Saúde de Lisboa

Master's degree in Clinical-Laboratory Technologies

Lisboa, 2024

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Resumo

Este relatório está integrado no âmbito da unidade curricular Projeto/Tese/Estágio do segundo ano, do Mestrado Tecnologias Clínico-Laboratoriais. O estágio de natureza profissional foi realizado de junho de 2023 a março de 2024, num laboratório de Microbiologia Ambiental e Ocupacional, pertencente ao Centro de Investigação em Saúde e Tecnologia (Health & Technology Research Center - H&TRC).

Durante os 10 meses, no laboratório, estiveram a decorrer 4 projetos de doutoramento, com o objetivo de avaliar a exposição ocupacional a agentes microbiológicos, nomeadamente fungos e bactérias, em diferentes ambientes (carpintarias, explorações avícolas e escolas primárias). Foram realizadas campanhas de amostragem, e as atividades de laboratório, para o tratamento de amostras, consistiu na aplicação de técnicas de microbiologia, e na utilização de métodos de biologia molecular.

Todas as atividades realizadas durante este estágio permitiram consolidar os conhecimentos adquiridos ao longo do primeiro ano curricular do Mestrado, bem como adquirir novos conhecimentos e novas competências práticas relacionadas com a área da microbiologia ambiental. Todo este conhecimento adquirido pode representar uma mais-valia para o futuro profissional.

Palavras-chave: Microbiologia, Saúde ocupacional, Exposição ocupacional

Abstract

This report is integrated within the scope of the Project/Thesis/Internship discipline of the second year of the master's degree in Clinical-Laboratory Technologies. The professional internship was carried out from June 2023 to March 2024, in an Environmental and Occupational Microbiology laboratory, belonging to the Health & Technology Research Center (H&TRC).

During the 10 months, in the laboratory, 4 doctoral projects were undergoing, with the aim of evaluating occupational exposure to microbiological agents, namely fungi and bacteria, in different environments (sawmills, poultries, and primary schools). Were performed sampling campaigns, and laboratory activities for post-sampling consisted of the application of microbiology techniques and the use of molecular biology methods.

All activities carried out during this internship allow to consolidate the knowledge acquired throughout the first year of the Master's degree, as well as acquire new knowledge and new practical skills related with environmental microbiology. All this knowledge acquired can represent added value for the future professional

Keywords: Microbiology, Occupational health, Occupational exposure

Table index

Figure Index

ATP	Adenosine Triphosphate
ARG	Antibiotic resistance genes
AMR	Antimicrobial Resistance
A. fumigatus	*Aspergillus fumigatus*
B	Bathroom
BZ	Bench zone
Ca	Cantinee
C	Classroom
CO_2	Carbon dioxide
CO	Carbon monoxide
CFU	Colony-Forming Units
DG18	Dichloran Glycerol Agar
dPCR	digital Polymerase Chain Reaction
DMSO	Dymethyl sulfoxide
E.coli	*Escherichia coli*
E	Exterior
EDC	Electrostactic Dust Cloth
EDCT	EDC on the t-shirt
G	Gymansium
H&TRC	Health & Technology Research Center
ICH	InChildHealth
IAQ	Indoor air quality
SHO 24	International Symposium Occupational Safety and Hygiene
IA	Invasive aspergillosis
ITZ	Itraconazole
L	Library
MAC	MacConkey agar
MZ	Machine zone
MEA	Malt Extract Agar
MM	Mastermix

MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MPG	Mechanical protective gloves
MRSA	Methicillin-Resistance *Staphylococcus aureus*
O	Office
OD	Optical Density
PM	Particulate matter
PBS	Phosphate Buffered Saline
POZ	Posaconazole
PF	Primer forward
PR	Primer reverse
P	Probe
qPCR	Real-Time Polymerase Chain Reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RT	Room temperature
SDA	Saboraud Dextrose Agar
S. aureus	*Staphylococcus aureus*
TSA	Tryptic Soy Agar
VRBA	Violet Red Bile Agar
VOZ	Voriconazole
W	Warehouse
WWTPs	Waste Water Treatment Plants

1. Introduction

This report is integrated within the scope of the second curricular year of the master's degree in Clinical-Laboratory Technologies in Escola Superior de Tecnologia da Saúde de Lisboa (ESTeSL) from Instituto Politécnico de Lisboa.

1.1. Internship objectives

According to the academic path, it is justified to undertake a professional internship, that allows to acquire practical and theoretical skills in environmental and public health.

The internship took place from June 2023 to March 2024 (as shown in Table 2 on Appendix 1), a total of 601 hours in an environmental/public health laboratory dedicated to environmental and occupational microbiology located at the Escola Superior de Tecnologia da Saúde de Lisboa. The supervisor responsible for this internship was Professor Carla Viegas.

The proposed objectives for carrying out this internship were:

- Carrying out studies in different occupational environments (sawmills, primary schools, and poultries) to characterize the distribution of azole-resistant *Aspergillus* section *Fumigati* isolates.

- Consolidate practice with the theory learned in the master's theoretical classes, such as, for example, in microbiology, the use of culture methods applied to public health, such as inoculation in different culture media (culturomics) of samples collected in primary schools, poultries and sawmills (settled dust, filters, swabs, etc.).

- Apply molecular biology techniques, such as PCR (targeting specific indicators of harmful fungal contamination).

1.2. Characterization of the internship location

The professional internship took place in an Environmental and Occupational Microbiology Laboratory from the Health & Technology Research Center (H&TRC) integrated in ESTeSL and followed 4 research works, done by 4 doctoral students (Bianca Gomes (BG), Marta Dias (MD), Pedro Pena (PP), and Renata Cervantes (RC)), that were undergoing during the internship period. All the doctoral students were supervised by Professor Carla Viegas.

As mentioned, there were 4 research projects ongoing during the internship period. Although all projects have the same objective, which resumes fungal and bacterial quantification and fungal characterization, they all focus on different occupational environments (sawmills, primary schools, and poultries), which will be described below. The **Figure 12** (on Appendix 2) shows a graphical summary of what was done in all projects, for better understanding.

All projects adopted a seasonal approach to know the influence of different temperatures and what humidity does on exposure to microorganisms.

Except for poultries, where the sampling campaign occurred in Madeira Island, all the sampling locations (in sawmills and in primary schools) were performed in the Lisbon metropolitan area.

2.1. Guidance for Microbial Occupational Exposure Assessment in Sawmills (MD)

This project proposed to assess and characterize the exposure of sawmill workers to microorganisms, such as fungi and bacteria, and their metabolites (endotoxins, cytotoxins, and mycotoxins), and the exposure to particulate matter.

a) Sampling sites

In sawmills, the sampling occurred in the next locations: the bench zone (BZ), machine zone (MZ), office (O), warehouse (W), and exterior (E).

b) Sampling methods

In every of the chosen locations, the following processes were completed: Lighthouse Handled Particle Counter, Coriolis µ air sampler, and MAS-100 air sampler with four distinct culture mediums (Tryptic Soy Agar (TSA), Violet Red Bile Agar (VRBA), Malt Extract Agar (MEA), and Dichloran Glycerol Agar (DG18)). In BZ, MZ, O, and W were performed both active and passive methods: floor surface swabs, Electrostatic Dust Cloths (EDCs) (left for 30 days on each location), and settled dust were collected. Andersen Six-Stage was performed with 3 different culture media (TSA, VRBA, and DG18 in duplicated).

In two sawmill workers, one from BZ area and the other from MZ area, was attached an EDC on the t-shirt (EDCT) near the breathing area, and in the same area, the Button personal air sampler was placed.

After finishing the sampling, the samples were accommodated in sterile bags and

transported to the laboratory between 0-4°C.

The following work after the sampling is the same in all settings and will be described in sections **3.2.2.** and **3.2.3.**

2.2. Identifying Determinants for Indoor Air Quality and their Health Impact in Environments for Children: Measures to Improve Indoor Air Quality and Reduce Disease Burdens (PP and RC)

The InChildHealth (ICH) project is integrated on HORIZON-HLTH-2021-ENVHLTH-02-02: Indoor air quality (IAQ) and health. This project proposes to determine the elements that affect indoor air quality and how they affect school-age children's health, including chemicals, particle concentrations, microorganisms, and physical properties.

a) Sampling sites

In primary schools, the sampling procedure was like the sawmills. Library (L), classrooms (C), cantinee (Ca), bathroom (B), gymnasium (G), and exterior were the chosen areas to perform the sampling.

b) Sampling methods

In all the locations, MAS-100 air sampler (with TSA, VRBA, MEA, and DG18), Lighthouse Handled Particle Counter, and Coriolis µ air sampler were used. Floor, door, and table swabs were done, was collected settled dust, Andersen Six-Stage with 3 different culture media (TSA, VRBA, and DG18 in duplicated) was performed, and an EDCs booklet (three 10 cm^2 pieces of EDC in a two paper sheet folded – **Figure 6**) was placed strategically in each location, at the height of 1.5-2.5 meters, and left for 30 days. An EDCT was put in two students per classroom, 2 Button personal air samplers were put in a teacher (in one of the classrooms sampled), and to a school auxiliary. EDCTs were collected at the end of the class, and the personal air sampler was performed for two hours. The samples were conditioned in the same way as the samples of sawmills.

c) Other assays

Additionally, in the primary schools, handle and table swabs were done for Methicillin-Resistance *Staphylococcus aureus* (MRSA) assessment, and the measurement of Adenosine Triphosphate (ATP) to assess the microbiological

contamination of cleaning practices, as mentioned in **sections 3.2.5.1**, and **3.2.5.2**, respectively.

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

This project aims to describe the potential health risks of poultry farm employees' microbial exposure, the impact on animal productivity, and the indirect environmental effects of Portuguese poultry pavilions, and if has a relationship with the different materials used in animal bedding.

As the sampling occurred outside of Portugal continental, it was only possible to participate in post-sampling procedures of samples from passive methods.

a) Other assays

Only this project assessed *Escherichia coli* (*E.coli*) and the procedure will be described in point **3.2.5.3**.

3. Background

3.1. Microbiologic agents

In the scenario of occupational exposure to microorganisms, it is important to know the microbial composition of the environment to enable taking precautions towards workers and other people who could happen to be in these environments (1). Among these microorganisms, bacteria, and fungi are the most present in different settings, such as healthcare facilities, schools, wood work or agricultural environments (2).

During the internship period, total and gram-negative bacteria were only quantified. However, *Escherichia coli* and Methicillin-Resistant *Staphylococcus aureus* were assessed in two of the current projects. *E.coli* was assessed in poultries and MRSA in primary schools.

In all projects, besides macro and microscopic identification of fungi, the major objective was to characterize the azole resistance of *Aspergillus* spp.

3.1.1. Escherichia coli

Escherichia coli is a gram-negative bacteria and belongs to the *Enterobacteriaceae* family. It can be found in the lower intestine tract of humans and animals, and it is the most common bacteria associated with opportunistic infections (3). *E.coli* can be discharged into the environment through wastewater or faeces (4,5), so it can be used as an indicator of faecal pollution (4).

Escherichia coli has previously been linked to poultry workers in the poultry industry, as demonstrated by a study (6). Animal production uses a lot of antimicrobial medicines, however, they are frequently administered inappropriately or at considerably larger doses than what is advised. (7). These behaviors, among others, tend to cause Antimicrobial Resistance (AMR) in meat-producing workers, customers, and the animals in question. AMR is seen as a serious problem in the One-Health concept and one of the main threats to food safety (7,8).

In the poultries project, *E.coli* was assessed, as well characterization of some antibiotic resistance genes (ARGs).

3.1.2. Methicillin-Resistant *Staphylococcus aureus*

Gram-positive *Staphylococcus aureus* (*S. aureus*) is a common bacteria found in healthy people's skin, skin membranes, and mucous membranes. It is one of the most prevalent infections in people and is also the source of infections acquired in hospitals and in the community (9,10). These bacteria can cause skin, bloodstream, and lower respiratory tract infections, among other organs (11).

However, compared to *S. aureus* infections, MRSA infections are more fatal and are strongly associated with hospital infections. This is concerning because MRSA infections not only are they nosocomial infections, they also can be acquired in the community (12). Portugal is considered the country with the highest prevalence of MRSA infection (11,13). Furthermore, it is critical to investigate the areas that raise the greatest concerns because MRSA can withstand environmental conditions for extended periods of time.

In InChilHealth project, MRSA was assessed on the contact surfaces, such as desks or handle doors.

3.1.3. *Aspergillus* spp.

Aspergillus species are the cause of the most common mold infections. *Aspergillus* spp. are saprophytic molds found worldwide, and typically they inhabit the environment, in soil and decaying organic matter. Infections by these species occur through the inhalation of airborne conidia and can cause invasive aspergillosis (IA), a life-threatening infection, that affects mostly the respiratory tract system (14,15).

The most frequent species of *Aspergillus* are the *Fumigati* section (the number one cause of IA), and the *Flavi* section (16). *Aspergillus* species can produce (under different environments, and up to 60°C) toxigenic metabolites (referred to as mycotoxins) that can be harmful to humans, and cause diverse health effects (17). The severity of these effects will depend on the amount of time of exposure to the mycotoxins, the quantity of the inhaled mycotoxin, and of the health conditions of the individuals. In immunocompromised individuals, *Aspergillus* section *Fumigati* infection represents an increase in morbidity and mortality, mostly due to delayed diagnosis and, consequently, delayed initiation of antifungal therapy (18).

The first line of therapy for aspergillosis is triazoles. The triazoles are composed of itraconazole (ITZ), voriconazole (VOZ), and posaconazole (POZ) (14). ITZ is used for chronic and non-invasive aspergillosis, VOZ is the first-line therapy against IA, and POZ has been shown to reduce invasive fungal infections (14,19).

3.1.3.1. Azole resistance

Azoles are antifungals that are used in a range of fields, including agriculture, wood working, and, as already mentioned, therapy against aspergillosis (14,20). However, with the extended use in a lot of fields, it is becoming a concern due to the ineffectiveness of azole therapy, i.e., the growing azole resistance (21).

Azole resistance occurs due to mutations in the *cyp*51A gene. These mutations will modify the structure of the CYP51A enzyme, causing alteration to azole affinity, and leading to azole resistance (22,23).

Since exposure to bacteria and fungi can result in illnesses, allergic reactions, or long-term health issues, it is important to assess the microbiological composition of the air in each location and implement measures to guarantee improved quality for the workers and people involved (24).

3.2. Exposure assessment

Occupational environments are prone to dust formation, resuspension of dust, and oscillations in temperature and humidity. These conditions facilitate exposure to fungi and to their metabolites, and also can potentially enhance their exposure (16). Since these environments represent an increased risk factor of exposure to work, it is important to evaluate these factors.

In the internship period, in all four projects, active and passive sampling techniques were employed, together with post-sampling laboratory procedures to enable microbiological characterization. It also studied the differences that temperature and humidity have on the exposure to fungi, so seasonal sampling was implemented in all three projects.

In this section sampling methods and post-sampling laboratory procedures will be discussed.

3.2.1. Sampling methods

3.2.1.1. Active sampling and particulate matter assessment

Active sampling consists of passing a predefined airflow volume through a collecting device, onto a liquid solution or solid media. As an advantage of linking the airborne concentrations with the obtained results, these approaches can characterize indoor microbial populations using culture-based methods and molecular assays,

because the volume of the air sampler is known. However, these methods can be expensive, because of the use of electric devices (25,26).

Impinger, impaction, and filtration systems were used during the sampling campaigns, and are all active sampling methods. **Coriolis µ air sampler** is an impinger method, the **Andersen six-stage air sampler** and **MAS-100 air sampler** are impaction methods, and the **Personal Button Air Sampler** is an example of a filtration system.

Particulate Matter (PM) can be present in indoor dust and can come from outdoor sources, from cleaning procedures, and can be influenced by the quality of ventilation. PM 2.5 (PM size up to 2.5 µm), due to its small size, can penetrate the lungs, causing different health conditions (27,28). So, to evaluate PM, a **Lighthouse Handled Particle Counter** can be used (29).

1) Impinger method

Coriolis µ air sampler consists of air being drawn into a liquid solution, that allows the sample to be inoculated, or to perform molecular tests (25,30).

Figure 1 - Impinger method: Coriolis µ air sampler

2) Impaction methods

MAS-100 air sampler and Andersen six-stage air sampler require an air pump that allow the airflow from the outside into a solid culture medium (25).

Figure 2 - Impaction methods: MAS-100 air sampler and Andersen six-stage air sampler, respectively.

3) Filtration method

Personal Button Air Sampler is an easy-to-use device and can use electrostatic attraction, inertial forces, or diffusion that often cause the air particles to gather onto the membrane (25,31).

Figure 3 - Filtration method: Person Button Air Sampler

4) Particulate matter

Lighthouse Handled Particle Counter is a sampling technique to measure the amount of dust that penetrates the respiratory system, as shown in a study (32) that made a positive association between particulate matter and the concentration of airborne microorganisms (25,29).

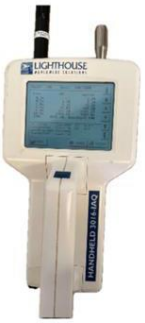

Figure 4 - Particulate matter: Lighthouse Handled Particle Counter

3.2.1.2. Passive sampling

Unlike active sampling, passive sampling can gather information on the microbial composition of the air for a longer time and evaluate possible changes over that time (25,33).

Surface swabs, Electrostatic Dust Cloths, Settled dust, and EDCs placed on a t-shirt are passive methods that were collected during the sampling campaigns.

Although not being considered passive methods, mops, and mechanical protective gloves (MPG) from primary schools and sawmills, respectively were recovered.

Passive sampling methods have more advantages than active sampling methods since they are cheaper and easier to perform than active methods. Also, as passive methods rely on gravitational force, and do not require a pump to collect samples, are more trustworthy since in active ones cell damage can occur due to high air velocity (25,30,33).

2) Surface swabs

The superficies were swabbed using a 10 × 10 cm square stencil, which was disinfected with a 70% alcohol solution between each sampling site. The swabs were kept at 4°C until arrived at the laboratory.

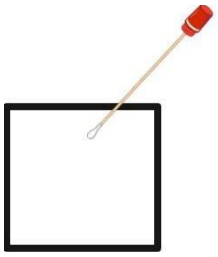

Figure 5 - Passive method: surface swabs

3) Electrostatic Dust Cloths

To collect dust, a piece of EDC (with 10 cm^2) or an EDC booklet (in the case of schools) (**Figure 6**) was placed strategically at each location on a place of difficult access, 1.5 meters above the floor, for a period of 30 days. At two people, was placed one EDCT (**Figure 7**), and it was collected at the end of the day. The pieces of EDC and EDCT were stored in a sterilized plastic bag until arrived at the laboratory.

Figure 6 - Passive method: example of an EDCT

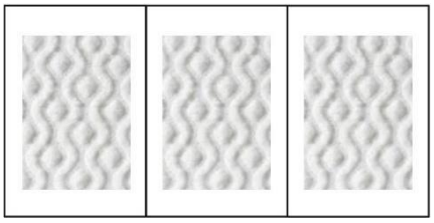

Figure 7 - Example of an EDC booklet

4) Settled Dust

Settled Dust was collected in each predefined location of each sampling setting, and it was collected with a vacuum cleaner with a sterilized coffee filter into the tube. The coffee filter with the dust was stored in a sterilized plastic bag until arrived at the laboratory.

3.2.2. Sample extraction

Passive and active samples were processed in different ways after arriving at the laboratory. The culture media resulted from active samples resulting from impaction methods were incubated for up to 5-7 days, at different temperatures (26 ± 1°C and 37 °C for fungi, 30°C for total bacteria, and 37°C for gram-negative bacteria).

All the plates resulting from the active impaction methods had two different ways to be incubated. Since the fungi prefer a humid environment for growth, the plates need to be incubated with the media lowered so that any condensation would fall on the medium. On the other hand, the media must be placed on top of the plates during incubation because the bacteria prefer dryer conditions to proliferate (34).

Active samples resulting from the impinger method, personal samples, and passive samples were processed in a similar way, which will be described below.

In this section, it is important to mention that the extracted samples used for mycotoxins assays and DNA extraction, were kept at -20°C, and the extracted samples for inoculation and endotoxin and cytotoxin assays were kept at -80°C. Note that cytotoxin, endotoxin, and mycotoxin assays are not done at the present laboratory, so the procedure will not be described.

Before starting the procedure, all the material, equipment, and work surfaces must be cleaned with 70% ethanol.

1) **Liquid samples (from Coriolis µ air sampler)**

The liquid was separated into two Eppendorf tubes, to do DNA extraction and cytotoxin assays, and the remaining liquid was divided into two Falcons tubes for mycotoxins and endotoxins assays.

2) Swabs

Each swab was cut into an Eppendorf with NaCl 0.9% + Tween 80 0.05%, agitated in the orbital shaker (*Light Duty Orbital Shaker*) at 250 rpm for 30 minutes,

and, after agitating, the swab was removed from the Eppendorf, and glycerol was added and frozen until DNA extraction.

3) EDCs, EDCTs, and Filters (from vacuumed dust)

All the samples were cut into 2 pieces. One of the half was cut again in half and put into sterile bags for mycotoxin and endotoxin assays. The other half was put in a falcon with NaCl 0.9% + Tween 80 0.05% and agitated at 250 rpm for 30 minutes. Half of the sample was "squeezed", and the remaining liquid was divided into two falcon tubes (one tube was for cytotoxic assays, and in the other glycerol was added and kept until inoculation).

The procedure of extraction of MPG from sawmills and the mops from schools was done in the same way.

4) Filters (from Button sampler)

The filter was put into a falcon tube with NaCl 0.9% + Tween 80 0.05% and agitated at 250 rpm for 60 minutes. The filter was "squeezed", and glycerol was added. The sample was kept until inoculation.

5) Settled dust

The dust was weighted into a tube. If possible, 2 g were put into two different tubes for mycotoxin and endotoxin assays. In schools, it was normal to have less than 2 g of dust, so a composite sample was made from all the sampled locations. For the remaining dust, was added NaCl 0.9% + Tween 80 0.05% to the tube, agitated at 250 rpm for 30 minutes, and half of the sample was kept for cytotoxic assays, and glycerol was added to the other half, and kept until inoculation.

3.2.3. Assays

To understand the fungi composition of the air samples these were submitted to different assays, which will be explained below.

3.2.3.1. Microbial contamination assessment

1) Inoculation

The extracted samples kept for inoculation (mentioned in **3.2.2**.) were inoculated into different media, TSA and VRBA to assess bacteria, for fungi, were inoculated onto MEA,

and DG18 (DG18 was done in duplicated), and to assess azole resistance, the samples were inoculated in Saboraud Dextrose Agar (SDA) media supplemented with Itraconazole (ITZ), Voriconazole (VOZ), and Posaconazole (POZ).

Before the inoculation, all the different media plates were identified, and the work surface was disinfected with 70% ethanol. The liquid samples were distributed among all media using a spreader. The plates were incubated as described in section **3.2.2**.

2) Macro and microscopically Identification of fungi

After the incubation time, for bacteria (TSA and VRBA), only the colonies were quantified, and for fungi (MEA, DG18, SDA, ITZ, VOZ, and POZ), the different colonies were counted and identified macroscopically using lactophenol cotton blue mount procedures, as is shown in **Figure 8**.

One little piece of the fungi was cut and sliced, with a sterilized scalpel (the scalpel was sterilized in a sterilizer between cuttings), on a slide with one drop of lactophenol cotton blue and then put on a coverslip. This procedure was done at the Laminar Flow Camera (*BioWizard Silver Line Biosafety* cabinet).

All the plates were covered with parafilm, and the excess of lactophenol was removed from the slides. After this process, the slides were observed under a microscope while the respective fungi were visualized macroscopically on the plate. All the fungi were identified, and after that, were conserved (isolates conservation, described below on point **3.2.3.2**.).

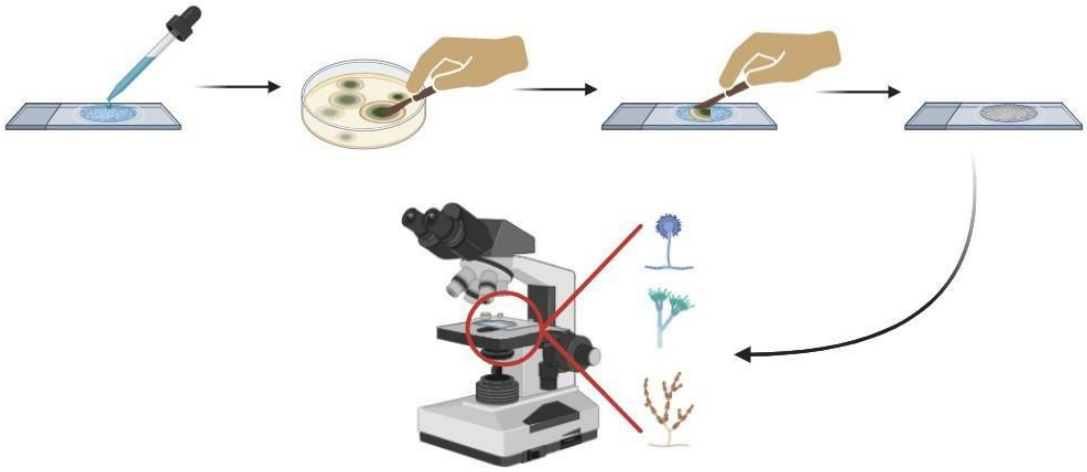

Figure 8 - Lactophenol cotton blue mount procedures, and microscopic identification of fungi.

3.2.3.2. Isolates conservation

After the identification of the fungi colonies (namely, *Aspergillus* sections *Circumdati, Flavi, Nidulantes, Fumigati,* and *Mucor* spp.), these were isolated into an Eppendorf with Phosphate Buffered Saline (PBS) solution. The piece of fungi was collected with a disposable inoculation loop and put onto the Eppendorf (35). It is important to mention that all *Aspergillus* section *Fumigati* and *Aspergillus* section *Flavi* isolates were collected in duplicate, in two different Eppendorfs.

It was added glycerol, and the samples were stored at -80°C, until further analyses.

3.2.3.3. DNA extraction

After the inoculation, the remaining sample was submitted to DNA extraction. Before starting the procedure, all the material, equipment, and work surfaces must be cleaned with 70% ethanol. The extraction procedure was adapted from the *Quick*-DNA™ Fungal/ Bacterial Microprep Kit (36):

1- The samples were defrosted.
2- Samples in Falcon tubes were centrifuged on Thermo Heraes Labofuge 400, and samples in Eppendorf were microcentrifuged on VWR® Micro Star 21/21R Microcentrifuge. After centrifuging, most of the supernatant was discarded and the pellet was kept.
3- To lysis the cell fungi, the pellet was resuspended, and the sample was put on ZR Bashing Bead Lysis tube with Bashing Bead Buffer. The tube was put on the vortex and then centrifuged (on VWR® Micro Star 21/21R Microcentrifuge).
4- Then, to remove the fungi's biggest organelles, part of the supernatant was added to a Zymo-Spin III-F Filter in a collection tube and centrifuged.
5- Then, the column was discarded, and the DNA Binding Buffer was added to the collection tube and mixed well.
6- Half of the mixture was transferred into a Zymo-Spin IC Column in a new collection tube and then centrifuged. This was done to facilitate the binding and recovery of DNA.
7- The liquid was discarded, and step 6 was repeated with the remaining mixture.
8- In a new collection tube, DNA Pre-wash Buffer was added in the same column in a collection tube and centrifuged. In this step, protein contaminants were removed.

9- The filtrated liquid was discarded, and before the DNA elution, salts and contaminants needed to be removed, so DNA Wash Buffer was added to the same column in a collection tube and centrifuged.
10- The same Zymo-Spin IC Column was transferred to an Eppendorf, and DNA elution Buffer was put into the column to purify the DNA. After 2-3 minutes, the Eppendorfs with the columns were centrifuged.
11- The eluted DNA was transferred again into the same column, and after 2-3 minutes, was centrifuged again.
12- The column was discarded, and the extracted samples were divided in three different Eppendorfs, one for Real-Time Polymerase Chain Reaction (qPCR) assays, one for fungal biomass assays (digital Polymerase Chain Reaction - dPCR), and the other for detection of toxigenic strains (Reverse Transcription Polymerase Chain Reaction - RT-PCR).
13- All the Eppendorfs were stored at -20°C until use.

3.2.3.4. qPCR for targeted specific fungal species/sections (37)

After DNA extraction, Real-Time Polymerase Chain Reaction was done to detect specific toxigenic sections of *Aspergillus*, namely sections *Circumdati, Flavi, Nidulantes,* and *Fumigati* (38).

Before starting the procedure, it was necessary to calculate the right amount of reagents (supermix, primers, and probe) to use. Table 3 (in Appendix 3) shows the calculation of reagents, template, and water for 110 samples, and for each reaction.

This procedure was done in three parts.

Before starting the procedure, all the material, equipment, and work surfaces must be cleaned with 70% ethanol.

<u>Part 1 - Preparation of primers (primer forward (PF) and primer reverse (PR)), probe (P), and mastermix (MM):</u>

i. 4 Eppendorf's were labeled with PF, PR, P and MM.
ii. On Eppendorf's primers were put each primer and water in a proportion of 1:10. For example, as shown in Table 3, for 110 samples, the amount of primer to add to the Eppendorf is 9 µL and the amount of water is 91 µL. Then, were taken 88 µL to the MM Eppendorf. This procedure was done as well with the probe.

iii. In the MM Eppendorf put the primers, the probe (as described above), supermix and the water.
iv. To homogenize the solution, with the micropipette doing “up and down”.

Part 2 – Plate preparation:

i. Set up the 96-well plate, as is shown in Table 4 (in Appendix 3).
ii. Dispense MM solution on each well (all reactions are done in duplicate).
iii. Water for negative control, positive template for positive control, and the samples were added in all respective wells.
iv. The plate was covered with an optical seal and centrifugated for 30 seconds.

Part III – Equipment preparation

i. After turning on the qPCR machine (CFX Connect Real-Time PCR Detection System), the place was put on.
ii. The settings and the cycling protocol were selected.
iii. Exported the data after the running cycle.

3.2.3.5. *Aspergillus* sp. screening of azole resistance and antifungal susceptibility testing

After the recovery of the *Aspergillus* spp. isolates, as mentioned in **3.2.3.2.** (that were confirmed by qPCR, in section **3.2.3.4**.), the antifungal susceptibility was tested (39):

1- To make pure colonies, the isolates were inoculated onto SDA culture media and incubated at 27°C for 2-5 days.
2- It was made a suspension for each isolate with NaCl 0.9% and half of the pure colony.
3- Part of the solution was transferred to a new Eppendorf.
4- To a cuvette, the solution with NaCl, in a portion of 0.9%, was added, and the Optical Density (OD) was read at 600 nm.
5- OD was adjusted to 0.6 and was prepared a new Eppendorf with adjusted OD.
6- *Aspergillus* purified colonies were inoculated into RPMI 1640 medium with 2% dextrose.

7- It was put onto the media E-test strips of ITZ, VOZ, POZ, and amphotericin (AMB), and were incubated at 35°C.
8- Minimal Inhibitory Concentration (MIC) was read at 24 and 48 hours, to know the resistance concentration of each antifungal agent.

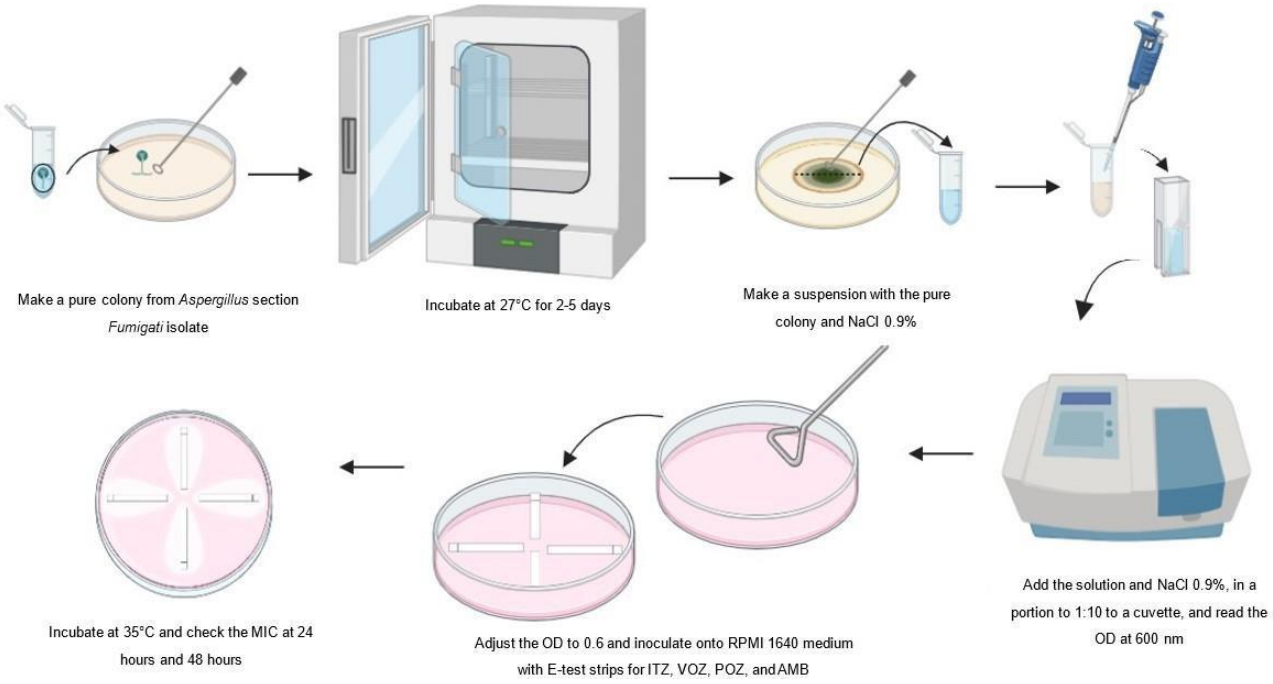

Figure 9 - Screening of *Aspergillus* section *Fumigati* isolates

3.2.4. Cytotoxin, Mycotoxin and Endotoxin assays

These studies were not performed in the present laboratory. They were carried out by partner laboratories, so the procedure will not be mentioned.

Samples extracted from EDCs (EDCTs, and EDCs from booklets), filters from Settled Dust, and the Settled Dust were kept for cytotoxicity and mycotoxins assays.

Settled Dust and Coriolis that were extracted, were kept for endotoxic assays.

3.2.5. Other assays

Here will explain how MRSA and *E.coli* were assessed, as well the ATP was measured.

3.2.5.1. MRSA assessment

For MRSA assessments were used EDCs (**Figure 6**), an EDCT (**Figure 7**), and a swab with a 10 × 10 cm square stencil, which was disinfected with a 70% alcohol solution between each sampling and was used Stuart transport media. The table and the handles of the door were the places that were swabbed because MRSA is transmitted through direct contact (11), so it is convenient to only swab in the places that are most touched by hands. All the sampling and extraction of the samples were done as mentioned above in sections **3.2.** and **3.2.2.1.**, respectively.

The extracted samples were inoculated onto CHROMagar MRSA and incubated at 37°C, and the colonies were observed at 24 and 48 hours. The colonies that grow with pink/red color are considered MRSA suspicious colonies. The suspicious colonies from EDC and EDCT samples were suspended in Brain Health Infusion Broth (BHI) and incubated at 37°C for 24 hours. After the incubation, an aliquot was inoculated in CHROMagar MRSA and again, incubated at 37°C, and the colonies were observed at 24 and 48 hours. Colonies with pink/red color were considered suspicious of being MRSA.

The isolates were conserved as shown in section **3.2.3.2**. and kept for further analyses.

3.2.5.2. ATP measurements for assessing cleaning effectiveness

To assess the cleaning effectiveness of superficies, such as classroom desks or door handles of bathrooms, the levels of ATP were measured. ATP has been used as a marker for the detection and quantification of bacterial and fungal contamination on surfaces, so the aim is that the levels of ATP are lower after the cleaning of the surfaces, which indicates lower microbial contamination (40,41).

An ATP swab was done on the surface before and after the cleaning. Before the cleaning process, with a 10 × 10 cm square stencil disinfected with a 70% alcohol solution. It was collected 8 samples per surface, each sample in different locations on the surface. After the school cleaning process, with a disinfected square stencil, 6 more samples were collected, on the same surface.

After collecting each sample, the ATP swabs were read with the Clean-Trace 3M (St. Paul, Minn.), and the data of the readings were analyzed to assess ATP levels by comparing the ATP measurements with the ATP relative light unit (RLU) range values established in standards that apply to the surface being tested, where high levels of RLUs are indicative of higher contamination (41).

3.2.5.3. *Escherichia coli* assessment

Escherichia coli identification starts with quantifying bacteria, colony-forming units (CFU), and CFU concentration ($CFU.m^{-3}/m^{-2}/m^{-2}.day/g^{-1}$). Gram-negative *E. coli* is genetically diverse and causes opportunistic human infections (3).

After being quantified, *E.coli* suspicious colonies (dark pinkish color and regular form colonies) were inoculated in MacConkey agar (MAC). After incubating for 24 hours at 37°C, the suspicious colonies were reisolated into an Eppendorf with 1 mL of PBS.

The following tasks were not in the internship period, but the isolates will be confirmed by a biochemical panel for identification and differentiation of members of the family Enterobacteriaceae (Analytical Profile Index 20E – bioMérieux API20E), and will be done a DNA extraction and *E.coli* antibiotic resistance genes characterization. It will use qRT-PCR to quantify the next priority ARGs: *blaNDM, blaKPC, blaOXA-48, blaIMP*, blaVIM, *mcr* and *armA.*

3.3. Citizen Science

Through a variety of activities that involve sampling as well as sample extraction, and even completing a walkthrough regarding indoor air quality - such as the number of windows, the type of furniture, or the number of students per room - citizen science projects allow students and school staff to actively participate in the InChildHealth project.

By doing these activities, the students, and school staff, can gain an understanding of the effects that air quality has on health, and how the work is done by researchers to analyze the microbiological particles in the air.

In ICH project, it was possible to help the students observe microscopically the most prevalent fungi in schools, help them to extract samples, like EDCs, and inoculate swabs onto culture media.

During the internship period, other than the projects already mentioned, it was possible to participate in other different projects, like the citizen and science campaigns in primary schools, from InChilHealth project, doing a scope review about Waste Water Treatment Plants (WWTPs), present a poster at International Symposium Occupational Safety and Hygiene (SHO 24) at Porto city, and participating on an evaluation of the indoor air quality of a nursing home, collecting samples to assess indoor air quality and search and control of *Legionella* spp..

4.1. Filling the knowledge gap regarding microbial occupational exposure assessment in Waste Water treatment plants – A scoping review (4)

This review adopted the Preferred Reporting Items for Systematic Review (PRISMA) checklist. It was intended to provide an overview of the assays and sample techniques used in WWTPs to evaluate worker exposure to microbiologic substances.

The acquired data may help uncover gaps in the knowledge regarding viral exposure at work or possibly help define future criteria and recommendations to guarantee a trustworthy microbiological characterization.

The full paper is available in Appendix 4.

4.2. Indoor Air Quality Evaluation

This evaluation had the objective of assessing the state and conformity of the nursing home installations regarding comfort, physical-chemical, and microbiologic parameters in terms of IAQ.

To assess comfort parameters, temperature, and relative humidity were measured, particulate matter ($PM_{2.5}$ and PM_{10}), carbon dioxide (CO_2), carbon monoxide (CO) and Total Volatile Organic Compounds to assess physical-chemical parameters, and bacteria and fungi to assess microbiologic parameters.

The sampling locations were chosen considering the priority of monitoring (rooms, living rooms, cantinee, secretary, office, gymnasium, and nursery). For *Legionella* spp. the sampling occurred in a shower of two rooms, and in shower of a locker room.

To study the microbiologic parameters, MAS-100 air sampler with solid culture media (TSA and VRBA for quantifying bacteria, and MEA and DG18 for fungi) was done, and

floor swabs with a 10 × 10 cm square stencil, which was disinfected with a 70% alcohol solution between each sampling.

For physical-chemical parameters was used Lighthouse Handled Particle Counter, and IAQ Wolfsense for CO and CO_2. IAQ Wolfsense was also used to measure comfort parameters.

To search for *Legionella* spp., it was collected water to sterilize water bottles (specific to Legionella). First, tap water was collected, then in a second bottle, was collected warm water. Additionally, the shower head fitting was swabbed.

The post-sampling laboratory work took place at the same laboratory of the internship (Environmental and Occupational Microbiology Laboratory from H&TRC, integrated in ESTeSL), and the methodology was done as shown in sections **3.2.2.** and **3.2.3**.

The report is available in Appendix 5.

4.3. Poster presentation at SHO 24

During this internship, it was proposed to create and present a poster entitled *Fungal Contamination in Lisbon's Primary Schools - Sampling Insights and Analytical* (Poster 1 from **Figure 13** on Appendix 6). Poster 1 was presented at SHO 24, carried out in Porto city. This poster aimed to present sampling methodologies applied in primary schools, located in the metropolitan area of Lisbon, as well as presenting analytical methods to assess fungal contamination in these settings.

4.4. Submitted work

During the internship, it was also possible to participate in some works that were submitted, such as Poster 2 (**Figure 14** on Appendix 7) and Poster 3 (**Figure 15** on Appendix 8), which were also presented at SHO 24, in Poster 4 (**Figure 16** on Appendix 9), that was presented at the 14th International Conference “Moulds and Mycotoxins”, in Bydgoszcz, Poland.

To Epidemiology in Occupational Health (EPICOH) 2024, with the title “**Mitigating Health Risks in Wastewater Treatment Plants: Identifying Key Microbial Contaminants and Protocols Needs**”, done by RC.

It was also possible to participate in “**The Power of Citizen and Science: Insights and Achievements from the InChildHealth Project**” by RC, and in “**Levels of fungi in**

the air of poultry farms following different stages of birds´ growth cycle", done and presented by BG.

5. Other laboratory activities

5.1. Preparation of sampling campaigns

Before the sampling campaign, it was important to ensure all the equipment's battery powers were fully charged (MAS-100 air sampler, Coriolis µ air sampler, Button personal air sampler, and the Lighthouse Handheld Particle Counter), and, a checklist was made with the aim of not missing anything before going to the campaign, like PBS solution for the Coriolis µ cups, sterilized water to wet the swabs (before swabbing the surfaces), and enough EDCs (to leave in all destined places and in the t-shirts of workers (in sawmills) and students (primary schools).

5.2. Culture media preparation

To prepare the culture media, all the quantities measured depended on the manufacturer's instructions, so in some culture media, the measurements may vary depending on the supplier, but for each media, it was always the same reagents. Were prepared media to quantify bacteria, fungi, and azole spiked media.

All the prepared media (TSA, VRBA, MAC, MEA, DG18, and SDA) were sealed with gauze and autoclave indicator tape. Then, the sealed Erlenmeyers were autoclaved at 120°C for 15 minutes. VRBA media was an exception, and it depended on the manufacturer's instructions. Some of them could be autoclaved, and others not. In these cases, the VRBA-prepared media was done on a heating plate until it started to bubble and was ready to pour on the plates.

a) Media to quantify bacteria

TSA and VRBA were used to quantify total bacteria and gram-negative bacteria, respectively. For both, TSA and VRBA, it was measured distilled water, and it was weighed TSA powder, and VRBA powder, respectively.

MacConkey Agar was used in two projects, one only quantified gram-negative bacteria, and the other quantified gram-negative bacteria and qualified possible *Escherichia coli* bacteria (42). For this media, only MAC powder and distilled water were needed.

b) Media to quantify and qualify fungi

To quantify and qualify fungi, it was used two different media, MEA a more generalist media, and DG18, which is more selective for fungi. For MEA media, it was needed distilled water, chloramphenicol (0.01%) diluted in ethanol 96% and glycerol. For DG18 media, DG18 powder was weighed, and glycerol was added to an Erlenmeyer with the desired distilled water.

c) Azole spiked media

Azole spiked media was used to quantify and qualify the azole resistance of fungi. Each azole solution (as described below, in section **5.3.**) was added to SDA media. To make SDA media were only needed distilled water and SDA powder.

After being autoclaved, azole solution (ITZ, VOZ, and POZ) was added to SDA liquid media. But in this case, the temperature of these had to be at around 37°C before putting the azole solutions in, because the azole solution could lose the active ingredient if the temperature was superior. If the temperature was inferior, the media started solidifying, and could not be put on the plates.

5.3. Azole solution preparation

For making the azole spiked media, the azole solution was added to the SDA media. The powders of ITZ were kept at 4°C, VOZ at room temperature, and POZ at -20°C.

The azoles were weighed, and then Dymethyl sulfoxide (DMSO) was added to each azole, in a sterilized falcon.

All the azole solutions were kept at -80°C until use, and valid for up to 6 months.

5.4. Solutions preparation

For the sample extraction, it was used NaCl 0.9% + Tween 80 0.05%. Distilled water, NaCl, and Tween 80 were needed to prepare the extraction solution.

Phosphate-buffered saline was used for the isolation of fungi colonies, and only PBS solution and distilled water were needed.

Both, PBS and NaCl 0.9% + Tween 80 0.05% were autoclaved at 120°C for 15 minutes and then kept at room temperature until use.

5.5. Sterilization of material supplies

Before the sampling, EDCs are sterilized at a Laminar Flow Camera (*BioWizard Silver Line Biosafety cabinet)* with UV lights for 30 minutes. Also, the filters for collecting settled dust (coffee filters), the filters for the person button sampler, and his attachments were sterilized with UV light.

The needed lab supplies, like Eppendorf's or unfiltered pipet tips, were prepared and autoclaved for 15 minutes at 120°C.

6. Timeline of Activities

Table 1 - Timeline of activities done during the internship period (from June 2023 to March 2024).

		2023							2024		
Project	**Task**	**June**	**July**	**August**	**September**	**October**	**November**	**December**	**January**	**February**	**March**
Guidance for Microbial Occupational Exposure Assessment in Sawmills	Sampling										
	Assays										
Identifying Determinants for Indoor Air Quality and their Health Impact in Environments for Children: Measures to Improve Indoor Air Quality and Reduce Disease Burdens	Sampling										
	Assays										
	Citizen and Science										
The Impact of Animals Bedding Material on the Sustainability of an Industrial Portuguese Poultry Farm through a One Health Perspective	Assays										

7. Discussion

The main objective of the projects ongoing during the internship period was to characterize the resistance of *Aspergillus* spp. to azole antifungals.

Aspergillus spp., namely, *Aspergillus fumigatus (A. fumigatus)* is one of the most common fungi in nature and can cause different health conditions, and compromise immunocompromised individuals, and its presence both, indoors and outdoors, may pose a significant risk to public health (43,44).

Continuous exposure to *A. fumigatus* in indoor environments can pose a health risk and can develop severe aspergillosis, which can be treated with azole antifungal (44). However, the emergence of azole resistance of *A. fumigatus* is leading to higher mortality and morbidity rates (45). So, it is crucial to understand how exposure to these microorganisms occurs, in a way to define and implement standardized protocols for effective risk assessments.

During the internship, air sampling methods were used to evaluate indoor air quality, namely exposure to fungi, in occupational environments. In sampling campaigns, active and passive approaches, and particulate matter were performed. The active methods are based on a pump that drowns the air into a solid or liquid media, and bioaerosol quantification can be calculated because the volume of sampled air is controlled. However, this method can be expensive due to the need for specialized equipment and maintenance. Passive methods use the gravitational force of microorganisms onto surfaces or collection media. This method is cheaper and easier to perform than active air sampling methods, and it is ideal for sampling for long periods, giving a broad spectrum of the microbial environment in each setting (46).

To evaluate microbial contamination in each setting, different culture media were used. The following culture media were employed to analyze bacteria: TSA for total bacteria, VRBA for gram-negative bacteria, MAC for *Escherichia coli*, and CHROMagar for Methicillin-Resistant *Staphylococcus aureus*. As may be expected, TSA media consistently had a higher bacterial load than VRBA, where most of the time there was nearly no bacterial growth. This is because practically all bacterial species can thrive in TSA medium, which is less selective than VRBA (47,48).

To evaluate fungi, only two media were used, MEA and DG18. MEA is a non-selective media, so there were, almost always higher loads of fungi, than in DG18 media, which limits the fast-growing colonies and makes easier quantification of fungi (49). DG18 was incubated at different temperatures, 27°C and 37°C, to evaluate the pathogenic potential.

Figure 10 shows the effect of the difference between the different media and different temperatures has on fungal growth.

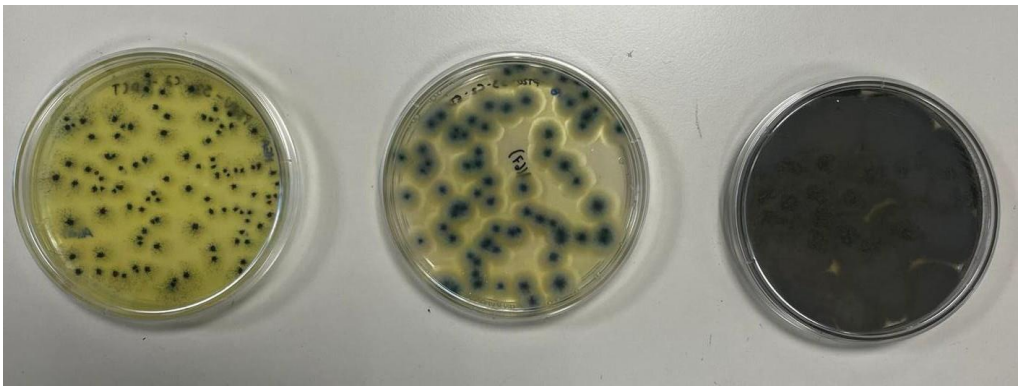

Figure 10 - From left to right, fungal growth of the same sample in MEA, DG18, and DG18 37°C

To evaluate antifungal resistance the samples were inoculated in azole-supplemented media, SDA, ITZ, VOZ, and POZ, and the isolates of *Aspergillus* section *Fumigati* that grow on these media were submitted to screening for azole resistance, measuring the Minimum Inhibitory Concentration referenced by microdilution methods (39).

Overall, the assessment of microorganism exposure in interior environments requires the selection of the most effective sampling methods (active and/or passive) and collection media for the isolation of bacteria and, particularly fungi, to assess their resistance mechanism to azole antifungals.

To reduce the risks of exposure to airborne pathogenic microorganisms that might negatively impact an individual's health, it is recommended to implement efficient sampling, culturing, and resistance monitoring protocols.

8. Conclusion

Indoor air quality is an important factor in ensuring the health and well-being of the surrounding area, which is why periodic monitoring of the biological, chemical and physical components of the environment is essential. Therefore, it is of great importance to choose good air sampling methods in occupational environments, as well as assay methods. Considering this, the area chosen to carry out the professional internship of the curricular unit Project/Thesis/Internship of the second curricular year of the Master's Degree in Clinical-Laboratory Technologies at the Escola Superior de Tecnologia da Saúde de Lisboa. The internship was carried out from June 2023 to March 2024 at the Environmental and Occupational Microbiology Laboratory of the Health and Technology Research Center (H&TRC).

During this internship period, several tasks were carried out, such as collecting air samples, in primary schools and sawmills, doing sample extraction, and carrying out different assays, such as DNA extraction, or PCR, and the interpretation of the results obtained, allowing a better understanding of the work carried out.

This internship allowed consolidate the theory learned throughout the first year of the master's degree and allowed acquire new theoretical and practical skills, thus fulfilling the objectives proposed for this internship.

All the knowledge acquired will be important for the future, especially in environmental microbiology.

9. Proposal for research work in the area

Based on the activities completed during the internship, "***Identification of Aspergillus spp. with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF)* mass spectrometry**" would be suggested as a potential research project to be implemented at the laboratory.

9.1. Introduction

The standard method for identifying *Aspergillus* spp. uses culture-based methods with molecular tools (50). These procedures involve macroscopic and microscopic identification of morphological characteristics (such as color, spores, mycelial structures, and shape of conidia) (43).

The conventional methods are laborious, but MALDI-TOF technology, which has recently been used in mycology, can identify fungi quickly and with a good price-quality ratio (51–53).

Matrix-assisted laser desorption ionization time-of-flight process is an analytical method for identifying and describing biomolecules, particularly proteins, peptides, and microorganisms. This technique combines soft ionization with mass spectrometry, to analyze the mass/charge ratio of biomolecules (54).

Comparing MALDI-TOF mass spectrometry with the standard meths for identification of fungi, MALDI-TOF is an all-around strong and adaptable technique that offers quick and precise analysis, and it is capable of detecting low concentrations of analytes and requires small amounts of sample (55).

9.2. Objective

This research work proposes a protocol to use MALDI-TOF in the identification of *Aspergillus* spp.

This proposed protocol was made after reviewing the literature on the application of MALDI-TOF on fungal identification (50,53–56).

9.3. Methodology

8.3.1. Preparation of the samples

1- Inoculate the extracted air samples in SDA media and incubate at 30°C for 2-3 days.
2- With a loop, collect part of the superior material of the fungi and put on a 1.5 µL Eppendorf, resuspended with 300 µL of distilled water and 900 µL of ethanol.
3- Vortex for 10 minutes and centrifuge at 13000 rpm/15000 rpm, for 1 minute.
4- Remove supernatant, and let the pellet dry at room temperature (RT) for 5-7 minutes.
5- Add 20 µL (or the quantity required to cover the pellet) of 70% formic acid and incubate at RT for 2-5 minutes.
6- Then, homogenize the pellet with 20 µL of acetonitrile (volume of formic acid = volume of acetonitrile).
7- Incubate at RT for 10 minutes, and centrifuge at 13000 rpm/15000 rpm, for 1 minute.
8- Deposit 1 µL of the final mixture onto the MALDI-TOF target plate.
9- Let dry, and add 1 µL of matrix solution (α-cyano-4-hydroxy-cinnamic acid matrix).
10- Before starting the MALDI-TOF analysis, let the mixture dry to allow crystal formation (crystals are essential for efficient ionization).

8.3.2. Mass spectrometer configuration and data analysis

1- Insert the MALDI-TOF target plate into the MALDI-TOF mass spectrometer.
2- Setting the configurations, such as laser parameters (e.g. wavelength and intensity), and defining the ionization and acceleration conditions.
3- Initiate the running.
4- Compare the obtained mass spectrometer with the reference databases to identify the molecules present.

9.4. Ethical issues

Ethic-related issues may not be considered because the samples that will be used come from an environmental source.

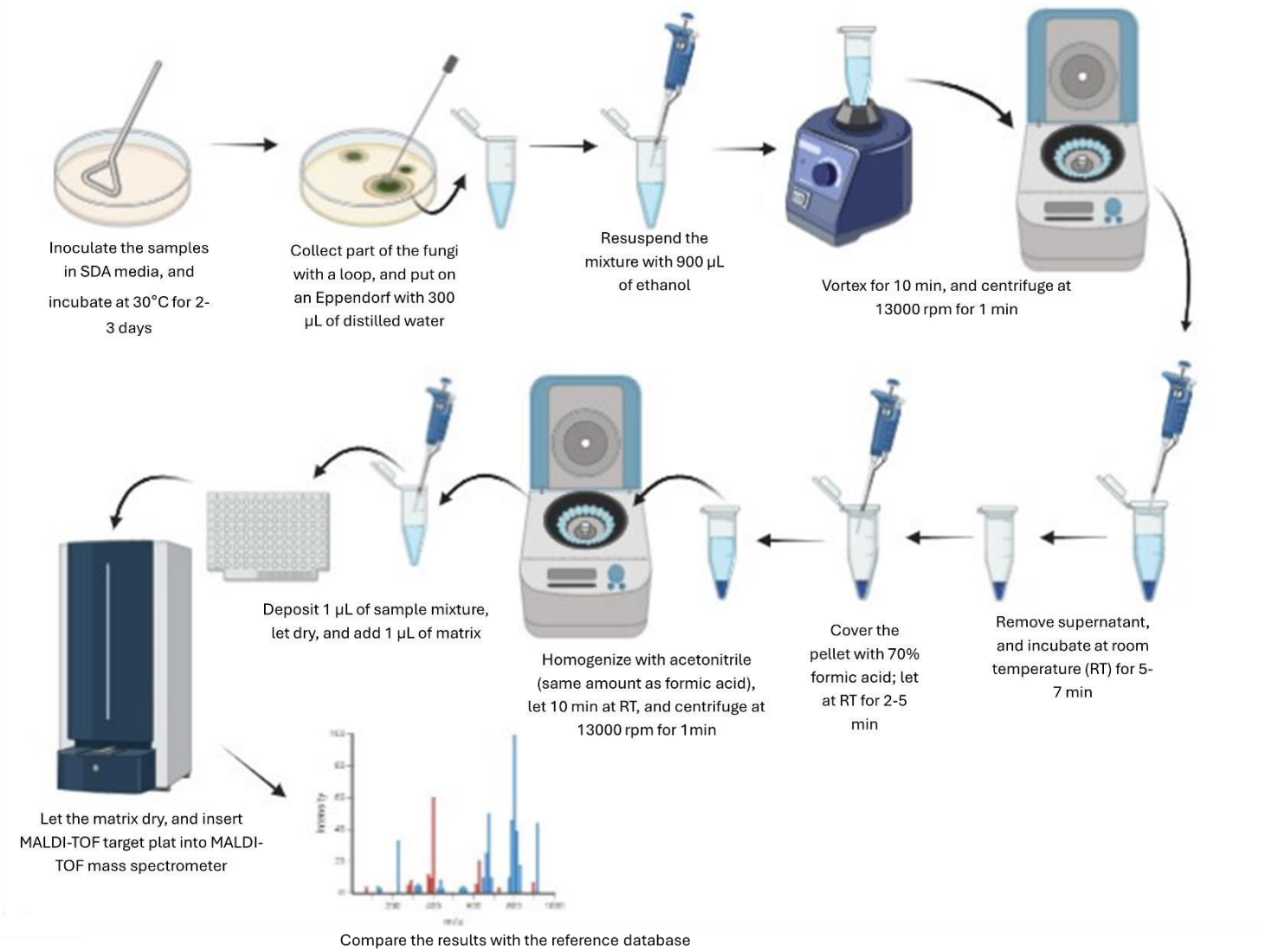

Figure 11 - Procedure for identification of fungi with MALDI-TOF MS

10. References

1. Viegas C, Cervantes R, Dias M, Gomes B, Pena P, Carolino E, et al. Unveiling the Occupational Exposure to Microbial Contamination in Conservation–Restoration Settings. Microorganisms. 2022 Aug 8;10(8):1595.

2. Smith DJ, Gold JAW, Benedict K, Wu K, Lyman M, Jordan A, et al. Public Health Research Priorities for Fungal Diseases: A Multidisciplinary Approach to Save Lives. J Fungi (Basel). 2023 Aug 3;9(8):820.

3. Mazumder R, Hussain A, Phelan JE, Campino S, Haider SMA, Mahmud A, et al. Non-lactose fermenting Escherichia coli: Following in the footsteps of lactose fermenting E. coli high-risk clones. Front Microbiol. 2022 Nov 3;13:1027494.

4. Riesenberger B, Rodriguez M, Marques L, Cervantes R, Gomes B, Dias M, et al. Filling the Knowledge Gap Regarding Microbial Occupational Exposure Assessment in Waste Water Treatment Plants: A Scoping Review. Microorganisms. 2024 Jun;12(6):1144.

5. Jang J, Hur H -G., Sadowsky MJ, Byappanahalli MN, Yan T, Ishii S. Environmental Escherichia coli: ecology and public health implications—a review. Journal of Applied Microbiology. 2017 Sep 1;123(3):570–81.

6. Aworh MK, Kwaga J, Okolocha E, Mba N, Thakur S. Prevalence and risk factors for multi-drug resistant Escherichia coli among poultry workers in the Federal Capital Territory, Abuja, Nigeria. PLOS ONE. 2019 Nov 21;14(11):e0225379.

7. Aworh MK, Kwaga JKP, Hendriksen RS, Okolocha EC, Thakur S. Genetic relatedness of multidrug resistant Escherichia coli isolated from humans, chickens and poultry environments. Antimicrob Resist Infect Control. 2021 Mar 23;10(1):58.

8. Gomes B, Dias M, Cervantes R, Pena P, Santos J, Vasconcelos Pinto M, et al. One Health Approach to Tackle Microbial Contamination on Poultries—A Systematic Review. Toxics. 2023 Apr 14;11(4):374.

9. Tong SYC, Davis JS, Eichenberger E, Holland TL, Fowler VG. Staphylococcus aureus Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. Clin Microbiol Rev. 2015 Jul;28(3):603–61.

10. Liu A, Garrett S, Hong W, Zhang J. Staphylococcus aureus Infections and Human Intestinal Microbiota [Internet]. 2024 [cited 2024 Jun 30]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC11053856/

11. Oliveira K, Viegas C, Ribeiro E. MRSA Colonization in Workers from Different Occupational Environments—A One Health Approach Perspective. Atmosphere. 2022 May;13(5):658.

12. Lakhundi S, Zhang K. Methicillin-Resistant Staphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology. Clin Microbiol Rev. 2018 Oct;31(4):e00020-18.

13. Serviço Nacional de Saúde (SNS). Programa Nacional para a Prevenção e Controlo de Infeções e de Resistência aos Antimicrobianos. 2017.

14. Rivero-Menendez O, Alastruey-Izquierdo A, Mellado E, Cuenca-Estrella M. Triazole Resistance in Aspergillus spp.: A Worldwide Problem? Journal of Fungi. 2016 Sep;2(3):21.

15. Verweij PE, Snelders E, Kema GH, Mellado E, Melchers WJ. Azole resistance in Aspergillus fumigatus: a side-effect of environmental fungicide use? The Lancet Infectious Diseases. 2009 Dec 1;9(12):789–95.

16. Viegas C, Faria T, Caetano LA, Carolino E, Gomes AQ, Viegas S. Aspergillus spp. prevalence in different Portuguese occupational environments: What is the real scenario in high load settings? Journal of Occupational and Environmental Hygiene. 2017 Oct 3;14(10):771–85.

17. Viegas C, Dias R, Gomes AQ, Meneses M, Sabino R, Viegas S. Aspergillus flavus contamination in two Portuguese wastewater treatment plants. J Toxicol Environ Health A. 2014;77(14–16):796–805.

18. Ráduly Z, Szabó L, Madar A, Pócsi I, Csernoch L. Toxicological and Medical Aspects of Aspergillus-Derived Mycotoxins Entering the Feed and Food Chain. Front Microbiol. 2020 Jan 9;10:2908.

19. Garcia-Rubio R, Cuenca-Estrella M, Mellado E. Triazole Resistance in Aspergillus Species: An Emerging Problem. Drugs. 2017 Apr 1;77(6):599–613.

20. Meneau I, Sanglard D. Azole and fungicide resistance in clinical and environmental Aspergillus fumigatus isolates. Med Mycol. 2005 Jan;43(s1):307–11.

21. Vermeulen E, Lagrou K, Verweij PE. Azole resistance in Aspergillus fumigatus: a growing public health concern. Curr Opin Infect Dis. 2013 Dec;26(6):493–500.

22. Pontes L, Beraquet CAG, Arai T, Pigolli GL, Lyra L, Watanabe A, et al. Aspergillus fumigatus Clinical Isolates Carrying CYP51A with TR34/L98H/S297T/F495I Substitutions Detected after Four-Year Retrospective Azole Resistance Screening in Brazil. Antimicrob Agents Chemother. 2020 Feb 21;64(3):e02059-19.

23. Berger S, El Chazli Y, Babu AF, Coste AT. Azole Resistance in Aspergillus fumigatus: A Consequence of Antifungal Use in Agriculture? Front Microbiol [Internet]. 2017 Jun 7 [cited 2024 Apr 29];8. Available from: https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2017.01024/full

24. Chawla H, Anand P, Garg K, Bhagat N, Varmani SG, Bansal T, et al. A comprehensive review of microbial contamination in the indoor environment: sources, sampling, health risks, and mitigation strategies. Front Public Health. 2023 Nov 23;11:1285393.

25. Whitby C, Ferguson RMW, Colbeck I, Dumbrell AJ, Nasir ZA, Marczylo E, et al. Compendium of analytical methods for sampling, characterization and quantification of bioaerosols. In: Advances in Ecological Research [Internet]. Elsevier; 2022 [cited 2024 Jan 23]. p. 101–229. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0065250422000320

26. Cox J, Mbareche H, Lindsley WG, Duchaine C. Field sampling of indoor bioaerosols. Aerosol Sci Technol. 2020;54(5):572–84.

27. Mao J, Tang Y, Wang Y, Huang J, Dong X, Chen Z, et al. Particulate Matter Capturing via Naturally Dried ZIF-8/Graphene Aerogels under Harsh Conditions. iScience. 2019 Jun 28;16:133–44.

28. Chalvatzaki E, Chatoutsidou SE, Almeida SM, Morawska L, Lazaridis M. The Representativeness of Outdoor Particulate Matter Concentrations for Estimating Personal Dose and Health Risk Assessment of School Children in Lisbon. International Journal of Environmental Research and Public Health. 2023 Jan;20(8):5564.

29. Viegas C, Faria T, Gomes AQ, Sabino R, Seco A, Viegas S. Fungal Contamination in Two Portuguese Wastewater Treatment Plants. Journal of Toxicology and Environmental Health, Part A. 2014 Feb 1;77(1–3):90–102.

30. Cervantes R, Dias M, Gomes B, Carolino E, Viegas C. Development of an Indexed Score to Identify the Most Suitable Sampling Method to Assess Occupational Exposure to Fungi. Atmosphere. 2022 Jul 15;13(7):1123.

31. Ghosh B, Lal H, Srivastava A. Review of bioaerosols in indoor environment with special reference to sampling, analysis and control mechanisms. Environment International. 2015 Dec 1;85:254–72.

32. Shintani H, Taniai E, Miki A, Kurosu S, Hayashi F. Comparison of the collecting efficiency of microbiological air samplers. J Hosp Infect. 2004 Jan;56(1):42–8.

33. Dias M, Gomes B, Cervantes R, Pena P, Viegas S, Viegas C. Microbial Occupational Exposure Assessments in Sawmills—A Review. Atmosphere. 2022 Feb 4;13(2):266.

34. Chen W, Mani S, Tang JX. An Inexpensive Imaging Platform to Record and Quantitate Bacterial Swarming. Bio Protoc. 2021 Sep 20;11(18):e4162.

35. Viegas C, Twarużek M, Almeida B, Dias M, Ribeiro E, Carolino E, et al. Cytotoxicity of Aspergillus Section Fumigati Isolated from Health Care Environments. JoF. 2021 Oct 7;7(10):839.

36. Yee DA, Tang Y. Investigating Fungal Biosynthetic Pathways Using Heterologous Gene Expression: Aspergillus nidulans as a Heterologous Host. Methods Mol Biol. 2022;2489:41–52.

37. Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J. The Ultimate qPCR Experiment: Producing Publication Quality, Reproducible Data the First Time. Trends Biotechnol. 2019 Jul;37(7):761–74.

38. Viegas C, Sousa P, Dias M, Caetano LA, Ribeiro E, Carolino E, et al. Bioburden contamination and Staphylococcus aureus colonization associated with firefighter's ambulances. Environmental Research. 2021 Jun 1;197:111125.

39. Guinea J, Verweij PE, Meletiadis J, Mouton JW, Barchiesi F, Arendrup MC, et al. How to: EUCAST recommendations on the screening procedure E.Def 10.1 for the detection of azole resistance in Aspergillus fumigatus isolates using four-well azole-containing agar plates. Clinical Microbiology and Infection. 2019 Jun 1;25(6):681–7.

40. Shaughnessy RJ, Cole EC, Moschandreas D, Haverinen-Shaughnessy U. ATP as a marker for surface contamination of biological origin in schools and as a potential

approach to the measurement of cleaning effectiveness. J Occup Environ Hyg. 2013;10(6):336–46.

41. van Arkel A, Willemsen I, Kluytmans J. The correlation between ATP measurement and microbial contamination of inanimate surfaces. Antimicrob Resist Infect Control. 2021 Aug 6;10(1):116.

42. Jung B, Hoilat GJ. MacConkey Medium. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 [cited 2024 Jun 2]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK557394/

43. Latgé JP. Aspergillus fumigatus and aspergillosis. Clin Microbiol Rev. 1999 Apr;12(2):310–50.

44. Arastehfar A, Carvalho A, Houbraken J, Lombardi L, Garcia-Rubio R, Jenks JD, et al. Aspergillus fumigatus and aspergillosis: From basics to clinics. Studies in Mycology. 2021 Sep 1;100(1):100115–100115.

45. Meis JF, Chowdhary A, Rhodes JL, Fisher MC, Verweij PE. Clinical implications of globally emerging azole resistance in Aspergillus fumigatus. Philos Trans R Soc Lond B Biol Sci. 2016 Dec 5;371(1709):20150460.

46. Rastmanesh A, Boruah JS, Lee MS, Park S. On-Site Bioaerosol Sampling and Airborne Microorganism Detection Technologies. Biosensors. 2024 Mar;14(3):122.

47. Kurm V, van der Putten WH, Hol WHG. Cultivation-success of rare soil bacteria is not influenced by incubation time and growth medium. PLoS One. 2019 Jan 10;14(1):e0210073.

48. Bonnet M, Lagier JC, Raoult D, Khelaifia S. Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical microbiology. New Microbes New Infect. 2019 Nov 30;34:100622.

49. Black WD. A comparison of several media types and basic techniques used to assess outdoor airborne fungi in Melbourne, Australia. PLoS One. 2020 Dec 18;15(12):e0238901.

50. Vidal-Acuña MR, Ruiz-Pérez de Pipaón M, Torres-Sánchez MJ, Aznar J. Identification of clinical isolates of Aspergillus, including cryptic species, by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Med Mycol. 2018 Oct 1;56(7):838–46.

51. Sanguinetti M, Posteraro B. Identification of Molds by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. J Clin Microbiol. 2017 Feb;55(2):369–79.

52. Dhiman N, Hall L, Wohlfiel SL, Buckwalter SP, Wengenack NL. Performance and cost analysis of matrix-assisted laser desorption ionization-time of flight mass spectrometry for routine identification of yeast. J Clin Microbiol. 2011 Apr;49(4):1614–6.

53. Bille E, Dauphin B, Leto J, Bougnoux ME, Beretti JL, Lotz A, et al. MALDI-TOF MS Andromas strategy for the routine identification of bacteria, mycobacteria, yeasts, *Aspergillus* spp. and positive blood cultures. Clinical Microbiology and Infection. 2012 Nov 1;18(11):1117–25.

54. De Carolis E, Posteraro B, Lass-Flörl C, Vella A, Florio AR, Torelli R, et al. Species identification of Aspergillus, Fusarium and Mucorales with direct surface analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clinical Microbiology and Infection. 2012 May 1;18(5):475–84.

55. Santos C, Paterson RRM, Venâncio A, Lima N. Filamentous fungal characterizations by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Appl Microbiol. 2010 Feb;108(2):375–85.

56. Zvezdanova ME, Arroyo MJ, Méndez G, Candela A, Mancera L, Rodríguez JG, et al. Detection of azole resistance in Aspergillus fumigatus complex isolates using MALDI-TOF mass spectrometry. Clin Microbiol Infect. 2022 Feb;28(2):260–6.

11. Appendices

11.1. Appendix 1

Table 2 - Recording the number of hours (dd/mm/yy format)

Day	Number of hours (h)	Day	Number of hours (h)	Day	Number of hours (h)
19/06/2023	4	12/10/2023	6	04/01/2024	5
20/06/2023	8	13/10/2023	3	08/01/2024	9
22/06/2023	4	16/10/2023	5	09/01/2024	7
26/06/2023	3	23/10/2023	4	11/01/2024	8
27/06/2023	7	24/10/2023	9	15/01/2024	6
28/06/2023	6	25/10/2023	6	16/01/2024	9
29/06/2023	6	26/10/2023	10	17/01/2024	8
30/06/2023	5	30/10/2023	6	18/01/2024	5
06/07/2023	5	31/10/2023	7	19/01/2024	3
11/07/2023	3	02/11/2023	6	22/01/2024	9
12/07/2023	6	06/11/2023	9	23/01/2024	7
13/07/2023	6	07/11/2023	7	24/01/2024	7
19/07/2023	7	08/11/2023	7	25/01/2024	3
20/07/2023	4	09/11/2023	6	29/01/2024	8
11/09/2023	7	10/11/2023	7	31/01/2024	7
12/09/2023	6	13/11/2023	7	01/02/2024	6
13/09/2023	5	14/11/2023	7	05/02/2024	7
14/09/2023	5	15/11/2023	5	06/02/2024	7
18/09/2023	5	16/11/2023	7	07/02/2024	8
19/09/2023	5	17/11/2023	7	08/02/2024	5
20/09/2023	6	20/11/2023	5	19/02/2024	7
21/09/2023	6	21/11/2023	9	20/02/2024	4
22/09/2023	5	22/11/2023	6	21/02/2024	4
25/09/2023	3	23/11/2023	6	26/02/2024	6
26/09/2023	6	27/11/2023	7	27/02/2024	5
27/09/2023	6	28/11/2023	10	28/02/2024	4
28/09/2023	6	29/11/2023	7	05/03/2024	4
02/10/2023	8	30/11/2023	7	06/03/2024	5
03/10/2023	7	04/12/2023	8	11/03/2024	2
04/10/2023	9	05/12/2023	7	12/03/2024	6
06/10/2023	7	07/12/2023	8	13/03/2024	4
10/10/2023	8	11/12/2023	7	14/03/2024	5
11/10/2023	4	03/01/2024			
Total	**188 hours**	**Total**	**411 hours**	**Total**	**601 hours**

11.2. Appendix 2

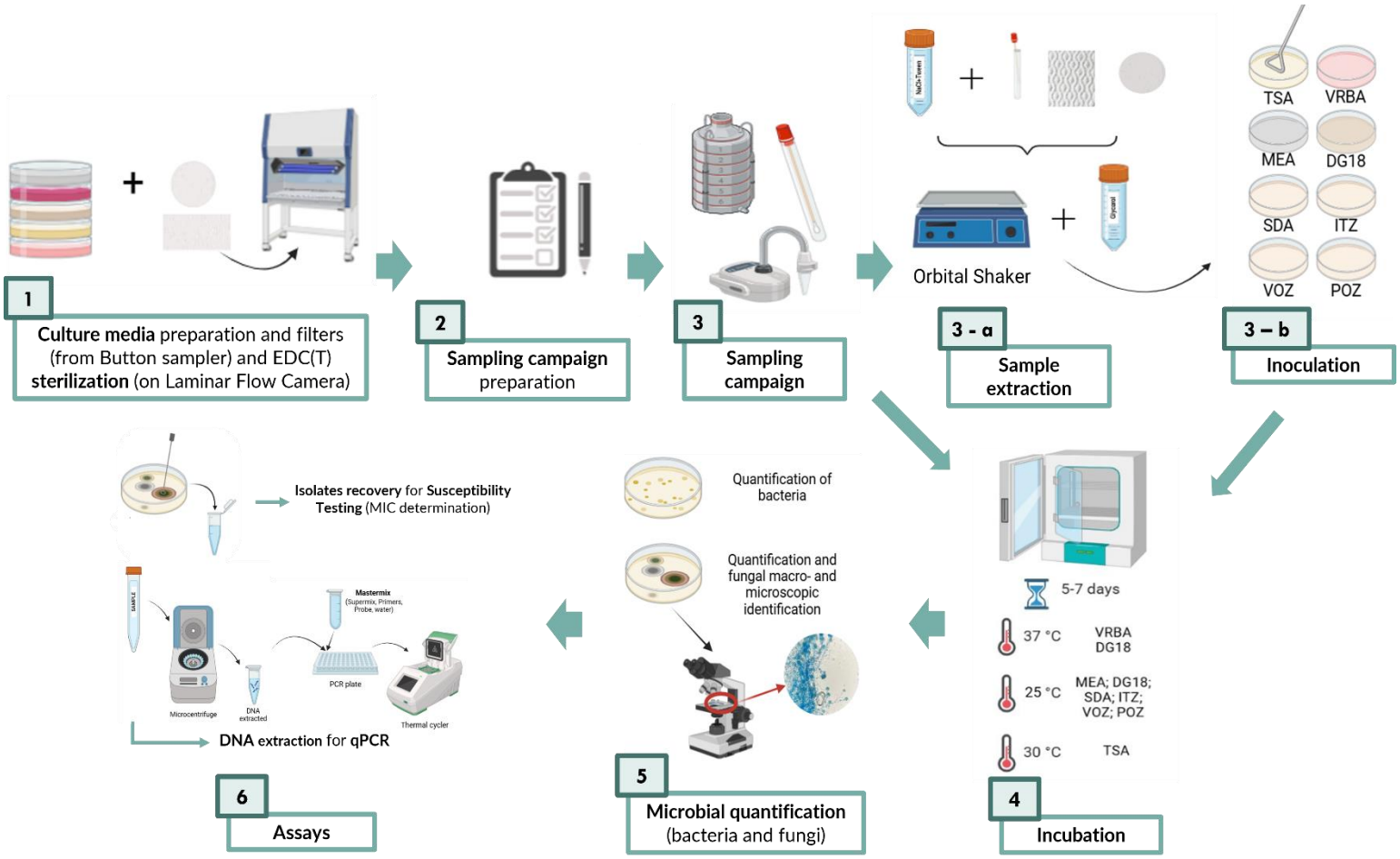

Figure 12 - Schematic representation from the sampling preparation to the assays

11.3. Appendix 3

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Table 3 - Quantities of reagents, template, and water for one reaction, and for 110 samples.

Reagent	Volume added per reaction (µL)	Volume added for 110 samples (µL)
Supermix	10	1100
Primer Forward (PF)*	0.8	88
Primer Reverse (PR)*	0.8	88
Probe (P)	0.2	22
Template	4	440
Water*	4.2	462
Total	**20**	**2200**

Table 4 - 96 well plate arrangement for 46 samples. Positive control (PC); Negative Control (NC); S1-S46 (samples).

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	NC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
B												
C	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22
D												
E	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34
F												
G	S35	S36	S37	S38	S39	S40	S41	S42	S43	S44	S45	S46
H												

11.4. Appendix 4

microorganisms

MDPI

Review

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

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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.

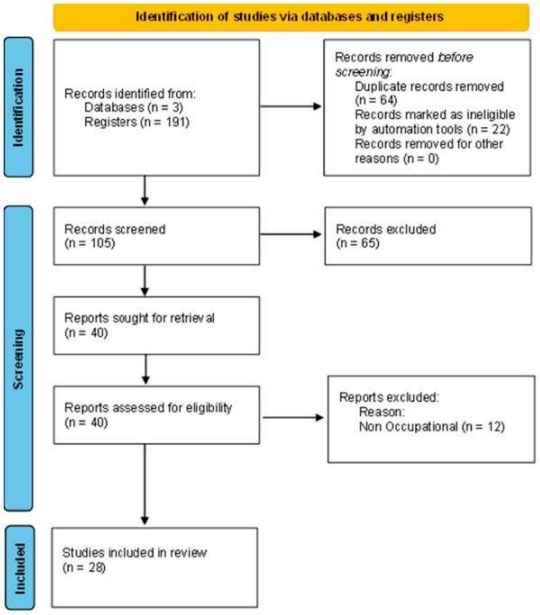

Figure 1. PRISMA methodology of selection of papers [12].

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 *Alicyclophilus* (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 deodorization 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 conventional 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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References

1. Koseoglu-Imer, D.Y.; Oral, H.V.; Coutinho Calheiros, C.S.; Krzeminski, P.; Güçlü, S.; Pereira, S.A.; Surmacz-Górska, J.; Plaza, E.; Samaras, P.; Binder, P.M.; et al. Current challenges and future perspectives for the full circular economy of water in European countries. *J. Environ. Manag.* **2023**, *345*, 118627. [CrossRef] [PubMed]
2. Ghimire, U.; Sarpong, G.; Gude, V.G. Transitioning Wastewater Treatment Plants toward Circular Economy and Energy Sustainability. *ACS Omega* **2021**, *6*, 11794–11803. [CrossRef] [PubMed]
3. Korzeniewska, E. Emission of bacteria and fungi in the air from wastewater treatment plants—A review. *Front. Biosci. Sch. Ed.* **2011**, *3*, 393–407. [CrossRef] [PubMed]
4. Li, J.; Zhou, L.; Zhang, X.; Xu, C.; Dong, L.; Yao, M. Bioaerosol emissions and detection of airborne antibiotic resistance genes from a wastewater treatment plant. *Atmos. Environ.* **2016**, *124*, 404–412. [CrossRef]
5. Li, Y.; Zhang, H.; Qiu, X.; Zhang, Y.; Wang, H. Dispersion and Risk Assessment of Bacterial Aerosols Emitted from Rotating-Brush Aerator during Summer in a Wastewater Treatment Plant of Xi'an, China. *Aerosol Air Qual. Res.* **2013**, *13*, 1807–1814. [CrossRef]
6. Talepour, N.; Hassanvand, M.S.; Abbasi-Montazeri, E.; Latifi, S.M.; Jaafarzadeh Haghighi Fard, N. Spatio-temporal variations of airborne bacteria from the municipal wastewater treatment plant: A case study in Ahvaz, Iran. *J. Environ. Health Sci. Eng.* **2020**, *18*, 423–432. [CrossRef] [PubMed]
7. Viegas, C.; Faria, T.; Gomes, A.Q.; Sabino, R.; Seco, A.; Viegas, S. Fungal Contamination in Two Portuguese Wastewater Treatment Plants. *J. Toxicol. Environ. Health A* **2014**, *77*, 90–102. [CrossRef] [PubMed]
8. Shi, B.; Zhao, R.; Su, G.; Liu, B.; Liu, W.; Xu, J.; Li, Q.; Meng, J. Metagenomic surveillance of antibiotic resistome in influent and effluent of wastewater treatment plants located on the Qinghai-Tibetan Plateau. *Sci. Total Environ.* **2023**, *870*, 162031. [CrossRef] [PubMed]
9. Gomes, B.; Dias, M.; Cervantes, R.; Pena, P.; Santos, J.; Vasconcelos Pinto, M.; Viegas, C. One Health Approach to Tackle Microbial Contamination on Poultries—A Systematic Review. *Toxics* **2023**, *11*, 374. [CrossRef]
10. Dias, M.; Gomes, B.; Cervantes, R.; Pena, P.; Viegas, S.; Viegas, C. Microbial Occupational Exposure Assessments in Sawmills—A Review. *Atmosphere* **2022**, *13*, 266. [CrossRef]
11. Daae, H.L.; Heldal, K.K.; Madsen, A.M.; Olsen, R.; Skaugset, N.P.; Graff, P. Occupational exposure during treatment of offshore drilling waste and characterization of microbiological diversity. *Sci. Total Environ.* **2019**, *681*, 533–540. [CrossRef] [PubMed]
12. Page, M.J.; McKenzie, J.E.; Bossuyt, P.M.; Boutron, I.; Hoffmann, T.C.; Mulrow, C.D.; Shamseer, L.; Tetzlaff, J.M.; Akl, E.A.; Brennan, S.E.; et al. The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *BMJ* **2021**, *372*, n71. [CrossRef]
13. Viegas, C.; Dias, R.; Gomes, A.Q.; Meneses, M.; Sabino, R.; Viegas, S. Aspergillus flavus contamination in two Portuguese wastewater treatment plants. *J. Toxicol. Environ. Health A* **2014**, *77*, 796–805. [CrossRef]
14. Viegas, C.; Faria, T.; Caetano, L.A.; Carolino, E.; Gomes, A.Q.; Viegas, S. *Aspergillus* spp. prevalence in different Portuguese occupational environments: What is the real scenario in high load settings? *J. Occup. Environ. Hyg.* **2017**, *14*, 771–785. [CrossRef] [PubMed]

15. Teixeira, J.V.; Miranda, S.; Monteiro, R.A.R.; Lopes, F.V.S.; Madureira, J.; Silva, G.V.; Pestana, N.; Pinto, E.; Vilar, V.J.P.; Boaventura, R.A.R. Assessment of indoor airborne contamination in a wastewater treatment plant. *Environ. Monit. Assess.* **2013**, *185*, 59–72. [CrossRef] [PubMed]
16. Cyprowski, M.; Stobnicka-Kupiec, A.; Ławniczek-Wałczyk, A.; Bakal-Kijek, A.; Gołofit-Szymczak, M.; Górny, R.L. Anaerobic bacteria in wastewater treatment plant. *Int. Arch. Occup. Environ. Health* **2018**, *91*, 571–579. [CrossRef]
17. Kozajda, A.; Jeżak, K. Occupational exposure to *Staphylococcus aureus* in the wastewater treatment plants environment. *Med. Pr.* **2020**, *71*, 265–278. [CrossRef]
18. Uhrbrand, K.; Schultz, A.; Madsen, A. Exposure to Airborne Noroviruses and Other Bioaerosol Components at a Wastewater Treatment Plant in Denmark. *Food Environ. Virol.* **2011**, *3*, 130–137. [CrossRef]
19. Lu, R.; Frederiksen, M.W.; Uhrbrand, K.; Li, Y.; Østergaard, C.; Madsen, A.M. Wastewater treatment plant workers' exposure and methods for risk evaluation of their exposure. *Ecotoxicol. Environ. Saf.* **2020**, *205*, 111365. [CrossRef]
20. Masclaux, F.G.; Hotz, P.; Gashi, D.; Savova-Bianchi, D.; Oppliger, A. Assessment of airborne virus contamination in wastewater treatment plants. *Environ. Res.* **2014**, *133*, 260–265. [CrossRef]
21. Haas, D.; Unteregger, M.; Habib, J.; Galler, H.; Marth, E.; Reinthaler, F.F. Exposure to Bioaerosol from Sewage Systems. *Water. Air. Soil Pollut.* **2010**, *207*, 49–56. [CrossRef]
22. Wang, Y.; Yang, L.; Wild, O.; Zhang, S.; Yang, K.; Wang, W.; Li, L. ADMS simulation and influencing factors of bioaerosol diffusion from BRT under different aeration modes in six wastewater treatment plants. *Water Res.* **2023**, *231*, 119624. [CrossRef]
23. Yang, K.; Li, L.; Wang, Y.; Xue, S.; Han, Y.; Liu, J. Emission level, particle size and exposure risks of airborne bacteria from the oxidation ditch for seven months observation. *Atmos. Pollut. Res.* **2019**, *10*, 1803–1811. [CrossRef]
24. Zhao, X.; An, D.; Liu, M.; Ma, J.; Ali, W.; Zhu, H.; Li, M.; Ai, X.; Nasir, Z.A.; Alcega, S.G.; et al. Bioaerosols emission characteristics from wastewater treatment aeration tanks and associated health risk exposure assessment during autumn and winter. *Sci. Total Environ.* **2022**, *851*, 158106. [CrossRef]
25. Han, Y.; Li, L.; Liu, J. Characterization of the airborne bacteria community at different distances from the rotating brushes in a wastewater treatment plant by 16S rRNA gene clone libraries. *J. Environ. Sci.* **2013**, *25*, 5–15. [CrossRef]
26. Wu, T.; Zou, H.; Xia, H.; Zhou, Z.; Zhao, L.; Meng, M.; Li, Q.; Guan, Y.; Li, X. Genomic insight into transmission mechanisms of carbapenem-producing *Citrobacter* spp. isolates between the WWTP and connecting rivers. *Ecotoxicol. Environ. Saf.* **2023**, *262*, 115150. [CrossRef] [PubMed]
27. Yang, K.; Li, L.; Wang, Y.; Xue, S.; Han, Y.; Liu, J. Airborne bacteria in a wastewater treatment plant: Emission characterization, source analysis and health risk assessment. *Water Res.* **2019**, *149*, 596–606. [CrossRef]
28. Xu, G.; Han, Y.; Li, L.; Liu, J. Characterization and source analysis of indoor/outdoor culturable airborne bacteria in a municipal wastewater treatment plant. *J. Environ. Sci.* **2018**, *74*, 71–78. [CrossRef] [PubMed]
29. Talepour, N.; Hassanvand, M.S.; Abbasi-Montazeri, E.; Latifi, S.M.; Jaafarzadeh Haghighi Fard, N.; Shenavar, B. Identification of airborne fungi's concentrations in indoor and outdoor air of municipal wastewater treatment plant. *Environ. Health Eng. Manag.* **2020**, *7*, 143–150. [CrossRef]
30. Niazi, S.; Hassanvand, M.S.; Mahvi, A.H.; Nabizadeh, R.; Alimohammadi, M.; Nabavi, S.; Faridi, S.; Dehghani, A.; Hoseini, M.; Moradi-Joo, M.; et al. Assessment of bioaerosol contamination (bacteria and fungi) in the largest urban wastewater treatment plant in the Middle East. *Environ. Sci. Pollut. Res.* **2015**, *22*, 16014–16021. [CrossRef]
31. Gholipour, S.; Mohammadi, F.; Nikaeen, M.; Shamsizadeh, Z.; Khazeni, A.; Sahbaei, Z.; Mousavi, S.M.; Ghobadian, M.; Mirhendi, H. COVID-19 infection risk from exposure to aerosols of wastewater treatment plants. *Chemosphere* **2021**, *273*, 129701. [CrossRef] [PubMed]
32. Moazeni, M.; Nikaeen, M.; Hadi, M.; Moghim, S.; Mouhebat, L.; Hatamzadeh, M.; Hassanzadeh, A. Estimation of health risks caused by exposure to enteroviruses from agricultural application of wastewater effluents. *Water Res.* **2017**, *125*, 104–113. [CrossRef] [PubMed]
33. Jari, H.; Maleki, A.; Dehestaniathar, S.; Mohammadi, E.; Darvishi, E.; Hedayati, M.; Marzban, N.; Van Tai, T.; Nouri, B. Airborne bacteria and fungi in a wastewater treatment plant: Type and characterization of bio-aerosols, emission characterization and mapping. *Aerobiologia* **2022**, *38*, 163–176. [CrossRef]
34. Rosenberg Goldstein, R.E.; Micallef, S.A.; Gibbs, S.G.; Davis, J.A.; He, X.; George, A.; Kleinfelter, L.M.; Schreiber, N.A.; Mukherjee, S.; Sapkota, A.; et al. Methicillin-resistant *Staphylococcus aureus* (MRSA) detected at four U.S. wastewater treatment plants. *Environ. Health Perspect.* **2012**, *120*, 1551–1558. [CrossRef] [PubMed]
35. Acer, P.T.; Kelly, L.M.; Lover, A.A.; Butler, C.S. Quantifying the Relationship between SARS-CoV-2 Wastewater Concentrations and Building-Level COVID-19 Prevalence at an Isolation Residence: A Passive Sampling Approach. *Int. J. Environ. Res. Public Health* **2022**, *19*, 11245. [CrossRef] [PubMed]
36. Ramos, G.E.; Pak, H.; Gerlich, R.; Jantrania, A.; Smith, B.L.; King, M.D. Aerosol partitioning potential of bacteria presenting antimicrobial resistance from different stages of a small decentralized septic treatment system. *Aerosol Sci. Technol.* **2023**, *57*, 517–531. [CrossRef]
37. Mbareche, H.; Dion-Dupont, V.; Veillette, M.; Brisebois, E.; Lavoie, J.; Duchaine, C. Influence of seasons and sites on bioaerosols in indoor wastewater treatment plants and proposal for air quality indicators. *J. Air Waste Manag. Assoc.* **2022**, *72*, 1000–1011. [CrossRef] [PubMed]

38. Cox, J.; Mbareche, H.; Lindsley, W.G.; Duchaine, C. Field sampling of indoor bioaerosols. *Aerosol Sci. Technol.* **2020**, *54*, 572–584. [CrossRef]
39. Oliveira, K.; Viegas, C.; Ribeiro, E. MRSA Colonization in Workers from Different Occupational Environments—A One Health Approach Perspective. *Atmosphere* **2022**, *13*, 658. [CrossRef]
40. Viegas, C.; Dias, M.; Pacífico, C.; Faria, T.; Clérigo, A.; Dias, H.; Caetano, L.; Carolino, E.; Gomes, A.; Viegas, S. Portuguese cork industry: Filling the knowledge gap regarding occupational exposure to fungi and related health effects. *Front. Public Health* **2024**, *12*, 1355094.
41. Khan, M.M.; Siddiqi, S.A.; Farooque, A.A.; Iqbal, Q.; Shahid, S.A.; Akram, M.T.; Rahman, S.; Al-Busaidi, W.; Khan, I. Towards Sustainable Application of Wastewater in Agriculture: A Review on Reusability and Risk Assessment. *Agronomy* **2022**, *12*, 1397. [CrossRef]
42. Chen, C.; He, R.; Cheng, Z.; Han, M.; Zha, Y.; Yang, P.; Yao, Q.; Zhou, H.; Zhong, C.; Ning, K. The Seasonal Dynamics and the Influence of Human Activities on Campus Outdoor Microbial Communities. *Front. Microbiol.* **2019**, *10*, 1579. [CrossRef] [PubMed]
43. Seidel, D.; Wurster, S.; Jenks, J.D.; Sati, H.; Gangneux, J.-P.; Egger, M.; Alastruey-Izquierdo, A.; Ford, N.P.; Chowdhary, A.; Sprute, R.; et al. Impact of climate change and natural disasters on fungal infections. *Lancet Microbe* **2024**. [CrossRef] [PubMed]
44. Ibáñez, A.; Garrido-Chamorro, S.; Barreiro, C. Microorganisms and Climate Change: A Not So Invisible Effect. *Microbiol. Res.* **2023**, *14*, 918–947. [CrossRef]
45. Mao, J.; Tang, Y.; Wang, Y.; Huang, J.; Dong, X.; Chen, Z.; Lai, Y. Particulate Matter Capturing via Naturally Dried ZIF-8/Graphene Aerogels under Harsh Conditions. *iScience* **2019**, *16*, 133–144. [CrossRef] [PubMed]
46. Anon International Labour Organization. *Encyclopaedia of Occupational Health and Safety*; International Labour Organization: Geneve, Switzerland, 1998.
47. Dias, M.; Viegas, C. *Fungal Prevalence on Waste Industry—Literature Review Encyclopedia of Mycology ed Ó Zaragoza and A Casadevall*; Elsevier: Oxford, UK, 2021; pp. 99–106.
48. Meadow, J.F.; Altrichter, A.E.; Kembel, S.W.; Kline, J.; Mhuireach, G.; Moriyama, M.; Northcutt, D.; O'Connor, T.K.; Womack, A.M.; Brown, G.Z.; et al. Indoor airborne bacterial communities are influenced by ventilation, occupancy, and outdoor air source. *Indoor Air* **2014**, *24*, 41–48. [CrossRef] [PubMed]
49. Emerson, J.B.; Keady, P.B.; Brewer, T.E.; Clements, N.; Morgan, E.E.; Awerbuch, J.; Miller, S.L.; Fierer, N. Impacts of flood damage on airborne bacteria and fungi in homes after the 2013 Colorado Front Range flood. *Environ. Sci. Technol.* **2015**, *49*, 2675–2684. [CrossRef]
50. Afanou, K.A.; Eduard, W.; Laier Johnsen, H.B.; Straumfors, A. Fungal Fragments and Fungal Aerosol Composition in Sawmills. *Ann. Work Expo. Health* **2018**, *62*, 559–570. [CrossRef]
51. Duchaine, C.; Mériaux, A.; Thorne, P.S.; Cormier, Y. Assessment of particulates and bioaerosols in eastern Canadian sawmills. *AIHAJ J. Sci. Occup. Environ. Health Saf.* **2000**, *61*, 727–732.
52. Gomes, B.; Pena, P.; Cervantes, R.; Dias, M.; Viegas, C. Microbial Contamination of Bedding Material: One Health in Poultry Production. *Int. J. Environ. Res. Public Health* **2022**, *19*, 16508. [CrossRef]
53. Foddai, A.C.G.; Grant, I.R. Methods for detection of viable foodborne pathogens: Current state-of-art and future prospects. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 4281–4288. [CrossRef] [PubMed]
54. Madsen, A.M.; Frederiksen, M.W.; Jacobsen, M.H.; Tendal, K. Towards a risk evaluation of workers' exposure to handborne and airborne microbial species as exemplified with waste collection workers. *Environ. Res.* **2020**, *183*, 109177. [CrossRef] [PubMed]
55. Viegas, C.; Viegas, S.; Gomes, A.; Täubel, M.; Sabino, R. *Exposure to Microbiological Agents in Indoor and Occupational Environments*; Springer: Cham, Swizerland, 2017.
56. Black, W.D. A comparison of several media types and basic techniques used to assess outdoor airborne fungi in Melbourne, Australia. *PLoS ONE* **2020**, *15*, e0238901. [CrossRef] [PubMed]
57. Viegas, C.; Dias, M.; Carolino, E.; Sabino, R. Culture media and sampling collection method for aspergillus spp. Assessment: Tackling the gap between recommendations and the scientific evidence. *Atmosphere* **2021**, *12*, 23. [CrossRef]
58. Thorne, P.S.; Kiekhaefer, M.S.; Whitten, P.; Donham, K.J. Comparison of bioaerosol sampling methods in barns housing swine. *Appl. Environ. Microbiol.* **1992**, *58*, 2543–2551. [CrossRef] [PubMed]
59. Kabir, M.S.; Hsieh, Y.-H.; Simpson, S.; Kerdahi, K.; Sulaiman, I.M. Evaluation of Two Standard and Two Chromogenic Selective Media for Optimal Growth and Enumeration of Isolates of 16 Unique Bacillus Species. *J. Food Prot.* **2017**, *80*, 952–962. [CrossRef]
60. Amrane, S.; Raoult, D.; Lagier, J.-C. Metagenomics, culturomics, and the human gut microbiota. *Expert Rev. Anti Infect. Ther.* **2018**, *16*, 373–375. [CrossRef] [PubMed]
61. Bonnet, M.; Lagier, J.C.; Raoult, D.; Khelaifia, S. Bacterial culture through selective and non-selective conditions: The evolution of culture media in clinical microbiology. *New Microbes New Infect.* **2019**, *34*, 100622. [CrossRef] [PubMed]
62. Franchitti, E.; Pascale, E.; Fea, E.; Anedda, E.; Traversi, D. Methods for Bioaerosol Characterization: Limits and Perspectives for Human Health Risk Assessment in Organic Waste Treatment. *Atmosphere* **2020**, *11*, 452. [CrossRef]
63. WHO. *WHO Updates List of Drug-Resistant Bacteria Most Threatening to Human Health*; WHO: Geneva, Switzerland, 2024.
64. EFSA Panel on Biological Hazards (BIOHAZ); Koutsoumanis, K.; Allende, A.; Álvarez-Ordóñez, A.; Bolton, D.; Bover-Cid, S.; Chemaly, M.; Davies, R.; De Cesare, A.; Herman, L.; et al. Role played by the environment in the emergence and spread of antimicrobial resistance (AMR) through the food chain. *EFSA J.* **2021**, *19*, e06651.

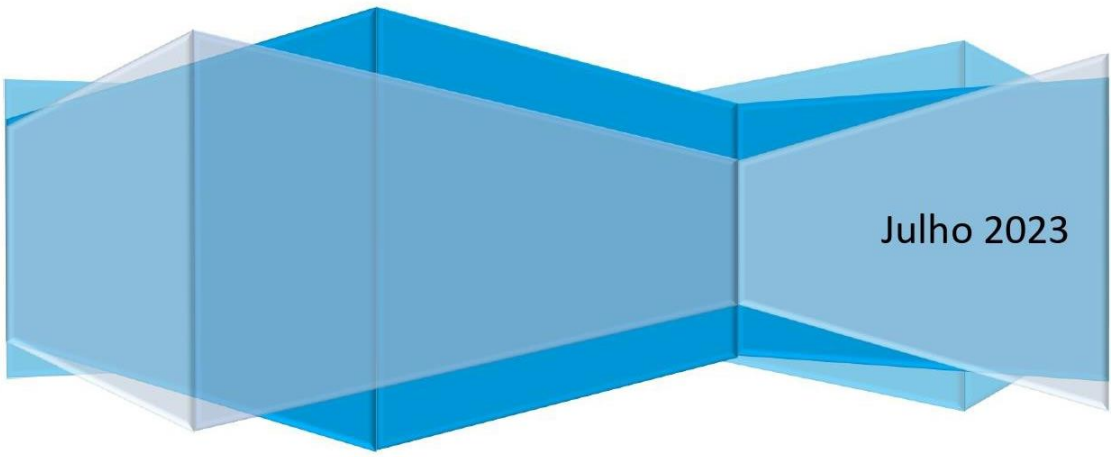
65. Yalin, D.; Craddock, H.A.; Assouline, S.; Ben Mordechay, E.; Ben-Gal, A.; Bernstein, N.; Chaudhry, R.M.; Chefetz, B.; Fatta-Kassinos, D.; Gawlik, B.M.; et al. Mitigating risks and maximizing sustainability of treated wastewater reuse for irrigation. *Water Res. X* **2023**, *21*, 100203. [CrossRef] [PubMed]
66. Yin, X.; Chen, X.; Jiang, X.-T.; Yang, Y.; Li, B.; Shum, M.H.-H.; Lam, T.T.Y.; Leung, G.M.; Rose, J.; Sanchez-Cid, C.; et al. Toward a Universal Unit for Quantification of Antibiotic Resistance Genes in Environmental Samples. *Environ. Sci. Technol.* **2023**, *57*, 9713–9721. [CrossRef]
67. Fatta-Kassinos, D.; Manaia, C.; Berendonk, T.U.; Cytryn, E.; Bayona, J.; Chefetz, B.; Slobodnik, J.; Kreuzinger, N.; Rizzo, L.; Malato, S.; et al. COST Action ES1403 New and emerging challenges and opportunities in wastewater reuse (NEREUS). *Environ. Sci. Pollut. Res. Int.* **2015**, *22*, 7183–7186. [CrossRef]
68. Ariyadasa, S.; Taylor, W.; Weaver, L.; McGill, E.; Billington, C.; Pattis, I. Nonbacterial Microflora in Wastewater Treatment Plants: An Underappreciated Potential Source of Pathogens. *Microbiol. Spectr.* **2023**, *11*, e0048123. [CrossRef]
69. Corbu, V.M.; Gheorghe-Barbu, I.; Dumbravă, A.S.; Vrâncianu, C.O.; Șesan, T.E. Current Insights in Fungal Importance—A Comprehensive Review. *Microorganisms* **2023**, *11*, 1384. [CrossRef] [PubMed]
70. Warnasuriya, S.D.; Udayanga, D.; Manamgoda, D.S.; Biles, C. Fungi as environmental bioindicators. *Sci. Total Environ.* **2023**, *892*, 164583. [CrossRef]
71. Viegas, C.; Eriksen, E.; Gomes, B.; Dias, M.; Cervantes, R.; Pena, P.; Carolino, E.; Twarużek, M.; Caetano, L.A.; Viegas, S.; et al. Comprehensive assessment of occupational exposure to microbial contamination in waste sorting facilities from Norway. *Front. Public Health* **2023**, *11*, 1297725. [CrossRef]
72. Marchand, G.; Wingert, L.; Viegas, C.; Caetano, L.; Viegas, S.; Twaruzek, M.; Lacombe, N.; Lanoie, D.; Valois, I.; Gouin, F.; et al. Assessment of waste workers occupational risk to microbial agents and cytotoxic effects of mixed contaminants present in the air of waste truck cabin and ventilation filters. *J. Air Waste Manag. Assoc.* **2024**, *74*, 145–162. [CrossRef] [PubMed]
73. Salambanga, F.R.D.; Wingert, L.; Valois, I.; Lacombe, N.; Gouin, F.; Trépanier, J.; Debia, M.; Soszczyńska, E.; Twarużek, M.; Kosicki, R.; et al. Microbial contamination and metabolite exposure assessment during waste and recyclable material collection. *Environ. Res.* **2022**, *212*, 113597. [CrossRef]
74. Viegas, C.; Pena, P.; Dias, M.; Gomes, B.; Cervantes, R.; Carolino, E.; Twarużek, M.; Soszczyńska, E.; Kosicki, R.; Caetano, L.A.; et al. Microbial contamination in waste collection: Unveiling this Portuguese occupational exposure scenario. *J. Environ. Manag.* **2022**, *314*, 115086. [CrossRef]
75. Viegas, S.; Viegas, C.; Martins, C.; Assunção, R. Occupational Exposure to Mycotoxins—Different Sampling Strategies Telling a Common Story Regarding Occupational Studies Performed in Portugal (2012–2020). *Toxins* **2020**, *12*, 513. [CrossRef] [PubMed]
76. Fisher, M.C.; Alastruey-Izquierdo, A.; Berman, J.; Bicanic, T.; Bignell, E.M.; Bowyer, P.; Bromley, M.; Brüggemann, R.; Garber, G.; Cornely, O.A.; et al. Tackling the emerging threat of antifungal resistance to human health. *Nat. Rev. Microbiol.* **2022**, *20*, 557–571. [CrossRef] [PubMed]
77. Rivero-Menendez, O.; Alastruey-Izquierdo, A.; Mellado, E.; Cuenca-Estrella, M. Triazole Resistance in *Aspergillus* spp.: A Worldwide Problem? *J. Fungi* **2016**, 2, 21. [CrossRef]
78. Macedo, D.; Brito Devoto, T.; Pola, S.; Finquelievich, J.L.; Cuestas, M.L.; Garcia-Effron, G. A Novel Combination of CYP51A Mutations Confers Pan-Azole Resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **2020**, *64*. [CrossRef] [PubMed]
79. Camps, S.M.T.; van der Linden, J.W.M.; Li, Y.; Kuijper, E.J.; van Dissel, J.T.; Verweij, P.E.; Melchers, W.J.G. Rapid Induction of Multiple Resistance Mechanisms in Aspergillus fumigatus during Azole Therapy: A Case Study and Review of the Literature. *Antimicrob. Agents Chemother.* **2012**, *56*, 10–16. [CrossRef] [PubMed]
80. Chowdhary, A.; Kathuria, S.; Xu, J.; Meis, J.F. Emergence of Azole-Resistant Aspergillus fumigatus Strains due to Agricultural Azole Use Creates an Increasing Threat to Human Health. *PLoS Pathog.* **2013**, *9*, e1003633. [CrossRef]
81. Garcia-Rubio, R.; Cuenca-Estrella, M.; Mellado, E. Triazole Resistance in Aspergillus Species: An Emerging Problem. *Drugs* **2017**, *77*, 599–613. [CrossRef]
82. Berger, S.; El Chazli, Y.; Babu, A.F.; Coste, A.T. Azole Resistance in Aspergillus fumigatus: A Consequence of Antifungal Use in Agriculture? *Front. Microbiol.* **2017**, *8*, 1024. [CrossRef]
83. Verweij, P.E.; Snelders, E.; Kema, G.H.; Mellado, E.; Melchers, W.J. Azole resistance in *Aspergillus fumigatus*: A side-effect of environmental fungicide use? *Lancet Infect. Dis.* **2009**, *9*, 789–795. [CrossRef]
84. Van Der Torre, M.H.; Novak-Frazer, L.; Rautemaa-Richardson, R. Detecting Azole-Antifungal Resistance in Aspergillus fumigatus by Pyrosequencing. *J. Fungi* **2020**, *6*, 12. [CrossRef]
85. Gonçalves, P.; Melo, A.; Dias, M.; Almeida, B.; Caetano, L.A.; Veríssimo, C.; Viegas, C.; Sabino, R. Azole-Resistant *Aspergillus fumigatus* Harboring the TR34/L98H Mutation: First Report in Portugal in Environmental Samples. *Microorganisms* **2020**, *9*, 57. [CrossRef] [PubMed]
86. WHO. *WHO Fungal Priority Pathogens List to Guide Research, Development and Public Health Action*; WHO: Geneva, Switzerland, 2022.
87. WHO. *WHO Bacterial Priority Pathogens List, 2024: Bacterial Pathogens of Public Health Importance to Guide Research, Development and Strategies to Prevent and Control Antimicrobial Resistance*; WHO: Geneva, Switzerland, 2024.

11.5. Appendix 5

HTRC Health and Technology Research Center
ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

RELATÓRIO DA AUDITORIA À QUALIDADE DO AR INTERIOR

[redacted]



1. ÍNDICE

2. OBJETIVO

O presente relatório tem como principal objetivo apresentar os resultados obtidos na auditoria de Qualidade do Ar Interior (QAI) realizada às instalações da [redacted], no dia 19 de julho de 2023. Uma avaliação do estado e conformidade da instalação quanto à QAI (parâmetros de conforto, físico-químicos e microbiológicos) será dada atendendo à conformidade legal, com base no Decreto-Lei n.º 101-D/2020 de 7 de dezembro – que estabelece os requisitos aplicáveis a edifícios para a melhoria do seu desempenho energético e regula o Sistema de Certificação Energética de Edifícios, transpondo a Diretiva (UE) 2018/844 e parcialmente a Diretiva (UE) 2019/944 – conjuntamente com o Despacho n.º 1618/2022 de 9 de fevereiro – que estabelece o regime de avaliação simplificada anual – e a Portaria n.º 138-G/2021 – que estabelece os requisitos para a avaliação da qualidade do ar interior nos edifícios de comércio e serviços, incluindo os limiares de proteção, condições de referência e critérios de conformidade, e a respetiva metodologia para a medição dos poluentes e para a fiscalização do cumprimento das normas aprovadas.

O presente relatório não serve para cumprimento do Decreto-Lei n.º 101-D/2020 de 7 de dezembro no que aos parâmetros microbiológicos diz respeito, pelo facto do laboratório não ser acreditado.

Ações de melhoria serão incluídas como sugestões à melhoria contínua da entidade, no que se refere a esta área de atuação.

3. EQUIPA TÉCNICA

Técnico de ensaios de campo:	Marina Almeida-Silva	Doutorada em Ciências do Ambiente, especializada em Qualidade do Ar e Licenciada em Saúde Ambiental
	Pedro Pena	Licenciado em Saúde Ambiental e Doutorando em Saúde Pública
	Carla Viegas	Doutorada em Saúde Pública e Licenciada em Saúde Ambiental
	Liliana Marques	Licenciada em Biotecnologia e Mestranda em Tecnologias Clínico-Laboratoriais
Relatório elaborado por:	Marina Almeida-Silva e Pedro Pena	
Instituição prestadora do serviço:	Centro de Investigação em Saúde e Tecnologia (H&TRC), Escola Superior de Tecnologia da Saúde de Lisboa	Av. D. João II, lote 4.69.01, Parque das Nações 1990-096 Lisboa Telefone: (+351) 218 980 400 Emails: marina.silva@estesl.ipl.pt

4. Caracterização das instalações

O edifício da [redacted] localiza-se [redacted]s. O edifício está inserido numa zona urbana, próximo de uma zona industrial (Figura 1), e é composto por quatro pisos:

- PISO -1: 1 lavandaria, 1 rouparia, 2 instalações sanitárias, 1 sala de pessoal, 2 salas de arrumos e um vestiário.

- PISO 0: 4 gabinetes, 1 refeitório, 1 cozinha, 1 sala centro de dia, 1 salas de estar, 1 sala de atividades, 2 salas de arrumos, vestiários e 5 instalações sanitárias.

- PISO 1: 1 sala de estar, 1 sala reuniões, 14 quartos e 16 instalações sanitárias (mais duas instalação sanitária que pertence à sala de enfermagem e sala dos sujos convertida).

- PISO 2: 2 gabinetes, 2 salas de estar, 17 quartos e 18 instalações sanitárias.

- PISO 3: 1 ginásio, 1 gabinete, 1 sala estimulação sensorial, 1 instalação sanitária, 1 sala de arrumos.

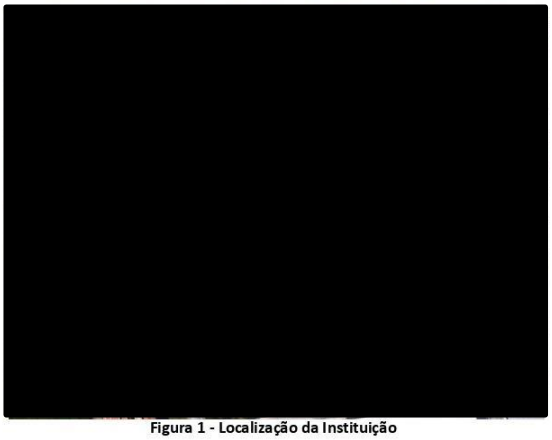


Figura 1 - Localização da Instituição

5. Caracterização geral do sistema AVAC

O sistema AVAC (aquecimento, ventilação e ar condicionado) existente é constituído por uma UTAN (unidade de tratamento de ar novo) que efetua a insuflação de ar novo nos espaços de ocupação através de difusores; unidades de expansão direta e ventiloconvectores. Existe, ainda, um ventilador dedicado à extração que efetua a extração do ar nos diversos espaços, através de grelhas.

O edifício é climatizado por um chiller/bomba de calor, associado a unidades interiores de expansão direta do tipo cassete 4 e 6 vias e ventiloconventores que efetuam a climatização nos diversos espaços com ocupação.

No âmbito da presente auditoria, as condições de higiene e a capacidade de filtragem do sistema AVAC do edifício foram avaliadas. Os requisitos de avaliação estão relacionados com:

- O nível da existência de um andar de filtragem composto por, pelo menos, um filtro com:
  - Classificação mínima F5 (EN 779), antes das baterias ou permutadores de calor;
  - Classificação mínima de F7, a jusante de ventiladores com motores e transmissão por correias em contacto com o ar circulante;
  - Classificação mínima de F7, a jusante de atenuadores acústicos, exceto nos casos onde se verifique a existência de um certificado que ateste a não desagregação do elemento acústico, emitido por laboratório acreditado, dispensando neste caso o referido na subalínea anterior.

6. Método

6.1. Parâmetros amostrados

A avaliação da Qualidade do Ar Interior é realizada para os poluentes mencionados na Portaria 353-A/2013 e especificados nas Tabelas I.08, I.09, I.10, I.11 e I.12, baseando-se assim nos seguintes parâmetros:

- Conforto:
  - Temperatura (T)
  - Humidade relativa (HR)
- Físico-químicos:
  - Partículas em suspensão no ar ($PM_{2.5}$ e PM_{10})
  - Dióxido de Carbono (CO_2)
  - Monóxido de Carbono (CO)
  - Compostos Orgânicos Voláteis Totais (COVs)
- Microbiológicos:
  - Bactérias (incluindo *Legionella spp.* e *Legionella pneumophila*)
  - Fungos

6.2. Locais de amostragem

Através do estudo e caracterização das instalações foram definidos 12 locais prioritários de monitorização (9 pontos de amostragem de parâmetros de conforto, físico-químicos e microbiológicos e 3 pontos de amostragem de *Legionella* spp. e *Legionella pneumophila* em águas de consumo humano). Os pontos de amostragem e a sua localização estão descritos na Tabela 1.

Tabela 1 - Identificação dos pontos de amostragem e localização

ID	Local	Piso	Informação complementar
P1	Exterior	Piso 0	Exterior próximo à secretaria
P2	Sala (a)	Piso 0	Ocupação: 4 pessoas
P3	Sala (b)	Piso 0	Ocupação: 8 pessoas; Porta aberta para o jardim + 1 fumador
P4	Refeitório (a)	Piso 0	Em serviço; Corrente de ar
P5	Secretaria	Piso 0	Ocupação: 2 pessoas
P6	Quarto 104	Piso 1	Ocupação: 2 pessoas
P7	Quarto 113	Piso 1	Vazio e Limpo
P8	Sala de Estar	Piso 1	Ocupação: 6 pessoas
P9	Escritório	Piso 1	Ocupação: 3 pessoas
P10	Sala de Enfermagem	Piso 1	Sem ocupação
P11	Sala – Snoezlen	Piso 3	
P12	Sala – Refeitório	Piso 2	Ocupação: 10 pessoas
P13	Quarto 203	Piso 2	Ocupação: 1 pessoa
P14	Quarto 212	Piso 2	Ocupação: 2 pessoas
P15	Ginásio	Piso 3	Sem ocupação
LP1	Chuveiro Quarto 103	Piso 1	-
LP2	Chuveiro vestiário feminino	Piso -1	-
LP3	Quarto 207	Piso 2	-

Os períodos de amostragem para os parâmetros químicos e físicos variaram entre 5 a 10 minutos.

As amostragens de *Legionella* spp. e *Legionella pneumophila* foram efetuadas em locais que seguiam os seguintes critérios:

- Ponto mais longe dos depósitos de AQS (águas quentes sanitárias);

- Locais onde poderão passar mais utentes;
- Localizações mais suscetíveis.

A amostragem dos parâmetros microbiológicos foi realizada de acordo com as informações apresentadas na Tabela 2.

Tabela 2 - Caracterização do processo de amostragem: Parâmetros microbiológicos

ID	Local	Parâmetros a amostrar	Duração de amostragem	Volume de amostragem
P1	Exterior	Fungos e Bactérias	1 min	200 L
P2	Sala (a)		1 min	200 L
P3	Sala (b)		1 min	200 L
P4	Refeitório (a)		1 min	200 L
P5	Secretaria		1 min	200 L
P6	Quarto 104		1 min	200 L
P7	Quarto 113		1 min	200 L
P8	Sala de Estar		1 min	200 L
P9	Escritório		1 min	200 L
P10	Sala de Enfermagem		1 min	200 L
P11	Sala – Snoezlen		1 min	200 L
P12	Sala – Refeitório		1 min	200 L
P13	Quarto 203		1 min	200 L
P14	Quarto 212		1 min	200 L
P15	Ginásio		1 min	200 L
LP1	Chuveiro Quarto 103	*Legionella* spp. e *Legionella pneumophila*	-	2 L
LP2	Chuveiro vestiário feminino		-	2 L
LP3	Quarto 208		-	2 L

6.3. Equipamentos e método de análise

Os equipamentos e métodos de análise utilizados estão descritos na tabela 3.

Tabela 3 - Identificação de equipamentos e métodos de análise

Parâmetros	Atividade	Variável	Equipamento	Marca	Método
Conforto	Medição	Humidade relativa (%)	IAQ Wolfsense	GreyWolf	NDIR; Leitura direta através das células eletroquímicas
	Medição	Temperatura (ºC)	IAQ Wolfsense	GreyWolf	
Físico-Químicos	Medição	CO (ppm)	IAQ Wolfsense	GreyWolf	NDIR; Leitura direta através das células eletroquímicas
	Medição	CO_2 (ppm)	IAQ Wolfsense	GreyWolf	
	Medição	COV (ppb)	Modelo 7545	IAQ-CALC™	
	Medição	$PM_{2.5}$ e PM_{10} (mg/m^3)	IAQ	LightHouse	Medição fotométrica
	Medição	Ozono (mg/m^3)	IAQ Wolfsense	GreyWolf	NDIR; Leitura direta através das células eletroquímicas
Biológicos	Colheita de amostra ambiental (Ar)	Fungos ($UFC.m^{-3}$)	M-Air Tester	MAS-100	Quantificação (Meio de cultura)
	Colheita de amostra ambiental (Ar)	Bactérias ($UFC.m^{-3}$)	M-Air Tester	MAS-100	
	Colheira de amostra ambiental (superfície)	Fungos ($UFC.m^{-2}$)	Zaragatoa	n/a	ISO 11731:1998

As amostras relativas aos parâmetros microbiológicos foram analisadas pelo Laboratório de Microbiologia Ambiental do H&TRC-ESTeSL-IPL. No procedimento de amostragem, a colheita é feita por impacto sobre uma superfície das partículas em suspensão no ar, em meio semi-sólido (agar). Durante o transporte, as amostras foram mantidas frescas e ao abrigo da luz solar. Para bactérias, a incubação nas placas foi feita a 30ºC, durante 7 dias, em meio de cultura Tryptic Soy Agar (TSA) com 0,2% de nistatina e a 37ºC, durante 7 dias, em meio de cultura Violet Red Bile Agar (VRBA). No que se refere aos fungos, estes foram incubados a 27ºC, durante 7 dias em meio de cultura Malt Extract Agar (MEA) e Dichloran-Glycerol Agar (DG18).

Adicionalmente, a avaliação da presença da bactéria *Legionella* spp. e *Legionella pneumophila* em amostras de água para consumo humano foi realizada. A colheita de água foi efetuada pela equipa técnica, tendo sido as amostras analisadas pelo Laboratório de Análises do Instituto Superior Técnico.

7. Resultados

7.1. Parâmetros de conforto

A nível do conforto térmico dos ocupantes de espaços interiores, segundo a ISO 7730:2005, a humidade relativa deve estar entre 30-70% e a temperatura deve ser de 23 a 26 °C. Tal como se encontra indicado na tabela 4, verificou-se que a humidade relativa e a temperatura do ar cumprem os valores requeridos.

Tabela 4 - Resultados obtidos para os parâmetros Temperatura (T) e Humidade Relativa (HR) e avaliação da sua conformidade legal

ID	Local	Piso	Temperatura (ºC)			Humidade Relativa (%)		
			Valor de referência[1]: 23 a 26 ºC			Valor de referência[1]: 30 a 70 %		
			Média	Max[2]	Conformidade	Média	Max	Conformidade
P1	Exterior	Piso 0	26,8	27,5	Não aplicável	49,0	59,1	Não aplicável
P2	Sala (a)	Piso 0	24,1	24,2	Conforme	59,9	60,2	Conforme
P3	Sala (b)	Piso 0	24,1	24,4	Conforme	60,8	62,4	Conforme
P4	Refeitório (a)	Piso 0	23,7	24,1	Conforme	62,2	63,7	Conforme
P5	Secretaria	Piso 0	23,9	23,9	Conforme	60,8	61,1	Conforme
P6	Quarto 104	Piso 1	23,5	23,6	Conforme	64,3	64,5	Conforme
P7	Quarto 113	Piso 1	24,7	24,8	Conforme	57,8	58,5	Conforme
P8	Sala de Estar	Piso 1	23,3	23,4	Conforme	61,5	61,9	Conforme
P9	Escritório	Piso 1	23,5	23,5	Conforme	62,0	62,9	Conforme
P10	Sala de Enfermagem	Piso 1	24,5	24,7	Conforme	57,9	59,2	Conforme
P11	Sala – Snoezlen	Piso 3	25,0	25,1	Conforme	58,1	58,6	Conforme
P12	Sala – Refeitório	Piso 2	23,7	24,3	Conforme	60,1	61,8	Conforme
P13	Quarto 203	Piso 2	23,1	23,1	Conforme	61,7	62,4	Conforme
P14	Quarto 212	Piso 2	24,5	25,0	Conforme	62,5	65,4	Conforme
P15	Ginásio	Piso 3	25,0	25,5	Conforme	55,3	56,7	Conforme

[1] Valores propostos através da ISO 7730:2005.

[2] O valor a sombreado corresponde a uma excedência pontual durante o período de amostragem

7.2. Parâmetros físico-químicos

Nas tabelas 5 a 7 apresentam-se os resultados obtidos para os parâmetros físico-químicos.

Tabela 5 - Resultados obtidos para os parâmetros $PM_{2.5}$ e PM_{10} e avaliação da sua conformidade legal

ID	Local	Piso	$PM_{2.5}$ ($\mu g\ m^{-3}$)			PM_{10} ($\mu g\ m^{-3}$)		
			Valor de referência[1]: 25 $\mu g\ m^{-3}$ [2]			Valor de referência[1]: 50 $\mu g\ m^{-3}$[2]		
			Média	Max	**Conformidade**	Média	Max	**Conformidade**
P1	Exterior	Piso 0	10	18	Não aplicável	20	31	Não aplicável
P2	Sala (a)	Piso 0	20	24	Conforme	23	32	Conforme
P3	Sala (b)	Piso 0	11	19	Conforme	24	38	Conforme
P4	Refeitório (a)	Piso 0	15	32	Conforme	30	45	Conforme
P5	Secretaria	Piso 0	11	14	Conforme	27	40	Conforme
P6	Quarto 104	Piso 1	8	13	Conforme	15	29	Conforme
P7	Quarto 113	Piso 1	5	10	Conforme	21	29	Conforme
P8	Sala de Estar	Piso 1	6	10	Conforme	19	27	Conforme
P9	Escritório	Piso 1	10	15	Conforme	28	30	Conforme
P10	Sala de Enfermagem	Piso 1	5	12	Conforme	15	22	Conforme
P11	Sala – Snoezlen	Piso 3	9	12	Conforme	14	25	Conforme
P12	Sala – Refeitório	Piso 2	17	30	Conforme	21	25	Conforme
P13	Quarto 203	Piso 2	10	12	Conforme	15	21	Conforme
P14	Quarto 212	Piso 2	8	11	Conforme	13	26	Conforme

[1] Limiares de proteção para poluentes físico-químicos estabelecidos na Portaria n.º 353-A/2013, de 4 de Dezembro.
[2] Valor médio.
[3] O valor a sombreado corresponde a uma excedência durante o período de amostragem

Relativamente aos critérios estabelecidos pela Portaria n. 138-G/2021 verifica-se a conformidade com os todos os valores estabelecidos.

X

Tabela 6 - Resultados obtidos para os parâmetros CO_2 e CO e avaliação da sua conformidade legal

ID	Local	Piso	CO_2 (ppm)			CO (ppm)		
			Valor de referência[1]: 1250 ppm [2]			Valor de referência[1]: 9 ppm [2]		
			Médio	Max	Conformidade	Médio	Max	Conformidade
P1	Exterior	Piso 0	416	450	Não aplicável	1,8	2,2	Não aplicável
P2	Sala (a)	Piso 0	500	650	Conforme	2,0	2,2	Conforme
P3	Sala (b)	Piso 0	460	526	Conforme	1,9	2,1	Conforme
P4	Refeitório (a)	Piso 0	634	700	Conforme	2,2	2,3	Conforme
P5	Secretaria	Piso 0	614	660	Conforme	2,2	2,3	Conforme
P6	Quarto 104	Piso 1	571	604	Conforme	2,1	2,2	Conforme
P7	Quarto 113	Piso 1	614	800	Conforme	2,1	2,2	Conforme
P8	Sala de Estar	Piso 1	560	670	Conforme	1,8	2,0	Conforme
P9	Escritório	Piso 1	630	890	Conforme	2,0	2,2	Conforme
P10	Sala de Enfermagem	Piso 1	501	631	Conforme	2,2	2,3	Conforme
P11	Sala – Snoezlen	Piso 3	676	733	Conforme	2,2	2,2	Conforme
P12	Sala – Refeitório	Piso 2	520	650	Conforme	2,2	2,3	Conforme
P13	Quarto 203	Piso 2	445	516	Conforme	2,1	2,2	Conforme
P14	Quarto 212	Piso 2	490	620	Conforme	2,2	2,2	Conforme
P15	Ginásio	Piso 3	460	485	Conforme	2,3	2,7	Conforme

[1] Limiares de proteção para poluentes físico-químicos estabelecidos na Portaria n.º 138-G/2021, de 1 de julho.
[2] Valor máximo.

Os resultados relativos aos parâmetros CO_2 e CO apresentam conformidade legal em todos os pontos. Estes resultados dever-se-ão às excelentes condições de ventilação natural existentes nos locais em análise.

Tabela 7 - Resultados obtidos para os parâmetros COV e avaliação da sua conformidade legal

ID	Local	Piso	COV (µg.m^{-3}) Valor de referência[1]: 600 µg.m^{-3} [2]		
			Médio	Max	Conformidade
P1	Exterior	Piso 0	200	200	Conforme
P2	Sala (a)	Piso 0	300	300	Conforme
P3	Sala (b)	Piso 0	320	500	Conforme
P4	Refeitório (a)	Piso 0	380	400	Conforme
P5	Secretaria	Piso 0	970	1800	Não-Conforme
P6	Quarto 104	Piso 1	1690	1800	Não-Conforme
P7	Quarto 113	Piso 1	400	500	Conforme
P8	Sala de Estar	Piso 1	379	600	Conforme
P9	Escritório	Piso 1	400	500	Conforme
P10	Sala de Enfermagem	Piso 1	596	700	Não-Conforme
P11	Sala – Snoezlen	Piso 3	690	1100	Não-Conforme
P12	Sala – Refeitório	Piso 2	440	500	Conforme
P13	Quarto 203	Piso 2	300	300	Conforme
P14	Quarto 212	Piso 2	500	600	Conforme
P15	Ginásio	Piso 3	450	500	Conforme

[1] Limiares de proteção para poluentes físico-químicos estabelecidos na Portaria n.º 138-G/2021, de 1 de julho.
[2] Valor máximo.

Os resultados relativos ao parâmetro COVs apresentam, na sua generalidade, não-conformidade legal tendo em conta que o limiar de proteção de 600 µg.m^{-3}. Os locais mais a Secretaria, um dos quartos e a dala de Snoezlen. Este valor pode ser resultado de uma ventilação deficiente a qual promove a acumulação deste poluente associado a episódios de limpeza/higienização dos locais, uso de perfumes

pelos utilizadores, do uso de desinfetantes para a realização de tratamentos do próprio mobiliário e materiais de decoração, entre outras fontes.

Ainda assim, os valores de COVs obtidos são muito inferiores comparativamente às últimas auditorias realizadas, pelo que se entende que se está a trabalhar corretamente no controlo deste poluente.

7.3 Parâmetros microbiológicos

Nas tabelas 8, 9 e 10 apresentam-se os resultados obtidos para os parâmetros biológicos.

Tabela 8 - Resultados obtidos para os parâmetros microbiológicos (bactérias e fungos) e avaliação da sua conformidade legal

ID	Local	Piso	Bactérias [UFC m^{-3}] Valor de referência(1): Concentração de bactérias totais no interior inferior à concentração no exterior, acrescida de 350 UFC m^{-3}			Fungos [UFC m^{-3}] Valor de referência(1): Concentração de fungos no interior inferior à detetada no exterior		
			Valor obtido		Conformidade	Valor obtido		Conformidade
			TSA	VRBA		MEA	DG18	
P1	Exterior	Piso 0	110	210	-	390	315	-
P2	Sala (a)	Piso 2	510	25	Não conforme	345	360	Não conforme
P3	Sala (b)	Piso 1	210	45	Conforme	135	535	Não conforme
P4	Refeitório (a)	Piso 3	355	140	Conforme	340	455	Não conforme
P5	Secretaria	Piso 0	160	150	Conforme	115	120	Conforme
P6	Quarto 104	Piso 2	650	355	Não conforme	30	35	Conforme
P7	Quarto 113	Piso 2	705	355	Não conforme	5	35	Conforme
P8	Sala de Estar	Piso 1	665	355	Não conforme	250	60	Conforme
P9	Escritório	Piso 1	845	355	Não conforme	20	65	Conforme
P10	Sala de Enfermagem	Piso 1	1005	685	Não conforme	35	20	Conforme
P11	Sala – Snoezlen	Piso 3	1305	355	Não conforme	200	210	Conforme
P12	Sala – Refeitório	Piso 2	1405	355	Não conforme	15	15	Conforme

P13	Quarto 203	Piso 2	905	355	Não conforme	-	40	Conforme
P14	Quarto 212	Piso 2	180	235	Conforme	30	50	Conforme
P15	Ginásio	Piso 3	260	35		415	470	Não conforme

(1) Condições de referência para parâmetros microbiológicos estabelecidos na Portaria n.º 138-G/2021, de 1 de julho.

Todos os resultados microbiológicos em amostras de ar apresentados na Tabela 8 estão conformes, com exceção do P2, P6-P13 para bactérias e do P2-P4 e P15 para fungos. Considerando o disposto no ponto 3 do artigo 5º da Portaria n.º 138-G/2021, de 1 de julho, após análise das espécies presentes nos pontos de amostragem, cuja quantificação supera o disposto no mesmo diploma legal, nos pontos P1 e P3 foi identificada a espécie *Aspergillus ochraceus* (section *Circumdati*) (DG18: 15 UFC/m^3 e 45 UFC/m^3, respetivamente) e no ponto P14, foi identificado *Aspergillus versicolor* (section *Nidulantes*) (MEA: 20 UFC/m^3; DG18: 35 UFC/m^3), todos os pontos com mais de 12 UFC/m^3.

A avaliação microbiológica em superfícies revelou resultados positivos em todos os pontos de 2 a 15 para bactérias totais ($8{,}30\times10^4$ – $3{,}42\times10^5$ UFC.m^{-2}) e para bactérias Gram- ($7{,}90\times10^4$ – $1{,}41\times10^5$ UFC.m^{-2}). Quanto aos resultados da avaliação microbiológica em superfícies para fungos revelaram-se positivos nos pontos 3, em meio DG18 (2×10^4 UFC.m^{-2}) e no ponto 15 em meio MEA (4×10^4 UFC.m^{-2}). Para uma melhor caracterização da contaminação fúngica, foram identificadas as espécies fúngicas observadas nos métodos de amostragem utilizados (Tabela 9).

No total foram identificadas 11 espécies fúngicas diferentes em cada meio de cultura. O fungo mais prevalente em ambos os meios de cultura foi *Cladosporium* sp. (86,9% MEA; 81,5% DG18). Além disso, em ambos os meios foi também identificado com elevada frequência *Penicillium* sp. (5,6 % MEA; 11,3% DG18) e *Aspergillus* sp. (2,2 % MEA; 6,5% DG18). Foram identificadas *Aspergillus* sections, das quais, *Nidulantes* (40 % MEA; 25 % DG18), *Nigri* (60 % MEA), *Aspergilli* (42 % DG18) e *Circumdati* (33 % DG18). De salientar, que estas espécies apesar de terem sido encontradas nas superfícies, dependendo das atividades realizadas e das caraterísticas fúngicas, poderão ser observadas no ar se não forem implementadas as medidas preconizadas.

Tabela 9 - Resultados obtidos relativos à distribuição fúngica (CFU. m^{-3}).

ID da Amostra	MEA			DG18		
	ID	CFU/m3	%	ID	CFU/m3	%
P1 (outdoor)	*Cladosporium* sp.	350	89,7	*Cladosporium* sp.	260	82,5
	Penicillium sp.	5	1,3	*Penicillium* sp.	35	11,1
	Outras espécies	35	9,0	*Aspergillus* sp.	20	6,3
	TOTAL	390	100	TOTAL	315	100

P2	*Cladosporium* sp.	315	91,3	*Cladosporium* sp.	300	83,3
	Penicillium sp.	10	2,9	*Penicillium* sp.	60	16,7
	Aspergillus sp.	10	2,9			
	Rhizopus sp.	10	2,9			
	TOTAL	345	100	TOTAL	360	100
P3	*Cladosporium* sp.	110	81,5	*Cladosporium* sp.	475	88,8
	Penicillium sp.	5	3,7	*Penicillium* sp.	15	2,8
	Aspergillus sp.	5	3,7	*Aspergillus* sp.	45	8,4
	Alternaria sp.	15	11,1			
	TOTAL	135	100	TOTAL	535	100
P4	*Cladosporium* sp.	320	94,1	*Cladosporium* sp.	395	86,8
	Penicillium sp.	10	2,9	*Penicillium* sp.	55	12,1
	Alternaria sp.	5	1,5	*Aspergillus* sp.	5	1,1
	Outras espécies	5	1,5			
	TOTAL	340	100	TOTAL	455	100
P5	*Cladosporium* sp.	95	82,6	*Cladosporium* sp.	90	75,0
	Penicillium sp.	15	13,0	*Penicillium* sp.	30	25,0
	Alternaria sp.	5	4,3			
	TOTAL	115	100	TOTAL	120	100
P6	*Cladosporium* sp.	25	83,3	*Cladosporium* sp.	15	42,9
	Aspergillus sp.	5	16,7	*Penicillium* sp.	20	57,1
	TOTAL	30	100	TOTAL	35	100
P7	*Penicillium* sp.	5	100,0	*Cladosporium* sp.	15	42,9
				Penicillium sp.	15	42,9
				Outras espécies	5	14,3
	TOTAL	5	100	TOTAL	35	100
P8	*Cladosporium* sp.	205	82,0	*Cladosporium* sp.	45	75,0
	Penicillium sp.	20	8,0	*Penicillium* sp.	15	25,0
	Alternaria sp.	20	8,0			
	Outras espécies	5	2,0			
	TOTAL	250	100	TOTAL	60	100
P9	*Cladosporium* sp.	10	50,0	*Cladosporium* sp.	40	61,5
	Penicillium sp.	10	50,0	*Penicillium* sp.	20	30,8
				Mucor sp.	5	7,7
	TOTAL	20	100	TOTAL	65	100
P10	*Cladosporium* sp.	35	100,00	*Cladosporium* sp.	20	100,00
	TOTAL	35	100	TOTAL	20	100
P11	*Cladosporium* sp.	140	70,0	*Cladosporium* sp.	150	71,4
	Penicillium sp.	50	25,0	*Penicillium* sp.	40	19,0
	C. sitophila	10	5,0	*Aspergillus* sp.	10	4,8
				Alternaria sp.	5	2,4

				Mucor sp.	5	2,4
	TOTAL	200	100	TOTAL	210	100
P12	*Cladosporium* sp.	5	33,3	*Penicillium* sp.	10	66,7
	C. sitophila	10	66,7	*Cladosporium* sp.	5	33,3
	TOTAL	15	100	TOTAL	15	100
P13				*Cladosporium* sp.	10	25,00
				Aspergillus sp.	30	75,00
				TOTAL	40	100
P14	*Cladosporium* sp.	10	33,3	*Cladosporium* sp.	10	20,00
	Aspergillus sp.	20	66,7	*Aspergillus* sp.	40	80,00
	TOTAL	30	100	TOTAL	50	100
P15	*Cladosporium* sp.	400	96,4	*Cladosporium* sp.	435	92,6
	Aspergillus sp.	10	2,4	*Penicillium* sp.	5	1,1
	Outras espécies	5	1,20	*Aspergillus* sp.	30	6,4
	TOTAL	415	100	TOTAL	470	100

A avaliação microbiológica em superfícies para fungos revelou resultados positivos nos pontos 3, em meio DG18 (*Cladosporium* sp. $1x10^4$ CFU/m^{-2} e *Penicillium* sp. $1x10^4$ CFU/m^{-2}) e no ponto 15 em meio MEA (*Cladosporium* sp. $1x10^4$ CFU/m^{-2}, *Penicillium* sp. $2x10^4$ CFU/m^{-2} e *Rhizopus* sp. $1x10^4$ CFU/m^{-2}).

Tabela 10 - Resultados obtidos para os parâmetros *Legionella (Legionella* spp. *e Legionella pneumophila)* e avaliação da sua conformidade legal

ID	Local	Piso	*Legionella spp*		*Legionella pneumophila*	
			Valor de referência[1]: Concentração inferior a 100 UFC L^{-1}		Valor de referência[1]: Ausência de UFC L^{-1}	
			Valor obtido	Conformidade	Valor obtido	Conformidade
LP1	Chuveiro Quarto 103	Piso 1	Não detetado	Conforme	Não detetado	Conforme
LP2	Chuveiro vestiário feminino	Piso -1	Não detetado	Conforme	Não detetado	Conforme
LP3	Quarto 207	Piso 2	Não detetado	Conforme	Não detetado	Conforme

[1] Limiares de proteção para poluentes físico-químicos estabelecidos na Portaria n.º 138-G/2021, de 1 de julho.

7.4 Avaliação das condições do sistema AVAC

As condições de conservação e limpeza do sistema AVAC foram avaliadas e as observações obtidas encontram-se na Tabela 10.

Tabela 10 - Estado de conservação do AVAC

	Componente	Observação
UTAN	Deflectores de entrada de ar novo	Bom estado e limpo
	Pré-filtro (G4)	Não foi possível observar, uma vez que o sistema estava em funcionamento
	Filtro	
	Interior	Não foi possível observar, uma vez que o sistema estava em funcionamento
	Baterias e permutadores de recuperadores de calor	
	Serpentina/Bandeja de condensados/sifão	
	Acesso à manutenção	Bom
Geral	Difusores	Limpo

É fundamental sublinhar as excelentes condições de limpeza das grelhas distribuídas por todo o edifício. Na sua maioria encontravam-se em devidas condições de limpeza. Algumas exceções foram evidências, como demonstrado na Figure 2.

Figura 2 – Grelhas de distribuição existentes no edifício: uma em devidas condições de limpeza e outra a necessitar de manutenção.

8. Conclusão, Medidas de Melhoria e Recomendações

A realização da presente auditoria permitiu concluir que a [redacted]" adota procedimentos de planos de ação eficazes, possuindo uma qualidade do ar interior aceitável, evidenciando apenas alguns pontos menos positivos referentes a alguns poluentes específicos. Os Compostos Orgânicos Voláteis são, de forma continuada, um problema recorrente de ambientes interiores. Sendo um poluente proveniente da utilização de produtos de limpeza e produtos de higiene pessoal torna-se difícil a sua remoção/eliminação. Contudo, com uma adequada ventilação dos espaços é possível garantir uma aceitável qualidade do ar interior. Salienta-se que ao longo das últimas avaliações este parâmetro tem, ainda assim, evidenciado melhorias significativas.

Numa perspetiva de melhoria contínua, bem como garantir o bom funcionamento das instalações, sugere-se que continue os mesmos procedimentos aplicados até à data e que não se esqueça de:

- Proceder à substituição dos filtros do sistema AVAC (aquando necessário e sempre que a empresa de manutenção assim o entenda);
- Proceder à manutenção periódica do sistema AVAC o que possibilita a minimização da existência ou alteração dos parâmetros físico-químicos e microbiológicos;
- Regular o uso de ar condicionado de forma a obter um bom conforto térmico;
- Deve-se fazer um uso adequado da ventilação natural através da através da abertura de janelas de forma rotineira e sem prejudicar os ocupantes do espaço. **Ter apenas em atenção a possível existência de correntes de ar como foi por demais evidente junto à zona do Refeitório**;
- Ajustar o plano de limpeza para que estas sejam feitas após os períodos de maior ocupação dos espaços, conduzir à ventilação dos espaços após limpeza e uso de produtos de limpeza inodoros e ecológicos procedendo à sua alternância de utilização de forma a não criar resistência por parte de microrganismos;
- Não utilizar vassouras;
- Selecionar os produtos de limpeza atendendo não só à sua eficiência, mas também à reduzida emissão de compostos orgânicos voláteis.
- Garantir a monitorização da barreira térmica para a prevenção do desenvolvimento de *Legionella* spp. nos extremos de rede (Despacho n.º 1547/2022).

Lisboa, 18 de outubro de 2023

A Responsável:

[redacted]

XVIII

Anexos

a. **Boletim de análise laboratorial de identificação de *Legionella* spp. e *Legionella pneumophila* – LP1**

[redacted]

Relatório de Ensaios

[redacted]

Cliente: [redacted]

Análise Bacteriológica de Água + Zaragatoa

Requisição nº 03623 **de** 2023-07-20
Receção da amostra em: 2023-07-20
Início da análise em: 2023-07-20
Conclusão da análise em: 2023-07-31

Dados da Amostra

Origem: ---

Matriz: Água de Consumo

Colheita

Data e hora: 2023-07-19
Ponto de colheita:
Efetuada por: Cliente (*)

Rótulo: LP1 A/B

Obs: ---

Resultados

Nota(s): Filtração por membrana com eluição. Meio BCYE e GVPC. Limite de Deteção 25 ufc/L.

Parâmetro		Resultado	Método
Legionella spp	Não detetado	ufc / 2L e zaragatoa	ISO 11731:2017
Legionella pneumophila	Não detetado	ufc / 2L e zaragatoa	ISO11731:2017/M.M.9.27(02-04-2018)

Observações

Portaria nº 25 2021 de 29jan classificação risco legionella

Lisboa, 2023-07-31

O Laboratório de Análises

[redacted]

(Responsável de Núcleo)

O ensaio assinalado com (*) não está incluído no âmbito da acreditação do [redacted]
No caso de colheita efetuada pelo cliente, os resultados reportados aplicam-se à amostra conforme rececionada no [redacted]
Este Relatório não contém todas as informações requeridas pela NP EN ISO/IEC 17025 (nomeadamente as incertezas dos ensaios/colheita), conforme acordado com o cliente, as quais poderão ser fornecidas a pedido deste.

Os resultados constantes neste Boletim referem-se exclusivamente à amostra e parâmetros analisados. Este Boletim só pode ser reproduzido na totalidade.
A apresentação de um resultado incluindo o símbolo < (menor) representa o limite de quantificação para esse parâmetro pelo método indicado. Lista de Métodos/Técnicas fornecida mediante solicitação.

XX

b. Boletim de análise laboratorial de identificação de *Legionella* spp. e *Legionella pneumophila* – LP2

[redacted]

Relatório de Ensaios

[redacted]

Cliente [redacted]

Análise Bacteriológica de Água + Zaragatoa

Requisição nº 03623 **de** 2023-07-20
Receção da amostra em: 2023-07-20
Início da análise em: 2023-07-20
Conclusão da análise em: 2023-07-31

Dados da Amostra

Origem: ---

Matriz: Água de Consumo

Colheita

Data e hora: 2023-07-19
Ponto de colheita:
Efetuada por: Cliente (*)

Rótulo: LP2 A/B

Obs: ---

Resultados

Nota(s): Filtração por membrana com eluição. Meio BCYE e GVPC. Limite de Deteção 25 ufc/L.

Parâmetro		Resultado	Método
Legionella spp	Não detetado	ufc / 2L e zaragatoa	ISO 11731:2017
Legionella pneumophila	Não detetado	ufc / 2L e zaragatoa	ISO11731:2017/M.M.9.27(02-04-2018)

Observações

Portaria nº 25_2021 de 29jan_classificação risco_legionella

Lisboa, 2023-07-31

O Laboratório de Análises

[redacted]

(Responsável de Núcleo)

O ensaio assinalado com (*) não está incluído no âmbito da acreditação [redacted].
No caso de colheita efetuada pelo cliente, os resultados reportados aplicam-se à amostra conforme rececionada [redacted].
Este Relatório não contém todas as informações requeridas pela NP EN ISO/IEC 17025 (nomeadamente as incertezas dos ensaios/colheita), conforme acordado com o cliente, as quais poderão ser fornecidas a pedido deste.

Os resultados constantes neste Boletim referem-se exclusivamente à amostra e parâmetros analisados. Este Boletim só pode ser reproduzido na totalidade.
A apresentação de um resultado incluindo o símbolo < (menor) representa o limite de quantificação para esse parâmetro pelo método indicado. Lista de Métodos/Técnicas fornecida mediante solicitação.

NP-Norma Portuguesa; EN-Norma Europeia; ISO-International Organization for Standardization; SMEWW-Standard Methods for the Examination of Water and Wastewater 23th Ed.; EPA-Environmental Protection Agency; LAB-L'Analyse de l'Eau; Rodier 10ème Ed.

XXI

AUDITORIA DE QUALIDADE DO AR INTERIOR	
[redacted]	

c. **Boletim de análise laboratorial de identificação de *Legionella* spp. e *Legionella pneumophila* – LP3**

[redacted]

Relatório de Ensaios

[redacted]

[redacted]

Cliente: [redacted]

Análise Bacteriológica de Água + Zaragatoa

Requisição nº 03623 **de** 2023-07-20
Receção da amostra em: 2023-07-20
Início da análise em: 2023-07-20
Conclusão da análise em: 2023-07-31

Dados da Amostra

Origem: ---

Matriz: Água de Consumo

Colheita

Data e hora: 2023-07-19
Ponto de colheita:
Efetuada por: Cliente (*)

Rótulo: LP3 A/B

Obs: ---

Resultados

Nota(s): Filtração por membrana com eluição. Meio BCYE e GVPC. Limite de Deteção 25 ufc/L.

Parâmetro		Resultado	Método
Legionella spp	Não detetado	ufc / 2L e zaragatoa	ISO 11731:2017
Legionella pneumophila	Não detetado	ufc / 2L e zaragatoa	ISO11731:2017/M.M.9.27(02-04-2018)

Observações

Portaria nº 25 2021 de 29jan classificação risco legionella

Lisboa, 2023-07-31

O Laboratório de Análises

[redacted]

(Responsável de Núcleo)

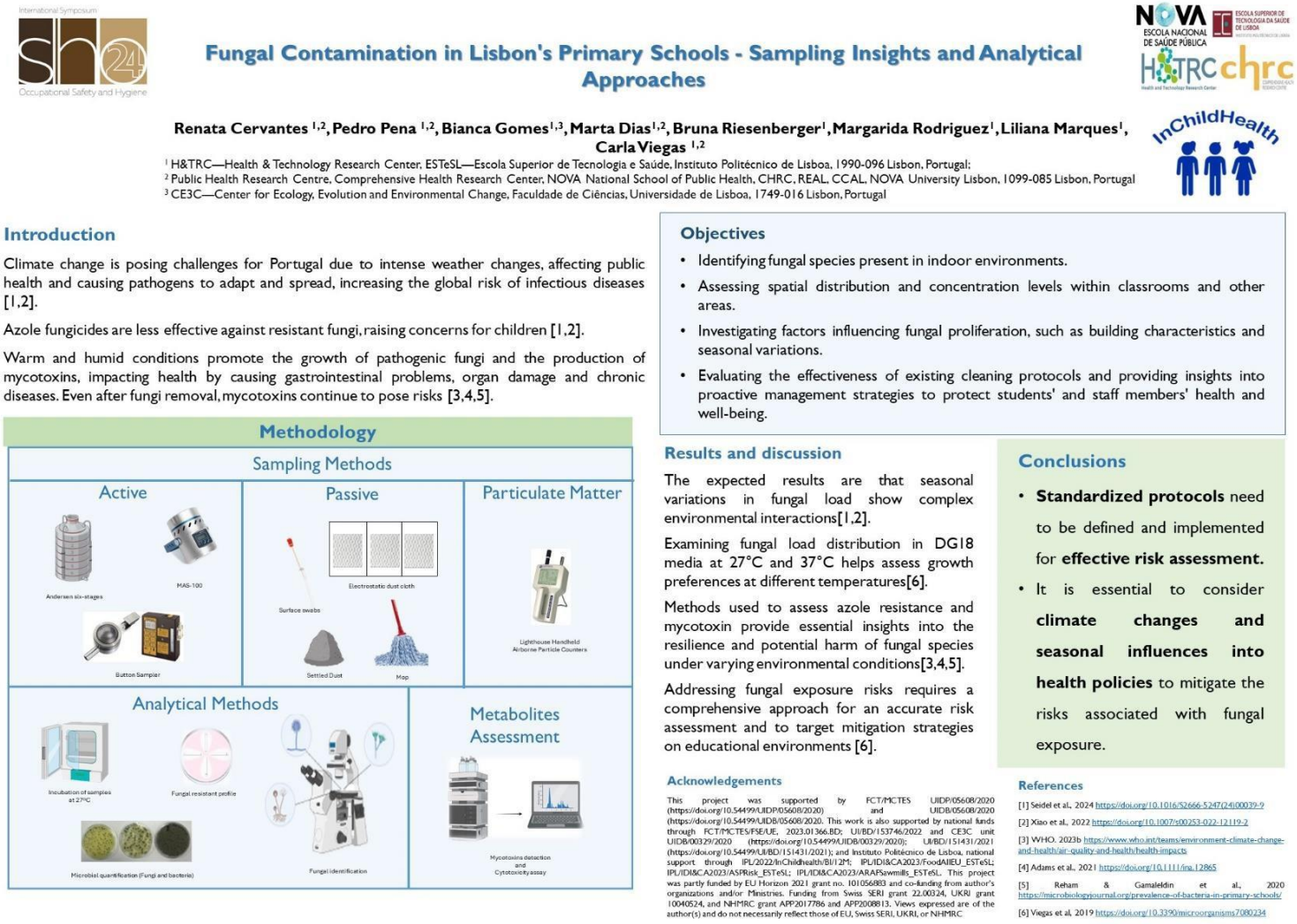
O ensaio assinalado com (*) não está incluído no âmbito da acreditação d[redacted].
No caso de colheita efetuada pelo cliente, os resultados reportados aplicam-se à amostra conforme rececionada n[redacted].
Este Relatório não contém todas as informações requeridas pela NP EN ISO/IEC 17025 (nomeadamente as incertezas dos ensaios/colheita), conforme acordado com o cliente, as quais poderão ser fornecidas a pedido deste.

Os resultados constantes neste Boletim referem-se exclusivamente à amostra e parâmetros analisados. Este Boletim só pode ser reproduzido na totalidade.
A apresentação de um resultado incluindo o símbolo < (menor) representa o limite de quantificação para esse parâmetro pelo método indicado. Lista de Métodos/Técnicas fornecida mediante solicitação.

MM: Método Interno. NP-Norma Portuguesa. EN-Norma Europeia. ISO-International Organization for Standardization. SMWW-Standard Methods for the Examination of Water and Wastewater 23th Ed.. EPA-Environmental Protection Agency. LAB-Laboratório de Água Rodão 13ème Ed.

XXII

11.6. Appendix 6



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]

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[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].

Methodology

Sampling Methods

Active

Andersen six-stages

MAS-100

Button Sampler

Passive

Electrostatic dust cloth

Surface swabs

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

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.

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.

Acknowledgements

This project was supported by FCT/MCTES UIDP/05608/2020 (https://doi.org/10.54499/UIDP/05608/2020) and UIDB/05608/2020 (https://doi.org/10.54499/UIDB/05608/2020. This work is also supported by national funds through FCT/MCTES/FSE/UE, 2023.01366.BD; UI/BD/153746/2022 and CE3C unit UIDB/00329/2020 (https://doi.org/10.54499/UIDB/00329/2020); UI/BD/151431/2021 (https://doi.org/10.54499/UI/BD/151431/2021); and Instituto Politécnico de Lisboa, national support through IPL/2022/InChildhealth/BI/12M; IPL/IDI&CA2023/FoodAllEU_ESTeSL; IPL/IDI&CA2023/ASPRisk_ESTeSL; IPL/IDI&CA2023/ARAPSawmills_ESTeSL. This project was partly funded by EU Horizon 2021 grant no. 101056883 and co-funding from author's organizations and/or Ministries. Funding from Swiss SERI grant 22.00324, UKRI grant 10040524, and NHMRC grant APP2017786 and APP2008813. Views expressed are of the author(s) and do not necessarily reflect those of EU, Swiss SERI, UKRI, or NHMRC

References

[1] Seidel et al., 2024 https://doi.org/10.1016/S2666-5247(24)00039-9

[2] Xiao et al., 2022 https://doi.org/10.1007/s00253-022-12119-2

[3] WHO. 2023b https://www.who.int/teams/environment-climate-change-and-health/air-quality-and-health/health-impacts

[4] Adams et al., 2021 https://doi.org/10.1111/ina.12865

[5] Reham & Gamaleldin et al., 2020 https://microbiologyjournal.org/prevalence-of-bacteria-in-primary-schools/

[6] Viegas et al. 2019 https://doi.org/10.3390/microorganisms7080234

Figure 13 - Poster 1: Fungal Contamination in Lisbon's Primary Schools - Sampling Insights and Analytical

11.7. Appendix 7

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]

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[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.

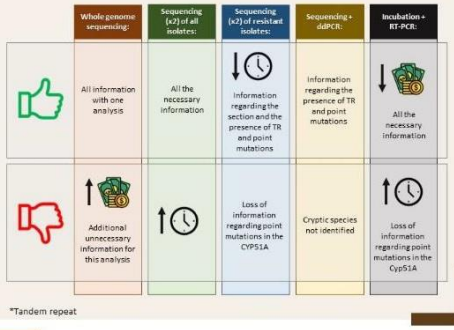
Objective

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

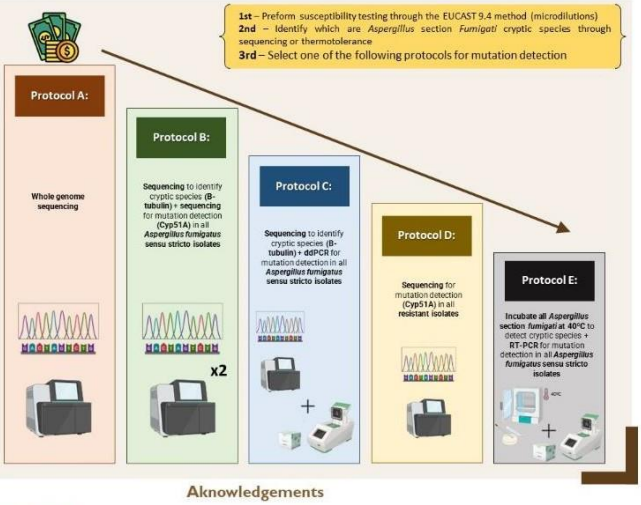
Hypotheses were determined based on the advantages and disadvantages of each suggested method, including its cost.



*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].



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

[1] Gonçalves et al. (2021) https://dx.doi.org/10.3390/microorganisms9010057
[2] Viegas et al. https://doi.org/10.1080/09603123.2020.1810210
[3] Van Der Torre et al. (2020) https://doi.org/10.3390/jof6010012
[4] WHO (2022) https://www.who.int/publications-detail-redirect/9789240060241
[5] Arastehfar et al. (2021) https://doi.org/10.1016/j.simyco.2021.100115

Aknowledgements

This project was supported by FCT/MCTES UIDP/05608/2020 (https://doi.org/10.54499/UIDP/05608/2020) and UIDB/05608/2020 (https://doi.org/10.54499/UIDB/05608/2020. This work is also supported by national funds through FCT/MCTES/FSE/UE, 2023.01366.BD; UI/BD/153746/2022 and CE3C unit UIDB/00329/2020 (https://doi.org/10.54499/UIDB/00329/2020); UI/BD/151431/2021 (https://doi.org/10.54499/UI/BD/151431/2021); and Instituto Politécnico de Lisboa, national support through IPL/2022/inChildHealth/BI/12M; IPL/IDI&CA2023/FoodAIREU_ESTeSL; IPL/IDI&CA2023/ASPRisk_ESTeSL; IPL/IDI&CA2023/ARAFSawmills_ESTeSL

Figure 14 - Poster 2: Budget-friendly protocol for TR34/L98H and TR46/Y121FT289A mutation

11.8. Appendix 8

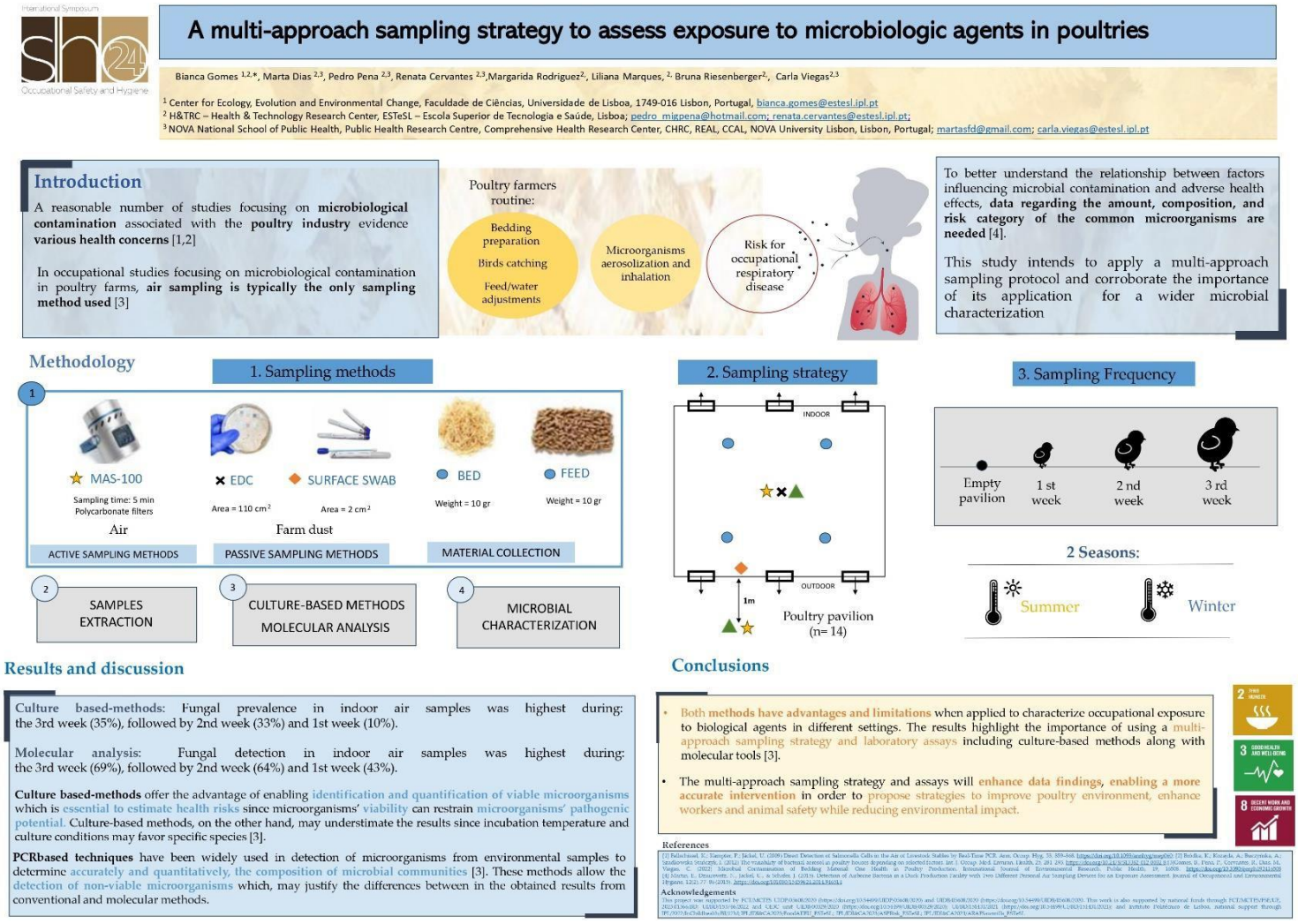


Figure 15 - Poster 3: A multi-approach sampling strategy to assess exposure to microbiologic agents in poultries

11.9. Appendix 9

First insights of Portuguese Primary schools' Fungal assessment – Is Indoor Air Quality legal framework suitable for this indoor setting?

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

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Introduction

The assessment of microbial indoor air quality in schools is vital for promoting student health. Portugal's regulations focus on commercial buildings (Ordinance nº 138-G/2021), neglecting standards for schools (1). Evidence suggests indoor/outdoor fungal ratio inadequacies in high-risk areas like schools (2).

Objective

To assess fungal threshold adequacy set by the Portuguese ordinance in different sites of schools located in the Lisbon area

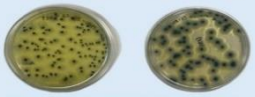
Methods

10 primary schools

- Bathrooms (N=6)
- Canteen (N=9)
- Classrooms N=10)
- Gymnasium (N=6)
- Library (N=8)
- Outdoor (N=10)

200L at 100 L/min

MEA | DG18



27 ºC for 7 days

Fungal identification

Results

- 9 / 10 did not comply with the Portuguese legal framework (I/O ratio) (Figure 1):
  - 5 / 6 in the bathroom
  - 4 / 9 in the canteen
  - 8 /10 in the classrooms
  - 3 / 8 in the library
  - 4 / 6 in the gymnasiums

Figure 1: Fungal quantification in MEA and DG18 in all the settings in each school and outdoor concentration.

- 1 / 10 (S7) complies with the Portuguese IAQ legal framework (I/O ratio).
- *Aspergillus* sections identified in all the schools (Table 1), including the one that complied with the legal framework (S7 - sections *Circumdati* and *Fumigati*)

Table 1: *Aspergillus* sections identified in each media per *school*.

	MEA	DG18
Circumdati	4/10	9/10
Flavi	2/10	6/10
Fumigati	3/10	3/10
Nidulantes	3/10	7/10
Nigri	9/10	5/10

Discussion

Although the quantitative cut-off complies in at least one school (S7), it does not meet the toxigenic species quantitative cut-off (1). The presence of critical species such as *Aspergillus* sections *Circumdati*, *Flavi*, *Nidulantes*, *Nigri*, and *Fumigati* in every school environment jeopardizes students' health and hampers learning conditions (3). Regarding *Fumigati* section, being classified as critical by WHO, its presence should be 0 CFU due to its pathogenic potential (4).

Conclusions

- It is crucial to perform microbial air quality surveillance in Portuguese schools.
- The current IAQ Portuguese legal framework is not suitable to apply in schools.
- The risk of exposure to toxigenic and with clinical relevance fungal species poses a major public health threat impacting also students' learning conditions and outcomes.

Acknowledgments:
This project was supported by FCT/MCTES UIDP/05608/2020 (https://doi.org/10.54499/UIDP/05608/2020) and UIDB/05608/2020 (https://doi.org/10.54499/UIDB/05608/2020. This work is also supported by national funds through FCT/MCTES/PIDE/UE; UIDB/153746/2022 and CE3C unit UIDB/00329/2020 (https://doi.org/10.54499/UIDB/00329/2020); UIDB/151431/2021 [illegible]; and by Instituto Politécnico de Lisboa, national support through IPL/2022/InCHdHealth/BI/12MI, IPL/ID&CA2023/FoodAIEU_ESTeSL, IPL/ID&CA2023/ASPRisk_ESTeSL, IPL/ID&CA2023/ARAFSwemills_ESTeSL.
Partly funded by the European Union's Horizon 2021 research and innovation program under grant agreement no. 101056883 and received co-funding from the author's organizations and/or Ministries. This work has received funding from the Swiss State Secretariat for Education, Research, and Innovation (SERI) grant number 22.00324, from the United Kingdom Research and Innovation (UKRI) grant number 10040524, and the Australian National Health & Medical Research Council (NMHRC) grant numbers APP2017786 and APP2008813.

References:
1. República D da. Diário da República. [cited 2023 Jun 18]. Portaria n.o 138-G/2021. Available from: https://diariodarepublica.pt/
2. Viegas C, et al. Bioburden in health care centers: Is the compliance with Portuguese legislation enough to prevent and control infection? Building and Environment. 2019;160:106226.
3. Norback D, et al. Endotoxin, ergosterol, muramic acid and fungal DNA in dust from schools in Johor Bahru, Malaysia — Associations with rhinitis and sick building syndrome (SBS) in junior high school students. Science of The Total Environment. 2016 Mar 1;545–546:95–103.
4. WHO. WHO fungal priority pathogens list to guide research, development and public health action [Internet]. 2022 [cited 2023 Mar 2]. Available from: https://www.who.int/publications-detail-redirect/9789240060241.

Figure 16- Poster 4: First insights of Portuguese Primary schools' Fungal assessment – Is Indoor Air Quality legal framework suitable for this indoor setting?