Universidade do Minho
Escola de Engenharia

Lívia Vanessa Ferreira de Aguiar

Indoor Biological Agents:
Evaluation of Primary Schools Environments

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Master Thesis for MSc Degree in Human Engineering

Work under the supervision of:
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RESUMO

A associação entre a saúde das crianças e a exposição a poluentes encontrados no ar interior das escolas tem sido estudada por diversos investigadores que referem fortes associações entre as diferentes exposições que caracterizam a qualidade do ar interior (QAI) e a prevalência da asma/alergia entre as crianças, que são consideradas uma população suscetível. Os estudos mais recentes sugerem que a diversidade de exposição a agentes microbiológicos poderá ter efeitos protetores para o desenvolvimento da asma. Este facto realça a importância de aumentar o conhecimento acerca da constituição microbiológica do ar interior.

O objectivo principal deste estudo é avaliar e caracterizar a exposição de crianças a agentes biológicos no ar interior de 20 escolas primárias do Porto, Portugal, através de um estudo transversal.

As avaliações decorreram em dois períodos de aquecimento, num total de 71 salas de aulas, incluindo amostragens de bactérias, fungos e endotoxinas no ar interior. As amostragens de bactérias e fungos foram realizadas com um amostrador microbiológico de ar. As avaliações de endotoxinas foram efetuadas através de um método ativo de recolha de ar e a sua análise através do método LAL (*Limulus Amebocyte Lysate*).

Em todas as escolas primárias avaliadas, as concentrações globais de bactérias e fungos foram superiores aos seus respetivos valores de referência, enquanto as concentrações de endotoxinas foram inferiores ao valor recomendado. *Penicillium* sp. e *Cladosporium* sp. foram os principais fungos identificados no ar interior. As concentrações de bactérias e endotoxinas variaram positivamente com a humidade relativa e o dióxido de carbono e, ao contrário do que seria esperado, não demonstraram qualquer interdependência entre si. As concentrações de endotoxinas revelaram ser mais dependentes das diferentes características estruturais dos edifícios escolares e das salas de aulas do que as concentrações de bactérias e fungos.

Para melhorar a QAI, devem ser adotadas estratégias de controlo na fonte e medidas de ventilação para a prevenção de efeitos negativos na saúde das crianças nas escolas, como o desenvolvimento e agravamento da asma, alergias e sintomas respiratórios.

PALAVRAS-CHAVE

Qualidade do Ar Interior, Escolas, Bactérias, Fungos, Endotoxinas

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ABSTRACT

The association between children's health and indoor air exposure in schools has been studied by several researchers, which documented strong associations between various exposures that characterize indoor air quality (IAQ) and the prevalence of asthma/allergy among children, considered a susceptible population. Recent findings suggest that the diversity of microbiological exposure seem to have protective effects on asthma development. This emphasizes the importance of increasing knowledge on indoor air microbiological characterization.

This study aim was to evaluate and characterize children's exposure to biological pollutants in indoor air of 20 primary schools in Porto, Portugal, through a cross-sectional study.

Indoor air biological assessment took place in two heating season periods, in a total of 71 classrooms, comprising bacteria, fungi and endotoxins air sampling. Bacteria and fungi air sampling was carried out with a microbiological air sampler. Endotoxin assessment was performed through active air sampling and analysis through LAL (Limulus Amebocyte Lysate) method.

Overall concentrations of bacteria and fungi were both higher than its respective reference values, while endotoxins concentrations were below the recommended value in all evaluated primary schools. *Penicillium sp.* and *Cladosporium sp.* were the prevalent fungi species found indoors. Bacteria and endotoxins concentrations were higher with higher levels of relative humidity and carbon dioxide and contrarily to what was expected, bacteria and endotoxins concentrations showed no relation between them. Endotoxins concentrations were found to be more influenced by different school building and classrooms characteristics than bacteria or fungi concentrations.

To improve IAQ, strategies of source control and ventilation measures should be adopted for the prevention of adverse health consequences to children in schools, like the development and aggravation of asthma, allergies and respiratory symptoms.

KEYWORDS

Indoor Air Quality, Schools, Bacteria, Fungi, Endotoxins

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List of Acronyms and Abbreviations

ACGIH	American Council of Government Industrial Hygienists
ARIA	How indoor air quality can affect children allergies and asthma
ASHRAE	American Society of Heating and Air-Conditioning Engineers
BiBa	Indoor Air in Primary Schools
CFU	Colony forming units
CFU/m^3	Colony forming units per cubic meter
CO_2	Carbon dioxide
DALY	Disability-Adjusted Life Year
DECOS	Dutch Expert Committee on Occupational Standards
EFA	European Federation of Asthma and Allergy Associations
EnVIE	Co-ordination Action on Indoor Air Quality and Health Effects
EN	European Standards
EU	Endotoxin Units
EU/g	Endotoxin Units per gram
EU/m^2	Endotoxin Units per square meter
EU/m^3	Endotoxin Units per cubic meter
EU/mL	Endotoxin Units per milliliter
EU/mg	Endotoxin Units per milligram
FCT	Portuguese Foundation for Science and Technology
FEUP	Faculty of Engineering of University of Porto
FMUP	Faculty of Medicine of University of Porto
GFA	Glass microfibre filter
HBROEL	Health-Based Occupational Exposure Limit
HESE	Health Effects of School Environment
HITEA	Health Effects of Indoor Pollutants: Integrating Microbial, Toxicological and Epidemiological Approaches
IAQ	Indoor Air Quality
IAIAQ	Promoting actions for healthy indoor air
IBM	International Business Machines

INEGI	Institute of Mechanical Engineering and Industrial Management
INSA	Portuguese National Institute of Health Doutor Ricardo Jorge
I/O	Indoor/Outdoor ratio
IOM	Institute of Medicine
IRSST	Institut de Recherche Robert-Sauvé en santé et en sécurité du travail
ISAAC	International Study of Asthma and Allergies in Childhood
ISPUP	Public Health Institute of University of Porto
ISO	International Standard Organization
L	Liters
L/min	Liters per minute
LAL	Limulus Amebocyte Lysate
LOQ	Limit of Quantification
LPS	Lipopolysaccharides
Max.	Maximum
MEA	Malt Extract Agar
Min.	Minimum
NIOSH	National Institute for Occupational Safety and Health
NOEL	No Observed Effect Levels
PATY	Pollution and the Young
OEL	Occupational Exposure Limit
OQAI	Observatory Network on Indoor Air Quality
OSHA	Occupational Safety and Health Administration
P25	25th Percentile
P75	75th Percentile
PCR	Polymerase Chain Reaction
ppm	Parts per million
RECS	Energy Performance Regulation for Buildings of Trade and Services
REH	Energy Performance Regulation for Residential Buildings
RLV	Relative Limit Values
RH	Relative Humidity
SCE	Building Energy Certification System
SD	Standard Deviation

SEARCH	School Environment and Respiratory Health of Children
SINPHONIE	Schools Indoor Pollution and Health: Observatory Network in Europe
SPSS	Statistical Package for Social Sciences
T	Temperature
TLV	Threshold Limit Values
TSA	Tryptic Soy Agar
TVOC	Total Volatile Organic Compounds
TWA	Time-Weighted Average
USA	United States of America
US EPA	United States Environmental Protection Agency
VOC	Volatile Organic Compounds
WHO	World Health Organization

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1. INTRODUCTION

The World Health Organization (WHO) has identified several risk factors for the development of disease and recognized that indoor air pollution is the 8th most important risk factor. In the World Health Report of 2014, it was estimated that 4.3 million deaths are caused annually by indoor air pollution (WHO, 2014). The United States Environmental Protection Agency (US EPA) has classified Indoor Air Quality (IAQ) among the main five environmental risks to public health (EPA, 2012).

The quality of the indoor environment is a major health concern due to the fact that the main indoor air source is outdoor air, plus additional pollutants emitted from building materials and consumer products. Most of the indoor air interacts directly with outdoor air through building openings that are apparently controlled (windows, doors and air-specific uptakes and exhausts), as well as some unwanted openings (leaks, cracks, etc.). If outdoor air is not clean it becomes a particularly critical and major source of pollution indoors (Jantunen *et al.*, 2011). Studies developed by US EPA indicate that indoor levels of pollutants can be two to five times - and occasionally more than 100 times - higher than outdoor levels. These contamination levels raise concern as people spend about 80-90% of their lifetime indoors and therefore indoor environment forms the basic breathing and dermal exposure background (Ashmore & Dimitroulopoulou, 2009; Herberger *et al.* , 2010; Wang *et al.*, 2007; Zhao & Wu, 2007). IAQ is also greatly affected by the level of economic development, with big differences between developing and developed countries (Etzel, 2007).

Based on the Eurostat demographic database, during the 2010/2011 academic year there were over 64 million students in Europe in kindergartens, primary and lower secondary education. There were about 15 million children in nursery schools, about 28 million pupils in primary schools, and about 22 million students in lower secondary schools. In most countries, students attend school for five or six days per week and spend on average from 700 to 900 hours per year in classrooms (Csobod *et al.*, 2014). Worldwide, the average number of years of schooling grew from 7.9 years in 1970 to 11.0 years in 2008; in Portugal, it already attained 12.9 years in 2008 (Macedo *et al.*, 2013).

This study was accomplished in the context of Human Engineering Masters' Degree, Department of Production and Systems from Engineering School, University of Minho, within the scope of ARIA Project: "*How indoor air quality can affect children allergies and asthma*"

(PTDC/DTP-SAP/1522/2012 from FCT - Science and Technology Portuguese Foundation), performed between 2013 and 2015. This project intended to explore associations between various exposures that children experience in their indoor environments (specifically their homes and primary schools) and their health. The targeted health issues were allergy, asthma and respiratory symptoms. This project is based upon a multidisciplinary team work and the institutions participating in the study were Faculty of Medicine (FMUP), Faculty of Engineering (FEUP), Institute of Mechanical Engineering and Industrial Management (INEGI) and Public Health Institute (ISPUP), all from University of Porto, with the collaboration of the Environmental Health Department of Portuguese National Institute of Health Doutor Ricardo Jorge (INSA).

This study aims to evaluate and characterize children's exposure to biological pollutants based in measurements performed in 20 primary schools' indoor air, located in Porto, from 3rd to 4th grades classrooms, and children with ages between 7 and 12 years old. To accomplish its aim, a cross-sectional epidemiological study was performed in order to investigate indoor biological levels, including fungi identification, and compare them with national and international guidelines. Possible correlations between indoor air biological agents with other chemical and physical parameters were explored as well as if buildings and classrooms' characteristics had influence on indoor air biological levels.

1.1. Indoor Parameters and Sources

IAQ in schools is generically characterized by a complex exposure to various indoor pollutants such as chemical (e.g. particulate matter, volatile organic compounds (VOCs), formaldehyde), physical (e.g. temperature, relative humidity) and microbiological (e.g. fungi and bacteria), sometimes in quite high concentrations. An overview of the main indoor pollutants in schools and related sources can be found in European Federation of Asthma and Allergy Associations (EFA) (2000) and Csobod *et al*. (2010).

Each indoor environment has unique characteristics that are influenced by a combination of pollution sources: (i) penetration of outdoor pollutants (local outdoor air, the type of activity in the surroundings and the environment quality); (ii) specific building characteristics (construction and covering materials, emissions from furniture and other materials, water leaks or damp surfaces and building maintenance procedures); (iii) indoor activities (cleaning practices, occupant behavior, level and length of occupancy); and (iv) ventilation system (Madureira, 2014). The few studies carried out on the effectiveness of remedial measures in Europe show that schools

frequently have IAQ problems due to poor building construction and maintenance, poor cleaning and poor ventilation. The studies also demonstrate that pollution at school is complex and variable and has clear impacts on health (Csobod *et al.*, 2014).

Poor IAQ in classrooms can lead to health problems for occupants in addition to reducing learning performance, attendance of students, and ambient comfort (Madureira *et al.*, 2015b). IAQ is characterized by physical factors (such as ambient temperature, humidity, ventilation rate), air pollutant factors (pollutant levels and exposure times) and human factors (activities and health status) (Bakke *et al.*, 2008; Dales *et al.*, 2008; Giulio *et al.*, 2010; OSHA, 2011).

Changes in construction designs in order to reduce energy demand in buildings resulted in better sealed buildings, lower ventilation rates and non-openable windows (Clausen *et al.*, 2011). Low ventilation rates have a negative effect on air renovation, due to less input of fresh air, and may cause increased concentrations of indoor-generated pollutants, which can be harmful to human health, and have been also associated with increased indoor air humidity and, therefore, higher risk of dampness and mold in buildings (Emenius *et al.*,1998; Sundell *et al.*, 1995).

1.2. Guidelines

In Portugal, Directive no. 2010/31/EU of the European Parliament and of the Council, of 19th May 2010, concerning buildings energy performance was transposed into national law by Decree-Law no. 118/2013 of 20th August, that replaced the existent national orientations for IAQ established at Decree-Law no 79/2006 of 4th April. This recent legislation aims to ensure and promote the improvement of energy performance of buildings through the Building Energy Certification System (SCE), which includes the Energy Performance Regulation for Residential Buildings (REH) and the Energy Performance Regulation for Buildings of Trade and Services (RECS). Regarding IAQ, the aforementioned law provides the establishment, in a specific ordinance, of minimum levels of fresh air rates by area, depending on the occupation, the characteristics of the building itself and its air conditioning systems, as well as protection thresholds for indoor air pollutants concentrations, in order to safeguard health protection and well-being of occupants. These requirements were established in Ordinance no. 353-A/2013 of 4th December. In this review of national legislation, it is important to notice that natural ventilation is privileged instead of mechanical ventilation equipment, in a perspective of resources optimization, energy efficiency and cost reduction. Adopted measures in this legislation intend to go towards the improvement of national buildings energy efficiency and create tools and

methodologies that support the definition of strategies, plans and mechanisms to encourage energy efficiency.

Worldwide, WHO IAQ guidelines represent the state-of-the-art in pollutants risk assessment (WHO, 2009, 2010). These guidelines act as limiting values for a group of pollutant concentrations or exposures, resultant from the best and most universally representative data currently available. The guidelines are a reference for risk assessment and risk management as they "are based on the accumulated scientific knowledge available at the time of their development". They have the character of recommendations and countries can refer to WHO guidelines as a scientific basis for legally compulsory standards or legislation (WHO, 2010).

1.3. Thesis outline

1. **Introduction**: hosts IAQ theme, along with its main parameters and sources. National and international IAQ guidelines are also presented.
2. **Relevance and Objectives**: contains the explanation of study relevance and its main and specific objectives.
3. **Literature Review**: it addresses the issues regarding bacteria, fungi and endotoxins characterization; the findings of respiratory health effects on children caused by IAQ status; the importance of walk-through of school building and classroom characteristics in IAQ studies; and the state-of-art of IAQ studies in schools.
4. **Methodology**: study design and the different steps taken at the cross-sectional study are described, as well as how analysis and calculations of all data were conducted.
5. **Results**: first, a characterization of the evaluated school buildings and classrooms is presented, followed by results presentation of the indoor air biological parameters assessment. Fungi genera and species identified in the evaluated classrooms and outdoors are also described. Influence of outdoor air on indoor air biological concentrations were presented, as well as correlations between indoor biological parameters and other selected parameters of IAQ (chemical and physical). Results of the investigation regarding possible differences in indoor air concentrations of biological parameters (bacteria, fungi and endotoxins) and school building/classroom characteristics are presented.

6. **Discussion**: discussion and study limitations are provided. Intervention and/or improvement measures, which are suggested in an attempt to contribute for better IAQ in primary schools, are included.
7. **Conclusion**: it includes a systematization of the study's main findings and it is also highlighted possible future investigations on this thesis subject.

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2. RELEVANCE AND OBJECTIVES

In this Chapter the importance of the study is described, along with its main purpose, research questions and specific objectives explaining how such questions are going to be answered.

2.1. Relevance of the study

Buildings such as offices, schools and homes differ so greatly in terms of scope, requisites and use that it makes sense to refer to schools as a special case in the context of IAQ policies. In Europe, there are over 64 million students and almost 4.5 million teachers who spend many hours per day inside kindergartens, primary and secondary schools (Csobod *et al.*, 2014). As children spend more time in schools than in any other environment except home, the levels of indoor air pollution in schools will significantly impact a child's total exposure to pollutants (Madureira, 2014). Another important impact of IAQ is on productivity, attendance and academic performance of students and teachers that can be affected by discomfort or illness caused by poor IAQ. Therefore, understanding the air pollution in these environments, documenting their concentrations and determining which factors influence these levels is very important (Madureira *et al.*, 2015b).

Although the home environment has been the focus of primary and secondary interventions, previous studies have shown that public areas, including schools, can also be a relevant source of exposure to biological agents, as schools present a particularly high risk due to overcrowding and to high incidence of infectious diseases among children (Tamburlini *et al.*, 2002). Since attendance within the school environment is compulsory, the school authorities should be under an obligation to provide a school environment that is appropriate for children, in particular to those with allergies or other kind of hypersensitivity (Csobod *et al.*, 2014).

A relevant factor that should be also considered is that primary school buildings have reduced budgets for design, construction and operation resulting in an inadequate maintenance of school buildings. The association between children's health and indoor air exposure in schools has been studied by several researchers (Clausen *et al.*, 2009; Mendes *et al.*, 2014; Simoni *et al.*, 2010), which documented strong associations between various exposures that characterize IAQ and the prevalence of asthma/allergy among children. Nevertheless, although the considerable interest in recent years for the health effects of indoor air pollution, issues such as specific school

environment determinants associated with the burden of these diseases remain poorly characterized. Notwithstanding, schools IAQ and its associated impacts on health, as well as the influence of building and classrooms characteristics and occupants' behavior on IAQ parameters levels, has been far less studied than in some other types of buildings (e.g. offices and homes).

Special attention needs to be taken due to the fact that children are considered a susceptible population: they are more susceptible to illnesses caused by air pollution because their immune system and organs are still developing and immature and, therefore, they are particularly vulnerable to the development of respiratory diseases, such as asthma. In fact, children breathe a greater volume of air relative to their body weight compared to adults and their organs are actively growing (Madureira *et al.*, 2014; Roda *et al.*, 2011). Moreover, children are less likely than adults to comprehend and clearly communicate their symptoms. Nevertheless, they spend much of their time inside crowded classrooms, in a confined atmosphere, reasons why they should deserve priority attention in IAQ studies.

The prevalence of asthma and allergic respiratory diseases among children has increased quite rapidly in the last decades (Sun *et al.*, 2009), at such a quick pace that the finding cannot be explained by genetic changes and it is more likely to be due to changes in environmental exposures and/or in lifestyle (Etzel, 2007). Recent findings suggest that the diversity of both fungal and bacterial exposure seem to have protective effects on asthma development, although the researchers were not able to specify the microorganisms that may confer protection (Ege *et al.*, 2011). This emphasizes the importance of increasing knowledge on indoor air microbiological characterization: investigation should focus on indoor air levels and types of microorganisms. As mentioned in Madureira *et al.* (2015a), the information about the concentrations and distribution of airborne biological agents is scarce and loose in Portugal. Therefore, studies on this subject are of extreme importance in order to bring more knowledge on the subject and conduct appropriate intervention intended to protect susceptible populations from being exposed to a hazardous indoor environment.

In Portugal, IAQ evaluation is an increasing and important subject, proved by the development of studies in this area, more sorely since the publication of the national legislation, Ordinance no. 353-A/2013 of December 4th, that establish reference values of maximum concentration for selected indoor air pollutants. These IAQ studies are important for this update and review of guidelines and they can also contribute to the awareness of the importance of proper

maintenance procedures, in order to reduce potential sources of indoor air contamination and leading to better schools indoor environments.

For all the previous reasons, it is of extreme importance to characterize indoor air levels of biological agents in primary schools and investigate possible associations with children's health, as well as its variation according with other indoor air pollutants, and building and classrooms characteristics.

2.2. Research Questions and Objectives

This study aims to answer the following research questions:

1. Do indoor levels of bacteria, fungi and endotoxins in primary schools exceed the respective national and international reference values?
2. Is outdoor air the major influence on indoor air biological levels at schools?
3. What are the predominant fungi genera/species identified in indoor environments of primary schools?
4. Indoor air biological parameters are influenced by the temperature (T) and/or the relative humidity (RH) of the indoor environment? And what about carbon dioxide (CO_2)?
5. Is there any relationship between the bacteria, fungi and endotoxin concentrations found indoors?
6. Are indoor biological concentrations affected by building and/or classrooms characteristics, including occupancy?
7. How can we improve IAQ of primary schools, regarding indoor air biological parameters?

To uncover answers to these questions this study aims to evaluate children's exposure to indoor air biological pollutants in primary schools of Porto, Portugal, with the following specific objectives:

1. to assess and quantify bacteria, fungi and endotoxins concentrations, in 20 primary schools, and to compare the attained results with national/international guidelines, as well as to compare with other national/international studies;
2. to assess the impact of outdoor bacteria and fungi concentrations in indoor air;
3. to identify the main fungi genera/species present;

4. to investigate the influence of CO_2, T and RH concerning the biological pollutants concentrations;
5. to determine if the concentration of bacteria, fungi and endotoxins varies according with each other's concentrations;
6. to investigate if primary schools' buildings/classrooms characteristics and occupants activities interfere with indoor air biological contamination levels;
7. to suggest intervention and/or improvement measures for a better IAQ in primary schools.

3. Literature Review

This chapter focuses on the characterization of the biological agents targeted in the study (bacteria, fungi and endotoxins) as well as on a brief description of asthma, allergies and respiratory symptoms. Moreover is also presented an overview of the influence of buildings and classrooms characteristics in IAQ and, finally, a state-of-art of IAQ studies in schools.

3.1. Biological Agents

Bioaerosols consist of aerosols containing microorganisms, like bacteria and fungi, or organic compounds derived from microorganisms, namely endotoxins (Cabral, 2010; Mandal & Brandl, 2011), which can penetrate into human body mainly through the nose, mouth, and eyes. In the human respiratory tract, the penetration depth depends mainly on the particle size (Cabral, 2010). Because of its biological complexity, bioaerosol is described as mixed biological material; chemical tests show that its composition is 70% of organic and 30% of inorganic components (Matković *et al.*, 2012). As aerosolization and indoor transport of airborne particles are largely governed by environmental conditions, their amount varies during the day, as well as over the year, within the same building (Matković *et al.*, 2012; Singh *et al.*, 2011). Consequently, the concentrations of airborne microbial contaminants represent transient and variable exposure levels (Singh *et al.*, 2011).

Outdoor airborne bacteria and fungi derive from soil, plants, and dead or decaying matter, and are present in every indoor living environment playing an ambivalent role in human health and disease, thus provoking both beneficial and adverse effects in exposed people (Csobod *et al.*, 2014; Jacobs, 2013). In fact, there are conflicting data about how early exposure may increase or may decrease the risk of future sensitization (Dales *et al.*, 2008): while Pegas (2012) affirms that exposure to bacteria and fungi may cause allergic reactions, asthma, and other respiratory complaints, excluding pathogenic bacteria that trigger specific diseases, on the other hand, Csobod *et al.* (2014) and Rintala *et al.* (2008) supports the finding that protective effects of exposure to elevated levels of microbial agents have been reported in many studies in rural environments. Adverse health outcomes are typically reported in connection with conditions of indoor dampness and associated microbial proliferation. Once again, contradictory statements can be found in the literature regarding health effects caused by exposure to indoor molds and

dampness. Dales *et al.* (2008) confirm that reports of indoor molds or dampness, or both, are consistently associated with increased respiratory symptoms but causality has not been established; in Csobod *et al.* (2014), the association between indoor moisture and dampness and health effects is well established; for Madureira (2014), dampness has been suggested to be a strong, consistent indicator of risk of asthma and respiratory symptoms (e.g. cough and wheeze). One thing that all have in common is that there are indications that dampness-related exposure affects human health; however, little is known on the underlying causal agents, which can be due to the lack of valid methods available for the exposure assessment (Jacobs, 2013). This ambivalence in the interaction between biological agents and humans calls for a more specific and detailed description of microbial exposures (Csobod *et al.*, 2014).

Indoor environments have been found to harbor microbial taxa not commonly found outdoors (Kembel *et al.*, 2012). In-depth studies of indoor air have shown that the concentration of bioaerosols on indoor air is significantly affected by moisture content of building material, RH and T, outdoors concentrations (particles are transferred to the inside through openings of the building envelope, such as windows and doors), indoor source strength, removal and deposition rate within the structure, indoor mixing, and chemical reaction, as well as air exchange rates, and number of occupants and pets (Mandal & Brandl, 2011; Mitchell *et al.*, 2007). Another important source of bioaerosols in indoor air includes furnishing and building materials, microbiological contamination within the walls and ceilings and floor activities. Notwithstanding, one of the most important factors affecting IAQ is how the building is heated, ventilated, air-conditioned (Mandal & Brandl, 2011; Meklin *et al.*, 2003; Tamburlini *et al.*, 2002). Factors that might determine the relative importance of these sources, within and among buildings, or which one is the most important, remains unclear (Kembel *et al.*, 2012; Meklin *et al.*, 2003).

3.1.1. Bacteria

Bacteria found in indoor environments are typically originated from human sources (shedding of skin and human oral and respiratory fluid emitted via coughing, sneezing, talking and breathing), pets, solids, plants or from outdoors (Hospodsky *et al.*, 2012; Madureira *et al.*, 2015a; Pegas, 2012). Bacteria living on the skin of individuals inhabiting a particular space define the microbial community observed in air samples taken from that same space; humans clearly contribute substantially and perhaps predominantly, to indoor microbial diversity (Kelley & Gilbert, 2013). Indoor settings also obtain a considerable fraction of their airborne bacteria populations from

environmental sources (Hospodsky *et al.*, 2012; Kelley & Gilbert, 2013; Pegas, 2012). The amount and type appears to depend on the degree of outdoor ventilation and the local climate (Kelley & Gilbert, 2013; Madureira *et al.*, 2015a). Kembel *et al.* (2012) found significant differences in the contribution of natural environmental bacteria in buildings that were well ventilated versus those with sealed windows and indicated that natural ventilation does influence airborne bacterial concentrations in the absence of active human occupants (Kelley & Gilbert, 2013; Madureira *et al.*, 2015a).

Gram-positive bacteria normally dominate the flora, at least in culture methods studies, comprising up to 75% of the bacteria present in the indoor air. Certain Gram-positive bacteria with strong immunogenic properties or potential toxin production, such as mycobacteria, streptomycetes or *Nocardiopsis sp.*, have also been shown to be present in indoor environments (Rintala *et al.*, 2008). Nevertheless, typical and most important bacterial strains found in indoor atmospheres are representatives of the genera *Bacillus*, *Micrococcus*, *Kocuria* and *Staphylococcus* (Mandal & Brandl, 2011).

Traditionally, infections are considered as the main health effect of bacteria; however, these microorganisms can affect our health in many other ways (Rintala *et al.*, 2008). The components of airborne bacteria in the indoor environment are linked to the development and exacerbation of chronic respiratory illness including asthma (Hospodsky *et al.*, 2012). In fact, several studies have reported that exposure to large concentrations of airborne microbes is often associated with asthma and rhinitis, hypersensitivity pneumonitis and sick building syndrome. Bacteria known to have allergenic or immunotoxic effects on human health, such as *Bacillus sp.*, *Streptomyces albus*, *Pantoea agglomerans, Pseudomonas chlororaphis*, *Arthrobacter globiformis*, *Thermoactinomycetes vulgaris*, and *Corynebacterium sp.*, were identified among various indoor environment (Mandal & Brandl, 2011). These associations are important in industrialized countries and in cities of emerging nations where people spend at least 85% of their time indoors (Hospodsky *et al.*, 2012).

3.1.2. Fungi

Many species of fungi live as commensal organisms in or on the surface of the human body. “Mold” is the common term for multicellular fungi that grow as a mat of intertwined microscopic filaments (hyphae). Exposure to molds and other fungi and their spores is unavoidable except when the most stringent of air filtration, isolation, and environmental sanitation measures are

observed, for example, in organ transplant isolation units (Hardin *et al.*, 2003). Fungi and their spores are more resilient than viruses and bacteria, being able to withstand greater stresses owing to dehydration and rehydration, as well as ultraviolet radiation (Tang, 2009).

In the atmosphere, fungi are present in bioaerosols. Fungal spores constitute a significant fraction of bioaerosol microbial particles and are often 100–1000 times more numerous than other bio-particles, like pollen grains. Until recently, it was thought that indoors, fungi existed mainly as spores (in groups or singly), but work carried out by several research teams showed that indoor fungi grown in culture media or building materials, and subjected to an air current, can release groups of spores, individual spores and fungal fragments. The concentration of fungal spores in bioaerosols depends on three important biological factors: (i) magnitude of sporulation; (ii) spore release from conidiophores; and (iii) conidia dimensions and weight (Cabral, 2010).

Sources for indoor airborne fungi can be outdoor air, since fungi spores have the potential to be blown into a building that uses natural ventilation, and indoor reservoirs (Madureira *et al.*, 2015a; Tang, 2009). Fungal spores in outdoor air are a major source for indoor fungi during the growing seasons (e.g., spring and summer) for naturally ventilated buildings. Potential promoting factors for fungal growth that are frequently found are excessive humidity and/or high water content of building materials, which are often caused by deficiencies in the buildings, such as lack of thermal insulation, as well as incorrect behavior of occupants (Madureira *et al.*, 2015a). Molds proliferate in environments containing excessive moisture such as from leaks in roofs and walls, and can also enter the building through heating and conditioning systems (Tamburlini *et al.*, 2002). Factors that might influence the types of fungi that can grow on particular materials include the nature of the material and the dynamics of water availability, temperature, and light. Fungi that are able to colonize indoor materials are generally those (i) with broad nutritional requirements, (ii) able to colonize very dry environments, or (iii) that readily degrade the cellulose and lignin present in many indoor materials. The apparent absence of visible or measurable indoor growth does not ensure absence of exposure (Spengler *et al.*, 2000).

Important fungal strains usually present in indoor air samples are comprised of the genera *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium* (Mandal & Brandl, 2011; Spengler *et al.*, 2000; Tamburlini *et al.*, 2002), although many different fungi have been dominant in different localities (Spengler *et al.*, 2000). Concentrations of both total and specific fungi in residential and office settings vary widely from study to study. Some of this variation is related to the types of

indoor environments studied (e.g., mechanically ventilated, air-conditioned, naturally ventilated), the climatic region in which the study was done, and variations in sample collection methods (Spengler *et al.*, 2000). Nevertheless, all fungal strains are able to form spores which are resistant to changing environmental conditions (Mandal & Brandl, 2011). Outdoors, *Cladosporium sp.*, *Alternaria sp.*, *Epicoccum nigrum*, and *Botrytis cinerea* are known to be integral part of the fungal air spora (Fischer & Dott, 2003).

Staying long periods of time in an indoor closed environment can contribute to infection since the lack of aeration, the poor conservation ventilation, temperature regulating systems and relative air humidity may contribute to the prevalence and multiplication of filamentous fungi in buildings, resulting in the spreading of spores in the environment, and opportunity for causing disease, whose gravity depends of individuals' immunological state, airborne spores concentration and exposition time (Barbosa *et al.*, 2012). Airborne fungal spores have been widely recognized as major allergens capable of causing asthma and allergic rhinitis as well as other allergic diseases, in building occupants (Cooley *et al.*, 1998; Douwes *et al.*, 2003; Hardin *et al.*, 2003; Levetin *et al.*, 1995; Madureira *et al.*, 2014). Species of *Penicillium*, *Aspergillus, Alternaria* and *Cladosporium* have been the most frequently associated with allergy and exist both in indoor and outdoor environments (Madureira, 2014; Madureira *et al.*, 2014; Tang, 2009). These fungal species have been found worldwide, in varying mixtures, in both indoor and outdoor environments, where airborne levels of fungi vary seasonally, usually being highest in autumn and summer and lowest in winter and spring (Tang, 2009).

Exposure to fungi can cause disease via three defined mechanisms: immune response (allergy), infection, and toxic-irritant effects by particular products generated (Roda *et al.*, 2011). Despite the absence of validated markers of exposure, efforts have been made to understand the relationship between mold exposures and chronic non-allergic health effects. There remains a lack of consensus regarding the systemic effects of mold exposures (Mitchell *et al.*, 2007).

Fungi from *Aspergillus* species are among the fungi considered to be most pathogenic (Barbosa *et al.*, 2012; Gniadek, 2012), and they also constitute the group of fungi most frequently isolated from the environment (Gniadek, 2012). The various species of *Aspergillus* produce large numbers of small conidia that become airborne and can be inhaled easily and remain in the air for prolonged periods due to their small size, being able to colonize upper or lower airways, possibly resulting in respiratory diseases (Barbosa *et al.*, 2012; Sabino *et al.*, 2014). Airborne *Aspergillus* species, like *Aspergillus fumigatus*, have been linked closely with exacerbation of

asthma and other allergic respiratory diseases, such as mycotoxicosis, allergy, and invasive infections, but can also cause symptomatic allergic lung disease and infectious mycosis (broncho-pulmonary aspergillosis) (Barbosa *et al.*, 2012; Douwes *et al.*, 2003; Sabino *et al.*, 2014). *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger* are among the most pathogenic to humans. Their taxonomic identification is still an open topic because of their morphological variability and ability to produce metabolites; new species, which exhibit adverse health effects to humans, are constantly being detected. *Aspergillus lentulus* is one of the recently detected species of considerable clinical importance; it reveals similarity to *Aspergillus fumigatus* (Gniadek, 2012).

Fungi can be useful indicators of IAQ and that is why it is important to deepen the studies of indoor atmospheres in order to promote air quality, health and well-being of all, and a better understanding of the biology of indoor fungi (Cabral, 2010).

3.1.3. Endotoxins

Endotoxins are well-known substances of the outer cell membrane of Gram-negative bacteria, like *Enterobacteriaceae* or *Pseudomonadaceae* that have high pro-inflammatory properties, and are present in normal indoor environments as constituents of organic dusts or aerosols. In its purified form, it is known as lipopolysaccharide, which is both toxic and immunogenic (Barnig *et al.*, 2013; Dales *et al.*, 2008; Delfino *et al.*, 2011; Douwes *et al.*, 2002; Jacobs *et al.*, 2013; Liebers *et al.*, 2008; Spaan *et al.*, 2007). They are mainly released during cell lysis (cell wall is damaged or when bacteria die), after which they exist in suspension in the atmosphere (Duquenne *et al.*, 2012; Jacobs, 2013).

Endotoxins are composed of proteins, lipids, and lipopolysaccharides (LPS) as shown in Figure 1. LPS of Gram-negative bacteria is an amphiphilic, heat-stable and water-soluble macromolecule responsible for most of the biological properties of bacterial endotoxins (Liebers *et al.*, 2008). LPS comprises three components or regions: Lipid A, an R polysaccharide and an O polysaccharide (Paba *et al.*, 2013). The lipid portion of LPS, lipid A, a phosphoglycolipid containing 3-hydroxy fatty acids, is chemically distinct from all other lipids in biological membranes and responsible for the molecule's characteristic toxicity (Su *et al.*, 2002), whereas the immunogenicity of the LPS is associated with the polysaccharidic fraction of the LPS-molecule (Duquenne *et al.*, 2012; Liebers *et al.*, 2008). The term LPS commonly refers to the

purified form; endotoxin refers to the LPSs attached to other elements of the bacterial membrane (Duquenne *et al.*, 2012).

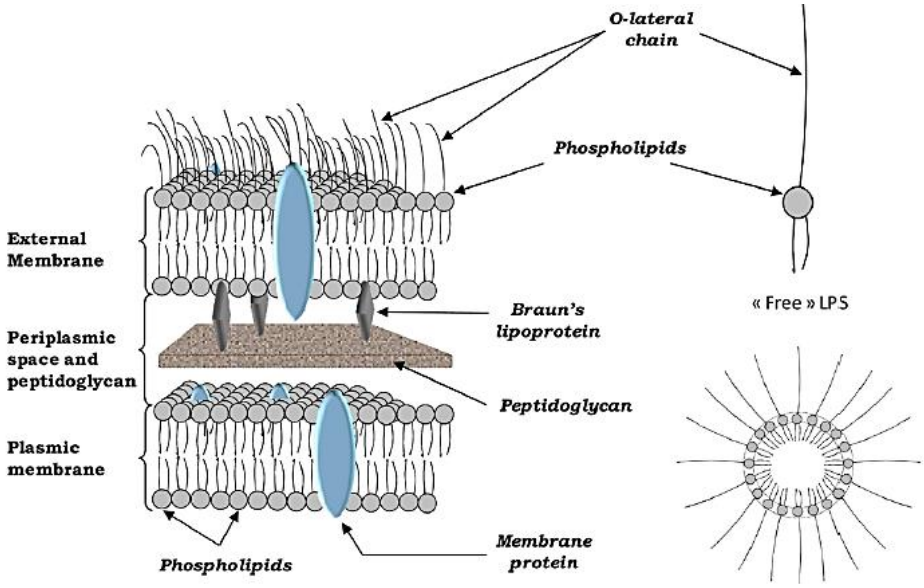

Figure 1 - Endotoxin structures and organization. Location of LPS's in outer membrane of Gram-negative bacteria (Duquenne *et al.*, 2012)

As previously mentioned, endotoxins are released into the environment after bacterial cell wall lysis, but they can also be released during the active growth of bacterial cells. However, their share in the total weight of microorganisms present in the air is relatively small, probably due to their sensitivity to environmental factors. For example, at a higher temperature and relative humidity, gram-negative bacteria phospholipid membranes lose thermodynamic stability and thus vitality (Matković *et al.*, 2012). Laitinen *et al.* (2001) believe that biological endotoxin activity depends on the bacteria types from which they originate. The aerodynamic particles size distribution for airborne endotoxin is also an important element in determining endotoxin toxicity and its health effects (Paba *et al.*, 2013). Once inhaled, these particles can deposit on the respiratory tract walls with a probability closely linked to their size. The depth of penetration and behavior of biological particles in the human respiratory system is strongly influenced by their size, shape, density, chemical composition, and reactivity (Duquenne *et al.*, 2012).

Although obvious in many industrial and agricultural environments, the source of endotoxin exposure may be less apparent in school settings. Cool-mist, ultrasonic, and recirculated spray humidifiers should be considered potential sources of high-level endotoxin exposure. Containing any other sources of water intrusion or condensation may also be useful in lowering endotoxin levels indoors (Spengler *et al.*, 2000). In urban home environments, increased endotoxin levels have been associated with several housing factors including presence of animals (cats and dogs),

pests, agriculture/farming activities, poor housekeeping, absence of central air conditioning, old floor coverings, contaminated air humidifiers, lower ventilation rates, storage of food waste, increased amounts of settled dust, carpeting, higher occupancy and even season (Dales *et al.*, 2008; Delfino *et al.*, 2011; Hyvarinen *et al.*, 2006; Jacobs, 2013; Paba *et al.*, 2013; Sheehan *et al.*, 2012). Dampness can increase endotoxin levels, but the relationship is not very clear as there were studies that did not find strong evidence for this association (Jacobs, 2013).

Given the pervasive presence of Gram-negative bacteria in household dust and air, everyone is exposed to at least low levels of environmental endotoxin (Horick *et al.*, 2006). In general, background levels of endotoxin in the environment are below 10 endotoxin units (EU) per cubic meter (m^3) of air (Liebers *et al.*, 2008). High levels of endotoxins exist in work environments such as agricultural areas, animal production facilities, waste processing, and textile production. Research is still necessary in certain areas where knowledge remains insufficient, such as in schools moreover because some studies have demonstrated the presence of endotoxins in these facilities (Sheehan *et al.*, 2012). However, the majority of studies in which indoor endotoxin was assessed to determine children's exposure involved solely the home environment (Jacobs *et al.*, 2014a). Other important factors for developing studies of endotoxins levels in schools relays on the fact that only few studies performed in schools attained to examine endotoxin levels mainly focused in allergen levels, along with a small number of endotoxin measurements (Jacobs *et al.*, 2013) and mainly assessed in floor dust samples (Barnig *et al.*, 2013; Jacobs *et al.*, 2013). According to Jacobs *et al.* (2014a), two recent studies indicated high endotoxin levels in schools of asthmatics in comparison to their home environment and, inclusively, in one of these, school endotoxin levels were associated with higher respiratory symptom rates.

Although the hazard of endotoxin is recognized, there is no internationally established threshold occupational exposure limit (OEL) (Duquenne *et al.*, 2012; Gioffre *et al.*, 2012). The lack of an OEL is mainly attributed to the absence of a standard protocol at international level for sampling and analysis of airborne endotoxins. The methods and protocols used for endotoxin sampling and analysis vary greatly from one study to another, and the lack of correlation between the data makes it impossible to establish a clear dose–effect relationship or an exposure limit for the workplace (Duquenne *et al.,* 2012). There is substantial agreement that standardization is needed so as to be able to compare results from studies investigating endotoxin exposure, related health effects and compliance with exposure limits (Paba *et al.*, 2013). For endotoxin "no observed effect levels" (NOEL) various health endpoints have been reported in the literature

ranging from 50 to several hundred EU/m^3 (Douwes *et al.*, 2003). Nevertheless, even lower concentrations, especially under chronic exposure, may disturb the respiratory ventilation function (Matković *et al.*, 2012). Because of the negative health implications associated with airborne endotoxin in occupational settings, in The Netherlands, the Dutch Expert Committee on Occupational Standards (DECOS) has proposed a health-based 8 hour time-weighted average (TWA) exposure limit of 50 EU/m^3 (Douwes *et al.*, 2003; Matković *et al.*, 2012; Spengler *et al.*, 2000). This limit was based on personal inhalable dust exposure (Spengler *et al.*, 2000). In the present study, endotoxin levels refer to this threshold limit value. In 2000, the Dutch Minister of Social Affairs considered adopting a legally binding limit of 200 EU/m^3 since a limit of 50 EU/m^3 was found not to be feasible because of economic effects for some sectors of the industry (Douwes *et al.*, 2003). The American Council of Government Industrial Hygienists (ACGIH), in 1999, has proposed a practical alternative to a limit value: use relative limit values (RLVs). The RLV makes use of an appropriate background endotoxin level and the presence of symptoms to determine an action level. When symptoms associated with endotoxin exposure are present (e.g., fatigue, malaise, cough, chest tightness, and acute airflow obstruction) and endotoxin levels exceed 10 times background levels, action should be taken to reduce endotoxin exposure. Therefore, 10 times background is proposed as a RLV action level in the presence of respiratory symptoms. In environments where the potential exists for endotoxin exposure but there are no complaints of respiratory symptoms, endotoxin levels should not exceed 30 times the background level. Thus, 30 times background is a maximum RLV in the absence of symptoms (Spengler *et al.*, 2000). With reference to occupational exposure limits, in 2010 DECOS recommended a health-based occupational exposure limit (HBROEL) of 90 EU/m^3 for both chronic and short-term exposure to inhalable endotoxins (Gioffre *et al.*, 2012; Paba *et al.*, 2013).

Endotoxin is probably the microbial agent most commonly measured from indoor samples in epidemiological studies on asthma and allergy, due to its known properties as an inflammatory agent and respiratory irritant (Csobod *et al.*, 2014). Already since the early 1960s endotoxin has been recognized as a component of house dust. Since then numerous studies were conducted on health effects related to endotoxin exposure. In fact, in these last years there has been a renewed interest in endotoxin, since European studies in children who were raised on a farm, where endotoxin exposure is higher than in urban areas, showed that early-life exposure to endotoxin may play an important role in the protection against atopic diseases like hay fever, asthma and sensitization in both children and adults (Barnig *et al.*, 2013; Dales *et al.*, 2008;

Hyvarinen *et al.*, 2006; Jacobs, 2013; Liebers *et al.*, 2008). This is consistent with one aspect of the "hygiene hypothesis", describing microbial exposures or infections associated with a lower incidence of atopic disease. The "hygiene hypothesis" has been proposed as a possible explanation for the increasing prevalence of allergic diseases in the western world over the last decades. However, even if children were protected from allergy development, they might develop other health impairments due to endotoxin exposure (Liebers *et al.*, 2008). Celedon *et al.* (2007) found among 500 children with risk of atopy an association of endotoxin exposure with a reduced risk of atopy but increased risk of wheeze.

Spengler *et al.* (2000) describes the acute, chronic and low-dose health effects caused by endotoxins inhalation. The latter, the type of exposure that children have in primary schools, is characterized by levels of airborne endotoxin that are similar or only slightly above normal outdoor background levels, except when heavily contaminated ultrasonic or cool-mist humidifiers are in use (Spengler *et al.*, 2000). Exposure to endotoxin in indoor environments has been linked to protection against the development of atopic disease in young children, like decreased rates of allergic sensitization, asthma and hay fever in early childhood. On the other hand, endotoxins have strong pro-inflammatory properties and are capable of inducing airway inflammation and worsening of non-allergic asthma, by increasing symptom prevalence, and it is also associated with fever reactions, impaired lung function and wheeze (Annesi-Maesano *et al.*, 2013; Dales *et al.*, 2008; Delfino *et al.*, 2011; Horick *et al.*, 2006; Jacobs, 2013; Jacobs *et al.*, 2013; Jacobs *et al.*, 2014a; Liebers *et al.*, 2008; Paba *et al.*, 2013; Roda *et al.*, 2011; Sheehan *et al.*, 2012; Spaan *et al.*, 2007). Nevertheless, these health mechanisms that are triggered by exposure to endotoxins are not well understood (Douwes *et al.*, 2003), and the individual immune response to endotoxins is the result of a complex interaction between dose and timing of exposure, additive or synergistic effects and genetic predisposition. Health effects of endotoxin exposure can best be described as paradoxical (Liebers *et al.*, 2008). However, it is important to note the inaccuracy in comparing data from studies that report results in weight units of endotoxin, since different endotoxin preparations do not necessarily have equivalent potencies. To address this concern the EU was implemented as a measure of activity or potency of endotoxin, as opposed to gravimetric methods (approximately 10 EU equals 1 ng i.e. 300 EU ≈ 30 ng) (Spaan, 2008).

3.2. Asthma, allergies and respiratory symptoms

Recent traditional estimates have shown that 1.5–2 million deaths per year worldwide could be attributed to indoor air pollution, ranking 10th among preventable risk factors contributing to the global burden of disease (Viegi *et al.*, 2004). An increase in the prevalence of bronchial asthma was documented in the final decades of the 20th century in the industrialized world, including Europe (Csobod *et al.*, 2014) and, according to Viegi *et al.* (2004), indoor air pollution may increase the risk of irritation phenomena, allergic sensitization acute and chronic respiratory disorders and lung function impairment. Nevertheless, IAQ is an increasing public health concern due to the amount of time spent indoors (70–90%), and the presence of biological contaminants liable to damage respiratory health (Roda *et al.*, 2011).

This subsection explores asthma, allergy and respiratory symptoms due to the importance and prevalence of these common chronic health problems among schoolchildren.

Several authors explains why children are an important vulnerable group to the effects of indoor air pollution: Madureira *et al.* (2014) and Roda *et al.* (2011) share the opinion that (i) children's bodies are still developing, (ii) they may have altered sensitivity to exposure to xenobiotics, and (iii) their immune systems are too immature to respond effectively to environmental attacks; Annesi-Maesano *et al.* (2013) affirms the same reasons of the two previous authors and includes more two facts: (iv) children breathe a larger volume of air than adults, and therefore the lungs of children are exposed to higher concentrations of air pollutants; and (v) children may be also more exposed to air pollution due to their behavior, as they are more physically active with an exploratory nature; on another level, Etzel (2007) refers that: (vi) children have a higher resting metabolic rate and rate of oxygen consumption per unit body weight than adults, due to a larger surface area per unit body weight; (vii) because their airways are narrower, irritation caused by air pollution that would produce only a slight response in an adult, can result in potentially significant obstruction in the airways of a young child; (viii) because children are short, they breathe closer to the ground than do adults, and so they have greater exposures than adults to pollutants that are heavier than air; and finally, (ix) because there is a considerable growth of children's lungs after birth, continuing well into adolescence. For all of the aforementioned reasons, indoor air pollutants have more impact on growing children than on healthy adults.

The health problems caused by IAQ problems on school building occupants are often non-specific symptoms rather than well-defined diseases and range from lower attendance, comfort and performance and increased rates of absenteeism among schoolchildren and staff, to acute

health effects (e.g. respiratory irritation), chronic diseases (e.g. asthma and allergies) and symptoms associated with the so-called "sick building syndrome" (eye irritation, headaches, etc.) (Kephalopoulos *et al.*, 2014). Symptoms commonly attributed to poor IAQ include: headaches; fatigue; shortness of breath; sinus congestion; coughing; sneezing; eye, nose and throat irritation; skin irritation; dizziness; and nausea (Csobod *et al.*, 2014). Due to varying sensitivities among school occupants, IAQ problems may affect a specific group of people and in variable ways. Individuals who may be particularly susceptible to the effects of indoor air pollution include, but are not limited to, people with: asthma, allergies, or chemical sensitivities; respiratory diseases; and suppressed immune systems (due to radiation, chemotherapy or disease) (Kephalopoulos *et al.*, 2014). Allergic individuals and asthmatics face a higher risk (Kephalopoulos *et al.*, 2014; Macedo *et al.*, 2013).

According to Macedo *et al.* (2013), health effects of exposure to air pollutants have been extensively documented and reviewed. There is strong evidence that poor IAQ may have respiratory and other health related outcomes and affects general well-being (Ferreira & Cardoso, 2014; Madureira, 2014), increasing the risk of irritations, acute respiratory symptoms, bronchial hyper responsiveness, respiratory infectious diseases, COPD and atopic sensitization (immunological disorders) (Annesi-Maesano *et al.*, 2013; Viegi *et al.*, 2004). Madureira (2014) cited the Promoting Actions for Healthy Indoor Air (IAIAQ) report, that declare the most important diseases which have been associated with (caused or aggravated by) indoor air exposures: allergic and asthma symptoms; lung cancer; cardiovascular diseases; chronic obstructive lung disease; (upper and lower) respiratory infections/symptoms, and acute toxication.

Another key aspect of indoor air pollution results from biological stressors. It is caused by hundreds of species of bacteria and fungi, in particular filamentous fungi (mold), which grow indoors when sufficient moisture is available. With reference to endotoxin, recent studies have shown that exposure levels in school classrooms are many times higher than in the home environment (Kephalopoulos *et al.*, 2014). From the literature review done, several authors reported that exposure to indoor air biological agents is often associated with respiratory symptoms, allergies and asthma, rhinitis, hypersensitivity pneumonitis and SBS symptoms, as well as perturbation of the immunological system (Annesi-Maesano *et al.*, 2013; Kephalopoulos *et al.*, 2014; Madureira *et al.*, 2015a; Niemeier *et al.*, 2006; Pegas, 2012; Salo *et al.*, 2009; Singh *et al.*, 2011). However, the relationship between specific health effects and indoor biological levels has not been well defined, due to the lack of a standard methodology of

sampling and analysis (Niemeier *et al.*, 2006). Notwithstanding, in Cabral (2010) the existence of an association between indoor airborne fungi and allergic sensitization to fungi and asthma was confirmed in the «Inner-City Asthma Study» carried out in seven low-income urban communities across United States of America (USA); Sahakian *et al.*, (2008) refers that Institute of Medicine (IOM) found sufficient evidence for associations between exposure to damp indoor environments or mold or other agents in damp indoor environments and cough, wheeze, and asthma symptoms in asthmatic persons, and limited or suggestive evidence for associations with asthma development and dyspnea (Sahakian *et al.*, 2008).

As already mentioned in the subsection 3.1, limited and inconsistent evidence suggests that moderate exposures to certain microbial agents, like endotoxins, bacteria and fungi, especially at early childhood when immune response is particularly susceptible, may prevent the development of allergies, atopy, asthma and hay fever, while it can be a risk factor for preliminary non-atopic conditions (Jacobs, 2013; Mendell *et al.*, 2011). Uncertainty remains as a factor in the causative role of indoor air pollution in asthma and respiratory allergies, but evidence suggests that several air pollutants may contribute to both asthma and allergies exacerbation and development (Madureira *et al.*, 2015c). People can be exposed to pollutants through inhalation, ingestion and dermal contact, but for most pollutants breathing is the most common pathway to exposure (Csobod *et al.*, 2014; Madureira, 2014). Csobod *et al.* (2014) states that in schools, respiratory health is particularly affected by air pollutants that enter the body through the inhalation route, with allergic subjects being at higher risk.

Asthmatic children are known to be exceptionally sensitive to poor IAQ. Asthma and allergic diseases are two of the most prevalent chronic diseases among children and it has been reported that more than one third of children in Europe have bronchial asthma or allergy (Madureira, 2014; Madureira *et al.*, 2015d). As mentioned by Fraga *et al.* (2008), asthma is the main cause of hospital admission for children in western countries and has a negative impact on schooling and school performance, being the leading cause of missed school days (Ferreira & Cardoso, 2014; Fraga *et al.*, 2008). It is, therefore, a global public health issue, affecting around 300 million people worldwide corresponding to 15 million disability-adjusted life year (DALY) per year, and it is estimated that there will be an additional 100 million persons with asthma by 2025. In Europe, between 1999 and 2004, asthma prevalence rates ranged from approximately 5% to 20% in children aged 6-7 years and from approximately 5% to 25% in children aged 13-14 years (Madureira, 2014).

Because much evidence points to environmental factors as triggers of the exuberant immune response, there has been much attention on identifying specific environmental factors that are most responsible for provoking asthma and developing strategies to minimize relevant exposures (Diette *et al.*, 2008). There are several studies regarding the prevalence and the state of art of asthma, allergies and respiratory problems among children. Evaluating the risk of developing childhood asthma is one of the four priority issues identified by the European Community, under the European Union Environment and Health Action Plan (Madureira *et al.*, 2015c). According to Madureira (2014), several studies have demonstrated a strong association between the exposure to poor indoor air and short and/or long-term health problems including asthma, allergic reactions and respiratory symptoms, even more significant in asthmatic children. For example, Clausen *et al.* (2009) reported strong associations between dampness, ventilation rate, concentrations of specific chemicals and the prevalence of asthma/allergy among schoolchildren. Schools Indoor Pollution and Health: Observatory Network in Europe (SINPHONIE) Project results showed multiple associations of selected microbial agents in indoor dust in schools with recent symptoms, past respiratory health symptoms and clinical measurements, indicating the relevance of microbial agents to the respiratory health of pupils and teachers (Kephalopoulos *et al.*, 2014). Jacobs (2013) states that many studies found consistent evidence for an association between dampness and mold observations in buildings and adverse effects on respiratory health in children and adults. PATY (Pollution and the Young) project compiled data on exposure and health status for 57161 children aged from 6 to 12 years in 12 countries and reported that indoor mold exposure was consistently associated with adverse respiratory health outcomes (wheeze, nocturnal cough, sensitivity to inhaled allergens and hay fever) (Madureira, 2014). From another point of view, Sahakian *et al.* (2008) states that some of the existing studies present evidence that exposures to specific microbial agents in damp indoor environments are associated with development of asthma and asthma-like symptoms in adults, and the studies among infants and young children have found adverse and protective effects.

In the study conducted by Kabir *et al.*, (2011), in Ireland, childhood asthma is still a growing public health concern due to an estimated increase of 39% in prevalence of symptoms of severe asthma in 2007 relative to the baseline year 1995, while it was observed a significant fall in allergies, especially symptoms of ever hay fever, and a rise of symptoms of ever eczema (Kabir *et al.*, 2011). Pegas (2012) describes the participation of Portugal in the International Study of Asthma and Allergies in Childhood Program (ISAAC), in 1993. It was created in 1991 with the

aim to assess the prevalence and progression of asthma and allergic diseases, through a standardized written questionnaire, translated and adapted to Portuguese, distributed to students between 13 and 14 years old in five geographic areas (Lisbon, Porto, Coimbra, Funchal and Portimão), and it pointed to significant regional differences in terms of prevalence of respiratory symptoms, recommending further studies to define evolutionary trends and identify risk factors.

In the study conducted by Tamburlini *et al.* (2002), in an overall perspective, the epidemiological research indicated relatively high rates and increasing trends of childhood asthma mostly in "western", industrialized and affluent countries, while in eastern European countries and in developing countries, the rates were generally lower. The authors also concluded that important differences in the development of atopic disorders may exist, with factors operating very early in life particularly relevant for the acquisition of childhood asthma, while the development of atopic sensitization and hay fever may also be affected by environmental factors occurring beyond infancy (Tamburlini *et al.*, 2002).

Further research is necessary to better evaluate the respiratory health effects of indoor pollution and to implement protective programs for public health (Viegi *et al.*, 2004).

3.3. Buildings and classrooms characteristics

The presence of unwanted and excessive moisture in buildings is known as dampness (Jacobs, 2013). Dampness is defined by WHO as "any visible, measurable or perceived outcome of excess moisture that causes problems in buildings, such as mold, leaks or material degradation, mold odor or directly measured excess moisture (in terms of relative humidity or moisture content) or microbial growth" (Borras-Santos *et al.*, 2013). There is evidence that moisture problems are prevalent in at least 20% from the buildings in Europe and Northern America (Jacobs, 2013), and that children in modern societies are spending more time indoors (Ashmore & Dimitroulopoulou, 2009). The majority of the studies in this area explored the effect of dampness in homes; the effect of dampness in schools has hardly been studied. However, as school buildings differ in size, construction, activities which take place in the building, and occupant density, there is a clear need to increase knowledge on the IAQ in school buildings (Jacobs, 2013).

Primary risk factors of dampness and moisture damage differ across climates, geographic area and building types and can be caused by leakage of rain or snow or moisture from the ground (outdoor sources), condensation due to inadequate ventilation, or by occupant behavior (indoor

sources). Water damage may also result from floods or failures of water piping (accidents) or from moisture within building materials and constructions (building sources). Dampness can be a concern, particularly in areas with a temperate damp climate, like Europe (Jacobs, 2013). Dampness and moisture in buildings can lead to microbial growth and harmful emissions into indoor air (Annesi-Maesano *et al.*, 2013; Borras-Santos *et al.*, 2013; Jacobs, 2013), but the mechanisms that explain the variety of health effects that can occur as result of exposure to dampness and microbial exposure are largely unknown (Borras-Santos *et al.*, 2013; Jacobs, 2013). Other important condition that favors temporary or permanent occurrence of mold is the technical condition of a school building which results from the construction technology, finish materials and its use (Ejdys, 2007).

The quality of the indoor air depends strongly, on one hand, on the interaction between the building and its outdoor environment, and, on the other hand, on the way the building is used, including the behavior of its occupants. Air pollutants in buildings are linked to various indoor factors (e.g. building structure, surfaces, furnishing, ventilation systems, etc.), the specific activities of the occupants, and the presence of nearby outdoor sources (Csobod *et al.*, 2014; Spengler *et al.*, 2000). Built environments are complex ecosystems that contain numerous organisms including trillions of microorganisms (Kembel *et al.*, 2012), and fungi, being ubiquous, are present in all indoor environments and cannot be eliminated from them (Hardin *et al.*, 2003). The aerosolization of biological particles from surfaces, that affects the concentrations of indoor microbial contaminants, is affected by relative humidity, temperature, ventilation, source of dust, type of flooring, season, housing conditions (e.g., water intrusion), the presence of carpeted floors, presence of dogs, and occupant density (Singh *et al.*, 2011).

Normal building materials and furnishings provide ample nutrition for many species of fungi, yet they can only grow and amplify indoors when there is an adequate supply of moisture. Where fungi grow indoors there is an inappropriate source of water that must be corrected before remediation of the mold colonization can succeed. Mold growth in the home, school, or office environment should not be tolerated because mold physically destroys the building materials on which it grows, is unsightly and may produce offensive odors (Hardin *et al.*, 2003). These are also reasons why there is a large variation in personal exposure between individual children (and classrooms), along with differences in building design, and activity patterns (Macedo *et al.*, 2013).

Mitchell *et al.* (2007) states that it is necessary to look at the relationship between the built environment and humans as a complex interplay between building occupants (who they are and what they do) and an array of physical, chemical, biological, and design factors. This evolution in understanding has profound implications for the design and operation of buildings, how the buildings are used, and the prevention and management of health problems that occur in building occupants (Mitchell *et al.*, 2007). Emissions from materials and products used indoors are meaningful pollutant sources, very often of greater importance to IAQ than either bio-effluents or pollutants from outdoor air (Clausen *et al.*, 2011). Outdoor pollutants including pollen and traffic and factory emissions enter buildings through open windows, ventilation system air intakes, and building leaks and cracks. These contaminants, along with those that arise inside the building, concentrate in tightly sealed buildings with inadequate ventilation (Annesi-Maesano *et al.*, 2013). Whether planned or not, buildings have multiple openings that allow the penetration and internal movement of air, water, and contaminants (*Green Schools: Attributes for Health and Learning*, 2006). Different building types and indoor air spore burden can be partly attributed to building age and construction materials. For example, old school buildings may have less insulation, and leakier structures (Turunen *et al.*, 2014); a wooden, organic frame may behave differently than a concrete frame (Meklin *et al.*, 2002a); some buildings have old ventilation systems, or perhaps rely only on natural ventilation (Turunen *et al.*, 2014). These background factors should be considered in order to understand the contribution of moisture damage to the microbial status of buildings (Meklin *et al.*, 2002a).

Moisture and mold damage of a building have been regarded as important factors modifying the microbial quality of the indoor air and have been strongly associated with adverse health effects (Meklin *et al.*, 2003). As dampness favors the development of fungi, it represents a risk factor for asthma and respiratory diseases (Tamburlini *et al.*, 2002), leading to sensitization and enhancing allergic responses in allergic and non-allergic subjects including infants (Hardin *et al.*, 2003; Jacobs, 2013; Mendell *et al.*, 2011). Moisture and mold were also associated with cough and wheeze in the general population as well as upper respiratory symptoms (nasal congestion, sneezing, runny or itchy nose) (*Green Schools: Attributes for Health and Learning*, 2006). As a matter of fact, toxicological evidence obtained *in vivo* and *in vitro* shows the occurrence of diverse inflammatory and toxic responses after exposure to microorganisms isolated from damp buildings, including their spores, metabolites and components (Madureira, 2014). The United States Institute of Medicine's Committee on Damp Indoor Spaces and Health found sufficient

evidence of an association between indoor dampness and several respiratory health outcomes, including asthma. However, the evidence was not strong enough to say there was a causal relationship, i.e., that dampness directly caused the respiratory health outcomes (Dales *et al.*, 2008; *Green Schools: Attributes for Health and Learning*, 2006; Meklin *et al.*, 2002a). Even though moisture and dampness have shown to be risk factors for health outcomes in buildings (Carrer *et al.*, 2015), it seems unlikely that dampness itself causes adverse effects on health (Jacobs, 2013), with the underlying mechanisms and specific causal agents of this relationship not completely understood and identified (Carrer *et al.*, 2015; Jacobs, 2013). WHO (2009) concluded that persistent dampness and microbial growth on interior surfaces and in building structures should be avoided or minimized, as they may lead to adverse health effects. Consequently, ventilation that controls moisture levels indoors can be expected to influence indirectly some of the outcomes, in particular those related to asthma, allergy and respiratory symptoms, but it may not always be effective (Carrer *et al.*, 2015). The conceptual framework for associations between dampness, microbial exposure and health is summarized in Figure 2 (Jacobs, 2013).

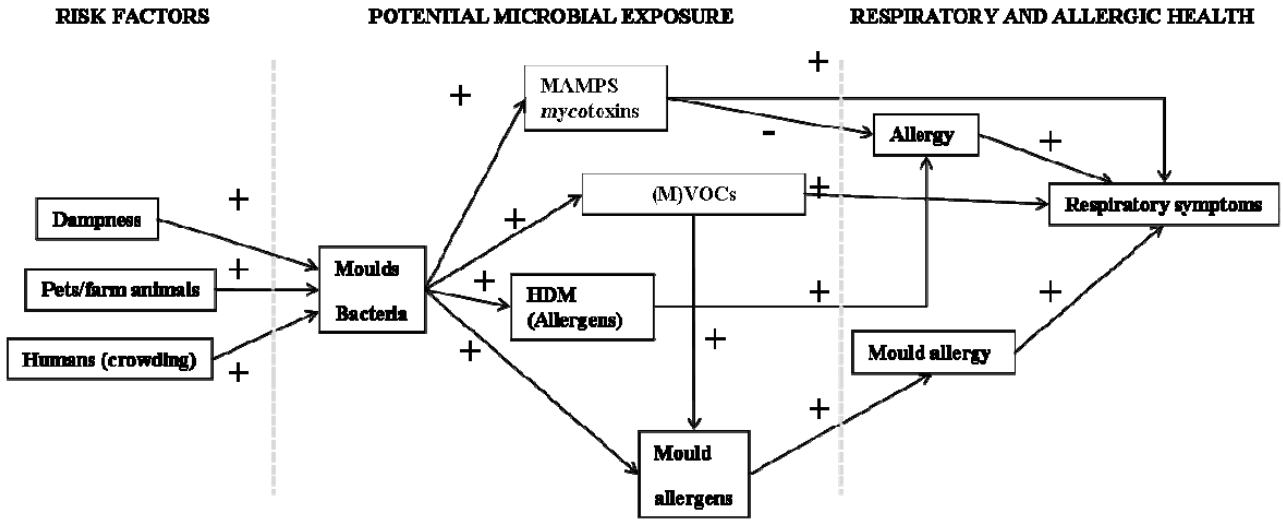

Figure 2 - Associations between dampness, microbial exposure and symptoms (Jacobs, 2013)

Madureira (2014) concluded that, from the literature review done, only a few studies were conducted to evaluate the associations of school characteristics and asthma, allergies and respiratory symptoms among the attending students or teachers, and that, the most commonly studied school characteristic was the presence of moisture and mold with inconsistent evidence found: two studies reported associations of some endpoints with the presence of molds in schools while in another study associations between asthma, allergies and respiratory problems

were not found. Jacobs (2013) mentioned a study in 32 school buildings in Finland that found an association between moisture damage in school buildings of concrete or brick construction and respiratory symptoms in children, as well as another study in 15 Danish schools that did not find a positive association between symptoms and extent of moisture and mold growth in the school buildings, but building-related and general symptoms, like eye-and throat irritation, headache and dizziness were increased in buildings with higher mold levels.

In the studies conducted by Meklin *et al.* (2002a) and Meklin *et al.* (2003), moisture damage in schools increased the respiratory symptoms of primary school children, but the effect was more evident in school buildings with concrete/brick frame than in wooden schools. In concrete/brick buildings, the effect of moisture damage was seen as elevated concentrations of airborne fungi, which may indicate that the building frame itself may act as a potential source and sink of microbial particles, thus masking the effect of moisture damage.

Although dampness may indicate potential mold growth and many studies have used reported dampness as a surrogate for exposure to fungi, it is also a likely indicator of bacterial growth (Hardin *et al.*, 2003; Spengler *et al.*, 2000). Nevertheless, ventilation and air-conditioning systems are more responsible for polluting the air with fungi than with bacteria (Gniadek, 2012). The maintenance of ventilation systems plays an important role in causality, as the systems themselves can become significant sources of pollution if they are poorly maintained (Annesi-Maesano *et al.*, 2013; Carrer *et al.*, 2015). Data from Park *et al.* (2001) did not show that reported mold/mildew was an important predictor of airborne endotoxin. The relative contribution of each is unknown, but mold, bacteria and endotoxins can all play a role in the reported spectrum of illnesses, and can all be minimized by control of relative humidity and water intrusion (Hardin *et al.*, 2003). The occurrence of certain microbes with high water activity demand, for example, *Aspergillus versicolor*, *Stachybotrys*, and *Actinobacteria* can be regarded as markers of moisture damage. However, there is only limited information on the microbial levels and microflora of indoor air in school buildings. *Penicillium*, *Aspergillus*, and *Cladosporium* have been reported to be common fungi in school environments, but there are large variations in total concentrations depending on geographical and seasonal weather conditions (Meklin *et al.*, 2003).

When mold colonization is discovered in home, school, or office environment, it should be remediated after the source of the moisture that supports its growth is identified and eliminated (Hardin *et al.*, 2003). Previous studies indicate a reduction of adverse health effects in pupils

after remediation of school buildings affected by moisture or mold (Jacobs, 2013). Prevention of fungal exposures in buildings requires that buildings are purposely designed and operated to minimize available moisture (Cabral, 2010; Spengler *et al.*, 2000). Direct penetration of aerosols is best prevented by design, and by filtration, and exposure from this source can be reduced using air-cleaning techniques. Aerosols released from occupants are difficult to control directly, but clean-air ventilation should provide dilution, and air cleaning may help to lower concentrations. Exposure to aerosols from indoor sources should be addressed primarily by source control (Spengler *et al.*, 2000), as mentioned in Co-ordination Action on Indoor Air Quality and Health Effects (EnVIE) project: the first strategy for good IAQ should be source control (Carrer *et al.*, 2015). To improve IAQ adequate outdoor air ventilation (filtered) throughout the year will help keep the indoor environment dry in winter and may dilute indoor aerosols and reduce exposure; dehumidification is necessary to keep water content of materials low enough to prevent fungal growth; and persistent condensation must be controlled (Spengler *et al.*, 2000). WHO points out that "building standards and regulations with regard to comfort and health do not sufficiently emphasize requirements for preventing and controlling excess moisture and dampness"; this needs to be carefully taken into account in the context of energy-efficiency measures in buildings (WHO, 2009).

Clausen *et al.* (2011) addresses the core question: "How do we ensure that energy performance goals for buildings can be met without jeopardizing indoor environment quality?". The challenge of today lies in the accomplishment of sustainable and low-energy built environment and at the same time healthy, comfortable, accessible and safe built environment. The path towards future low-energy use, or even energy autonomy or energy-positive buildings is seriously hampered by the fear of introducing a negative impact on human health (Bluyssen, 2014). One approach to lower the energy use in buildings is to reduce air exchange rates. Without due consideration of IAQ, such a change can increase the indoor concentrations of some pollutants and degrade public health (Clausen *et al.*, 2011). It was therefore concluded that there is a need to develop a strategy for using health rather than comfort as the major outcome for determining indoor environmental requirements, which would be a major departure from most present ventilation standards. Regarding this subject, studies like IAIAQ and HealthVent were conducted: IAIAQ project intended to estimate the potential effects of ventilation on burden of disease; HealthVent was funded by the European Commission with the overarching goal of creating health-based ventilation guidelines for Europe, in order to ensure adequate IAQ, tangible health benefits for the

occupants of buildings and a reduction in the burden of disease in the general population (Carrer *et al.*, 2015).

There is no consensus regarding this relationship between energy efficiency and IAQ (Bluyssen, 2014). Worldwide programs and requirements for energy certification of buildings have been established. A few countries (e.g. Portugal and the Netherlands) have – on a national level – combined the energy certification process with indoor environmental certification (Clausen *et al.*, 2011). The built environment and its indoor environment with occupant is a complex system. For the (re)design of indoor environments, it is clear that the current approach based on single-dose–response relationships is no longer appropriate to apply. It is required a view in which the focus is on users (situations) instead of single components and in which the focus is an improving quality of life as opposed to only prevent people from getting ill or feeling bad. A view in which IAQ is approached in an integrative multi-disciplinary way, taking into account possible problems, interactions, people and effects, focusing on situations rather than single components (Bluyssen, 2014). Collaborations between microbiologists and building scientists will be especially important for understanding the impact of materials and machine-aided ventilation (Kelley & Gilbert, 2013).

3.4. Indoor Air Quality Studies in Schools

Primary schools accommodate children for about 8 hours daily, which makes them the second most time-spent indoor environment after homes (Madureira, 2014). In many of the European Union countries, schoolchildren aged 7-10 years are taught for more than 800 hours per year and students aged 10 years or more spend a further 50-100 hours in classrooms (Annesi-Maesano *et al.*, 2013). Notwithstanding, indoor air of primary schools is cause for concern because schoolchildren represent a particularly susceptible group of the population, due to the reasons already listed in subsection 3.2, and asthmatic children are exceptionally sensitive to effects of poor IAQ. Therefore, schools constitute a particularly critical setting for this susceptible population group as air quality in schools can have an impact on children's health, attendance and learning performance (Csobod *et al.*, 2014). Various studies on air quality and children's health indicate that indoor residential risk factors of primary interest for asthma, allergies, and respiratory health include biological agents (Madureira *et al.*, 2015a). There is strong evidence of the potential detrimental role to health of a variety of indoor pollutants commonly found in school environments, originating in the outdoor air or associated with indoor materials, products or activities with indoor air pollutant levels two to five times higher than outdoor levels (Madureira,

2014). IAQ problems in schools may be even more serious than in other categories of buildings, due to higher occupant density and insufficient outside air supply, aggravated by frequent poor construction and/or maintenance of school buildings (Pegas *et al.*, 2011b).

Recently, many European projects have been carried out to characterize indoor and outdoor environments, possible pollutant sources and relationships between pollutants and health in schools (Pegas, 2012). In the following paragraphs is presented a brief description of some of these European Projects.

In 2000, the EFA and the Airways Diseases Patients Association implemented the European Union-funded project Indoor Air Pollution in Schools, that found evidence that the right to breathe clean air in schools was not widely recognized in Europe, and that there was a need for further research to evaluate the impact of air pollutants in schools on children's well-being, health and performance and to provide a sound basis for promoting European regulations and campaigns directed at a better school environment (Franchi & Carrer, 2002).

Between 2004 and 2005, the European Union-funded pilot study Health Effects of the School Environment (HESE) provided data on indoor air pollutants and health effects in schools in five European countries (Italy; France; Norway; Sweden; Denmark) (Simoni *et al.*, 2006). Besides promoting awareness of the importance of school environmental air quality on the health of children and providing practical tools for evaluating, improving, and maintaining good air quality in schools, the aims of the HESE included the evaluation of IAQ in schools and the impact of pollutants in the school environment on the respiratory health of European children, in particular on the aggravation of symptoms among asthmatic children (Madureira, 2014). HESE results revealed a number of typical IAQ problems in schools, particularly due to poor ventilation, and a widespread lack of awareness and preparedness to cope with environmental problems and take care of more vulnerable children, such as those suffering from asthma. HESE has also identified health impacts among children exposed to higher levels of indoor pollution at school, in the form of respiratory disturbances and reduced nasal patency (Simoni *et al.*, 2006).

The School Environment and Respiratory Health of Children (SEARCH) project, performed from 2006 until 2009, had as main objectives to assess the association between the school environment and children's respiratory health, and to draft recommendations for improving air quality in the school environment (Csobod *et al.*, 2010).

In 2008, the "Health Effects of Indoor Pollutants: Integrating Microbial, Toxicological and Epidemiological Approaches – HITEA" was started, with the aim of investigate the health effects

of dampness-related indoor air pollutants, in both homes (European Birth and Adult Cohorts) and European schools (Jacobs, 2013). This project studied the relationship between the role of biological agents present in indoor air and long term respiratory, inflammatory and allergic health impacts among children and adults. HITEA focused on many indoor exposures and factors, like allergens, chemicals, cleaning agents, traffic exhaust and poor ventilation, but the main objective focused on microbial exposures due to dampness and moisture problems of buildings (Pegas, 2012).

SINPHONIE project was the first Europe-wide pilot project to monitor the school environment and children's health in parallel in 25 European countries, in the period between 2010 and 2012 (http://sinphonie.eu/). It was a milestone project which has provided standardized methodologies and tools for better characterizing schools' indoor environments, assessing the health risks to schoolchildren and staff, and also developed guidelines and recommendations for healthy school environments covering a wide array of situations in Europe. The ultimate objective of SINPHONIE was to produce recommendations and guidelines on remedial measures in the school environment to cover a wider array of situations in Europe and to disseminate these guidelines to stakeholders able to take action (Csobod *et al.*, 2014). The project contributed to the European legal and policy framework for sustainability in schools, since children's health and educational potential depend on the quality of the school environment. In Portugal, six elementary schools and two kindergartens have been studied in Aveiro and Porto cities (Pegas, 2012).

In SINPHONIE are also mentioned other projects addressing IAQ in schools that have been carried out at a national level of European countries, as Indoor Air in Primary Schools (BiBa) project in Belgium (https://esites.vito.be/sies/BIBA/EN/home/Pages/home.aspx) and Observatory Network on Indoor Air Quality (OQAI) in France (http://www.airinterieur.org/). According to Madureira (2014), the latter highlighted that the status of the school buildings and irrespective ventilation levels are two of the major problems in schools, and these findings were supported by a recent literature review in school environment and IAQ in particular (Annesi-Maesano *et al.*, 2013).

Despite the fact that, recently, numerous IAQ studies were performed, the majority focused on chemical pollutants, without biological assessment. Children's homes are the preferred target study; however kindergartens and primary schools are also becoming an interesting field of

concern, proved by the development of new literature in this area presented in the following paragraphs.

The main conclusion of Cooley *et al.* (1998) study, performed in the USA during 22 months in 48 schools, was that *Penicillium* and *Stachybotrys* species may be associated with sick building syndrome. Also in United States, Levetin *et al.* (1995), 145 classrooms of 13 schools were examined from September 1991 to April 1992 and results showed that concentrations of airborne fungi in schools are quite variable depending on the type of ventilation systems as well as outdoor concentrations.

Meklin *et al.* (2002a) developed a study where microbial IAQ and respiratory symptoms of children were investigated in 24 schools with visible moisture and mold problems, and in eight non-damaged schools (total 32 schools), with the purpose of determine whether microbial IAQ and associated health status of children in schools with visible moisture and mold problems differed from those in non-damaged schools. In 2003, the same authors, Meklin *et al.* (2003), investigated the effect of building frame (concrete/brick or wood) and moisture damage on microbial IAQ on 17 wooden and 15 concrete or brick school buildings (total: 32 schools, 10 classrooms), in Finland. Mean concentrations of viable airborne fungi were significantly higher in wooden schools than in concrete schools, showing that the frame material was a determinant of concentrations of airborne fungi, and higher concentrations were found of the most common fungi, that is, *Penicillium*, Yeasts, *Cladosporium*, and nonsporing isolates. Moisture damage of the building did not change the fungal concentrations in wooden school buildings, whereas, in concrete schools the effect of moisture damage was clearly seen as higher concentrations compared with the reference schools. *Aspergillus versicolor*, *Stachybotrys*, and *Acremonium* were detected only in samples from moisture damaged buildings, and can be considered marker fungi of such damage in school buildings, both in concrete and wooden school buildings. In addition, elevated concentrations of *Cladosporium* and *Actinobacteria* were associated with moisture damage in concrete schools (Meklin *et al.*, 2003).

Ejdys (2007) analyzed the species composition of fungi occurring on wall surfaces and in the air in school buildings, in May 2005 after the heating season was over, and states that possible toxins penetrating the bodies of the children may cause various toxicoses, but, on the other hand, antibiotics produced in low concentrations may induce resistance of the bacterial flora of school occupants. In Figure 3 is presented the fungi genera identified in air and walls of schools buildings by Ejdys (2007).

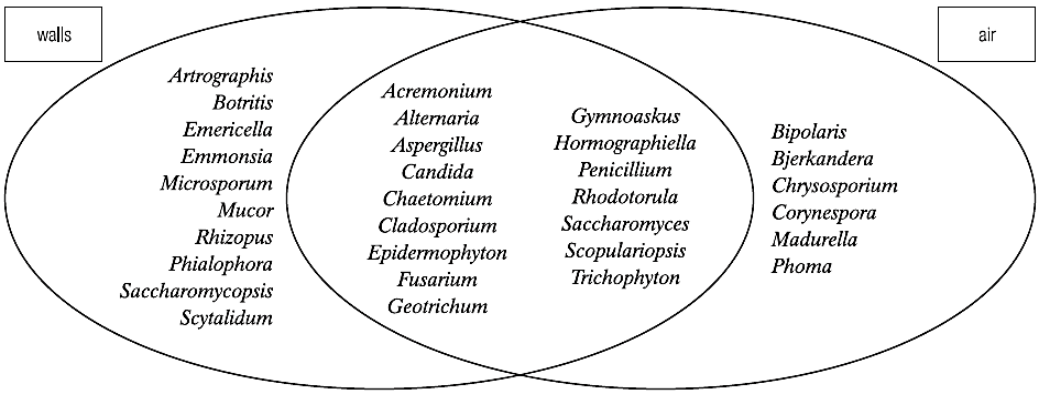

Figure 3 - Occurrence of fungal genera on the walls and in the air in the school buildings (Ejdys, 2007)

Twice as many species were identified on the walls as in the air. Only 6 genera were characteristic of the air: *Bipolaris, Bjerkandera, Chrysosporium, Corynespora, Madurella* and *Phoma*; nevertheless, *Penicillium*, *Cladosporium* and *Aspergillus* were recorded most frequently. The same genera also dominated on wall surfaces although the genus *Aspergillus* unquestionably dominated; of the 32 species that occurred only on the surfaces in the school buildings, 16 species represented the genus *Aspergillus*. Yeast-like fungi, mostly of the genera *Candida, Rhodotorula* and *Geotrichum*, were frequently recorded in both sample types and the prevalence in the air did not differ significantly from that on the wall surfaces (Ejdys, 2007).

Roda *et al.* (2011), among other parameters, assessed fungi and endotoxin concentrations in Paris child day care centers and compared with indoor levels found in Paris dwellings. Airborne endotoxin levels in child day care centers were higher than those found in Paris dwellings and *Penicillium* and *Cladosporium* were the most commonly identified genera fungi.

Daisey *et al.* (2003) focused specifically on school environment by reviewing the literature published until 1999 on IAQ, ventilation, and building characteristics in schools and identified commonly reported school building-related health symptoms. A more recent literature review was done by Annesi-Maesano *et al.* (2013), that provided a state of the art of the literature on adverse health effects, particularly respiratory health, through a review of the scientific literature (written in English) available on the PubMed website, from 1992 to 2012, on school environment, IAQ and associated adverse health effects. The main focus was on respiratory health because of the inhalation route of air pollutants that are associated with IAQ and/or building characteristics in schools. Analyzing the compilation of studies on IAQ and related health effects at school presented in this literature review, in which biological parameters were assessed, a total of 20 studies arise, from 1997 until 2011, at a worldwide level with countries like United States, China,

Sweden, Norway, Finland, Denmark, France, Italy and Portugal is also included. This finding is in agreement with Madureira (2014) and Pegas (2012): both refers that most studies in schools have been performed in northern Europe, Central Europe; USA and China, and also that significant differences among indoor environments from different regions have been found. In the literature review conducted by Annesi-Maesano *et al.* (2013), the main targeted health problems were allergic and respiratory problems, like asthma and atopy. A wide array of aims were found among the different studies, including 2 intervention studies; at a global level, the goal was to assess the effects of IAQ and dampness/moisture/molds in buildings on respiratory health of schoolchildren; some studies had more specific aims like, for example, study the influence of ventilation on IAQ and on health and exposure of pupils.

Within HITEA study, Jacobs published 3 articles regarding endotoxins levels in homes and classrooms of children, collected though the sampling of airborne settle dust with the Electrostatic Dust fall Collectors (EDC), during an 8-week sampling period, and the analysis was performed through Limulus Amebocyte Lysate (LAL) method. The first, published in 2013 (Jacobs *et al.*, 2013), comprised the analysis and comparison of the results of endotoxin levels in primary schools and homes of children in The Netherlands, during March and April 2010. Endotoxin was also studied in relation to asthma and sensitization. The second, in 2014 (Jacobs *et al.*, 2014a), endotoxin levels from a total of 25 primary schools in three European countries, from three different geographical regions, with different climates, were measured (Spain – 8 schools; The Netherlands – 11 schools; Finland – 6 schools). Measurements were conducted in two sampling periods: during winter/spring of 2009 and winter/spring of 2010. In general, endotoxin levels were higher in lower grades, potentially reflecting differences in physical activity, and in classrooms with higher occupancy. The third, also published in 2014 (Jacobs *et al.*, 2014b), associations between school dampness, microbial exposure and respiratory health in children were studied in 15 Spanish, Dutch and Finnish primary schools with and 10 without moisture, dampness and visible mold. Associations between school dampness and respiratory symptoms were found, but no significant effects on lung function and these associations were most pronounced in Finland, although microbial levels were considerably lower compared to Spanish and Dutch schools, indicating that associations between moisture, microbial exposure and health may vary between regions and countries. All of the aforementioned articles are gathered on José Jacobs Doctoral Thesis (Jacobs, 2013), published in 2013, plus one more article published by Borras-Santos *et al.* (2013), also in the scope of HITEA study.

IAQ of Finnish elementary school buildings and its influence on health and academic performance of students was assessed by Turunen *et al.* (2014). 4248 health questionnaires were answered, comprising 37 questions; weekly symptoms in the spring were fatigue, stuffy nose, and headache, whereas specific symptoms varied considerably. On the group level, the prevalence values most frequently found were wheezing, cough with wheezing, and fever.

Although there have been multiple studies worldwide to evaluate IAQ in school environments, in Portugal data and information about IAQ and health is scarce (Madureira, 2014). Among the few Portuguese studies available in literature, recently more and new researches have been conducted in this area.

Fraga *et al.* (2008) evaluated the association between the IAQ in nine secondary schools in the Porto area and the prevalence of allergic and respiratory symptoms in adolescents. The ISAAC questionnaire was applied to evaluate respiratory and allergic symptoms and their impact on adolescents' lives.

Borrego *et al.* (2008), through SaudAR - Health and The Air We Breathe, evaluated the relation between outdoor and IAQ and children's health in primary schools of Viseu. This project pointed out that the state of school buildings and inappropriate ventilation levels are major contributors to poor IAQ and negative health effects in schools (Csobod *et al.*, 2014).

Madureira *et al.* (2009) characterized the IAQ in eleven Porto secondary schools and evaluated the health symptoms among teachers and investigated the impact of pollutants on the prevalence of those symptoms.

Pegas has publications regarding IAQ in Portuguese schools between 2010 and 2012, where biological compounds were assessed through liquid impinger sampling. In 2010, studied the relationship between outdoor and IAQ of 3 primary schools in Lisbon, during December 2008 (Pegas *et al.*, 2010). In 2011, indoor and outdoor microbiological levels of a study performed in May-June 2009, also 3 primary schools of Lisbon, were published (Pegas *et al.*, 2011b), along with the results obtained through the comparison of the assessment's performed in these 3 primary schools of Lisbon during spring (May and June 2009), autumn (November 2009) and winter (February 2010) (Pegas *et al.*, 2011a). In 2012, the article provided information about an investigation of pollutant concentrations at 2 school buildings of different locations (city centre and suburban) in Aveiro, Portugal, between April and June 2010 (Pegas *et al.*, 2012). The Doctoral Thesis entitled "Indoor Air Quality in Elementary Schools of Lisbon and Aveiro" gathered all the aforementioned articles; overall, it was assessed indoor pollutant concentrations in sixteen

elementary schools in Lisbon and Aveiro and estimated the actual occurrence of asthma/rhinitis in Lisbon primary school population (Pegas, 2012).

Assessment of filamentous fungi and identification of *Aspergillus sp*. in indoor air of 34 classrooms of seven infant and elementary Portuguese schools, situated in Vila Nova de Gaia, from December 2010 to January 2011 (cooling season), was performed by Barbosa *et al*. (2012). Filamentous cultures were more prevalent than yeasts, being *Ascomycetes*, more widespread than *Zygomycetes* and elementary classrooms were more environmental contaminated than infantry level classrooms. *Aspergillus terreus* and *Aspergillus fumigatus* cultures isolation occurred from samples exposed in two elementary classrooms and were related with school habitability and hygienisation conditions.

A study conducted by Macedo *et al*. (2013), from May to October 2010, in 21 primary schools, 39 classrooms, during cooling season, where bacteria and fungi concentrations were assessed through impaction method, aimed at characterizing indoor environmental air of primary classrooms in the city of Maia, Portugal, and assessed the variability in pollutant levels between classrooms and correlations with classroom ventilation rates, occupancy rate, and other factors. This study also aimed at checking the impact of IAQ awareness as a public health problem in the environmental quality of schools.

In the city of Coimbra, Portugal, 1,019 students of 51 elementary schools were evaluated by Ferreira and Cardoso (2014), in order to determine if IAQ in schools was associated with the prevalence of allergic and respiratory diseases in children, through the application of a questionnaire and the assessment of school's IAQ, performed in the fall/winter (between November of 2010 and February of 2011) and in the spring/summer (between March of 2011 and June of 2011). The most prevalent symptoms and respiratory diseases identified in the children were sneezing, rales, wheezing, rhinitis, and asthma and the concentrations of various pollutants, suggest the need for corrective interventions, such as reducing air pollutant sources and improving ventilation.

The research project conducted by Madureira (2014), entitled "On the Contribution of Schools to Children's Overall Indoor Air Exposure" has similar aspects compared with the present investigation. Its main objective was to study IAQ in schools and homes, in order to understand its impact on children's health, also targeting asthma, allergy, and respiratory symptoms. The research project was designed in two phases. In the first phase, the study comprised 20 schools, 73 classrooms and microbiological air sampling (bacteria and fungi) was performed using a

single-stage microbiological air impactor, in two heating season periods: November 2011 to March 2012 and November 2012 to December 2012. Endotoxins concentrations were not evaluated. School's IAQ was assessed through the measurement of a set of indoor air parameters; indoor air levels were associated with building characteristics and occupant behavior as in this study; and it was evaluated the association between indoor air exposure and children's health outcomes focused on asthma, allergies and respiratory symptoms, out of the scope of this thesis. In the second phase, a subsample of 68 children was enrolled for further studies, including a walk-through inspection and checklist and an extensive set of IAQ measurements in their homes. The acquired data are relevant to assess children's environmental exposures and health status (Madureira, 2014; Madureira *et al.*, 2015c). This home assessment was also performed in ARIA Project but it is also out of the scope of the present thesis.

In 2014, a study performed by Madureira *et al.* (2014) aimed to characterize airborne fungi populations present in public primary schools in Porto, Portugal, during winter. Results showed a wide range of indoor fungi levels, with indoor concentrations higher than outdoors and the most prevalent fungi found indoors were *Penicillium sp.* and *Cladosporium sp*.

In the current year, 2015, 3 articles regarding IAQ in primary schools of Porto city were published. The first study concerned the assessment and determinants of airborne bacteria and fungi concentrations in 4 different indoor environments: homes, child day-care centers, primary schools and elderly care centers (Madureira *et al.*, 2015a). Air samples were collected during winter season in 20 primary schools (children aged 8-10 years old) of Porto city, in 73 classrooms, with a microbiological air sampler. Children's had two times higher dose rate to biological pollutants when compared to adult individuals, results that indicate, due to children's susceptibility, that special attention should be given to educational settings in order to guarantee their healthy future development. The main purposes of the second study (Madureira *et al.*, 2015b) were: to assess IAQ parameters and compare them with previous studies and guidelines; to study potential sources of indoor pollutant levels, such as building/classroom characteristics and occupant behavior; and to assess the variability in pollutant levels within and between schools. Concentrations of culturable bacteria were frequently higher than guidelines/reference values and the variability of bioaerosol concentrations between schools exceeded the variability within schools. Third study published (Madureira *et al.*, 2015d) was a cross-sectional survey, conducted to characterize the IAQ in schools and its relationship with children's respiratory symptoms, where concentrations of bacteria and fungi were assessed in 73 classrooms of 20

public primary schools located in Porto and children who attended the selected classrooms (n=1134) were evaluated by a standardized health questionnaire completed by the legal guardians, along with spirometry and exhaled nitric oxide tests. Bacteria levels exceeded the WHO air quality guidelines or national limit values, indicating that indoor air pollutants can be related with the development of respiratory symptoms, indicating that it is crucial to take into account the unique characteristics of the public primary schools, to develop appropriate control strategies in order to reduce the exposure to indoor air pollutants and, therefore, to minimize the adverse health effects.

Analyzing the Portuguese studies it is possible to conclude that data was collected in a few number of schools and/or assessed a small set of parameters. Until now, from the existent literature there are no performed studies in Portuguese primary schools that managed to evaluate, in a large sample of classrooms, building and classrooms characteristics, monitored biological agents levels (including bacteria, fungi and endotoxins), and studied the relationship between the concentrations of these microbiological agents and other chemical (CO_2) and physical parameters (T and RH), as well as with another important factors that may influence indoor biological levels (for example, occupant density and presence of dampness and mold).

4. METHODOLOGY

A description of the study design and a detailed explanation of the cross-sectional study of children's indoor air exposure in primary schools that set the foundations of this investigation are presented in this chapter. It is also enlightened how calculation and data analysis was performed in the present study.

4.1. Study design

In order to fulfill the objectives of this study a cross-sectional study to investigate the association between the IAQ in school and children's health was carried out. The contact with schools was allowed by Direção Geral dos Estabelecimentos Escolares, the official entity that assures regional policies for all schools in Porto. Nevertheless, public primary schools are run by the local municipality administration.

Porto is located in the North of Portugal (41°08'58.9"N, 8°36'39.1"W) and it is the second largest Portuguese city. The city heads the Porto Metropolitan Area, which includes, in 2015, according to the Statistics Portugal data, 18 municipalities, about 218 231 residents and a population density of 5270 habitants per square kilometer (km^2) (Statistics Portugal, 2015). The city of Porto is located along the Douro river estuary, featuring the Mediterranean climate (Köppen climate classification=Csb) with warm and dry summers and mild and wet winters (Aguiar *et al.*, 2014; Mendes *et al.*, 2014). The research team considered that all schools included in the study were exposed to the same climate.

Out of a total of 53 public primary schools located in Porto urban area, the 20 schools with the highest number of students and considered representative of the building stock were invited to participate in this study and all of them agreed. The selected number of studied schools correspond to approximately half of the public primary schools in Porto and about of 3500 children. In each school, four classrooms of 3rd and/or 4th grades were selected, among classrooms with similar conditions and considered to be representative of the school building. Exceptions were made in 3 schools where there were only 3 classrooms designed to the 3rd and 4th grades, and other 3 with only 2 classrooms. Preferably, classrooms should have high density of occupation and full weekly occupation time by the same class; and, if possible, at different floor levels. Therefore, the sample in study is considered a semi-random sample of children. In

this study, a total of 71 classrooms were assessed, comprising a universe of 1523 students attending daily classes.

4.2. Cross-Sectional Study of Children's Indoor Air Exposure in Primary Schools

Cross-sectional studies are frequently exploited in health research fields, due to its observational characteristics and the ability to collect, analyze and compare several data from different population groups at a specific point in time. There is no experimental procedure, so no variables are manipulated by the researcher. Another important characteristic is that this type of study provides information and describes certain variables in a group. As an observational study it is out of its scope the establishment of cause-and-effect relationships.

This cross-sectional study gathered data collected in two heating season periods: January to April 2014 and October 2014 to January 2015, as these two periods provides the worst case scenario of exposure due to the enclosed environments. Ten schools were evaluated in the first heating season period, corresponding to 35 classrooms, and 10 other schools in the second period campaign, with 36 classrooms. Figure 4 shows the geographical distribution of the selected public primary schools. Measurements were made during normal activities and under representative conditions of occupancy and use of the classrooms, and conducted in a discreet fashion in order not to disturb occupants' normal behavior.

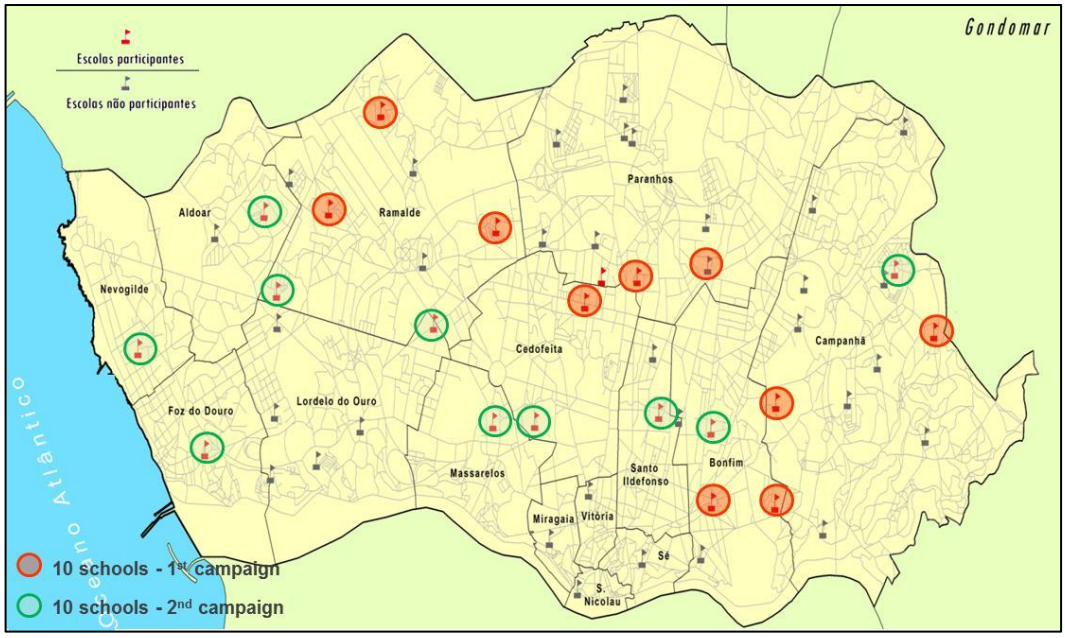

Figure 4 - Geographical distribution of the selected 20 public primary schools

In each school, along with the classrooms, one outdoor location was identified as the main indoor air supply and simultaneously studied. Samplers were placed at a height of 1-1.5 m above the floor, approximately at the breathing zone level, and as close as possible to the center of the room, away from walls, windows, doors or an active heating system.

In the present study, the following parameters were studied: (i) building and classroom characteristics; (ii) biological parameters, such as total bacteria count, fungi count and identification, and endotoxin concentrations; (iii) chemical parameter CO_2; and (iv) physical parameters: air T and RH. Each of these parameters was sampled with a specific sampling strategy for each pollutant (e.g. equipment, protocols, sample collection and analysis). An interdisciplinary work was undertaken supported on multiple field assessments to characterize children's exposure to indoor air and related health effects. Biological parameters determination and analysis were responsibility of ISPUP and INSA and were performed using methodologies accredited by International Standard Organization (ISO) guideline NP EN ISO/IEC 17025:2005 "General requirements for the competence of testing and calibration laboratories" (ISO, 2005). Chemical and physical parameters sampling were conducted by INEGI.

It is also important to refer that no intervention or improvement measures were applied in classrooms where biological levels were above the reference values. Tough, and in the scope of ARIA Project, an intervention study was conducted, but once again out of the scope of this thesis. The results of this intervention study will be the target of an independent publication, already submitted in 2015 to the Journal of Toxicology and Environmental Health, Part A.

4.2.1. Buildings and classrooms walk-through and checklist

A walkthrough survey was completed for each building school and individual rooms, by a trained researcher to gather information on building structure; age and size; refurbishment; number of floors; finishing materials; floors, walls and ceilings conditions; windows; areas; heating and ventilation systems; past occurrences and visible problems; potential indoor sources; and school and classroom number of occupants. Walkthrough inspection was made using a standardized validated checklist (Annex I) that was previously field-tested in previous European Projects, such as SINPHONIE (www.sinphonie.eu), and was fulfilled with the aid of principals and teachers. Air sampling and walkthrough inspections were performed simultaneously.

4.2.2. Bacteria and Fungi

Bacterial and fungal air samples were obtained using a single stage microbiological air impactor (Merck Air Sampler MAS-100) (Figure 5), following National Institute for Occupational Safety and Health (NIOSH) Method 0800:1998 - Bioaerosol Sampling (Indoor Air) (NIOSH, 1998) and EN 13098:2000 (European Standards, 2000). The equipment is calibrated annually in an external and accredited calibration laboratory. Tryptic Soy Agar (TSA) (supplemented with 0.25% cicloheximide) and Malt Extract Agar (MEA) (supplemented with 1% of chloramphenicol) were used as culture media for bacteria and fungi, respectively. Air was drawn through the sampler at a 100 liters per minute (L/min) rate and sequential duplicate air samples of 250 L were collected both indoors and outdoors. The used sampling volume (and consequently the duration) was the same within all rooms studied in a specific building. For each sampling day, four field blanks, two sterility blanks, one positive control and one negative control, per culture medium, was used. The air sampler was cleaned between each sample collection with cotton wipes wetted with isopropyl alcohol. After sampling, the agar media plates were sealed, marked and transported to the laboratory in a thermal bag for processing.

Figure 5 - Microbiological air impactor (Merck Air Sampler, MAS-100)

To quantify bacteria and fungi concentrations, samples were incubated at 37±1°C for 48±3 h and at 25±3°C for 72±3 h, respectively (European Standards, 2000). Quantification was performed by naked eye count in accordance to the methodologies expressed in ISO 4833:2013 (ISO, 2013) and EN 13098:2000 (European Standards, 2000). The number of colonies recovered on each plate was adjusted using a positive-hole correction factor, based on the Fellers law, that takes in account the probability that more than one particle containing a cultivable microorganism, passes through the same hole (Andersen, 1958). Results were expressed as

number of colony forming units per cubic meter of air (CFU/m^3). The quantification limit was established as 10 CFU per plate. Fungal identification was performed either on the original sampling media-MEA plates or after subculturing procedures, whenever colony isolation and growth observation were needed. Subculture was made on MEA plates and incubated, at 25±3°C, for periods ranging from 3 days to 3 weeks.

Identification of fungal colonies was based upon phenotypic characteristics and followed standard mycological procedures based on their micro and macro-morphological characteristics (Fisher & Cook, 1998).

4.2.3. Endotoxins

Air samples were collected with a portable high flow pump (Gilian, GilAir3) connected to a button aerosol sampler (SKC, Cat-no 225-360) containing a 25 mm glass microfibre filter (GFA Whatman, Cat-no 1820-025). Prior to sampling, pumps were always calibrated to a flow of 2L/min using a primary flow calibrator (Gilian, Gilibrator 2), that was calibrated annually at an external and accredited calibration laboratory. After sampling, pumps' flow was verified in order to check if its variation was between the accepted range (±5% of 2L/min). Time for sampling was established in 4 hours according to the Analytical Method 332 "Endotoxin analysis", from *Institut de Recherche Robert-Sauvé en Santé et en Sécurité du Travail* (IRSST) (IRSST, 2009). All materials used for sampling (button aerosol sampler, glass microfibre filter and connecting tube) were treated at 180°C for at least 4 hours prior to sampling. Samples were kept frozen at -20°C until analysis.

Endotoxin analysis comprises 2 steps: extraction and quantification. For extraction, sample filters were eluted in 5 ml extraction solution (Pyrogen Free Water plus 0.05% Tween 20) and rocked vigorously for 1 h at room temperature on a horizontal shaker. After 10 minutes of centrifugation at 1000 g, total supernatant per sample was collected and analyzed. Endotoxin quantification was performed using the LAL Kinetic-QCL™ (Lonza®) following the manufacturer's guidelines. Endotoxin concentrations were expressed as EU/m^3. The limit of detection for the LAL Kinetic-QCL™ is 0.005 EU/mL, corresponding to 0.025 EU/m^3 under the adopted procedure, which means that this concentration was the minimum measurable airborne endotoxin concentration.

4.2.4. Carbon Dioxide, Temperature and Relative Humidity measurements

The CO_2 concentrations, T and RH levels were continuously measured for a period of 5 days (school week, from Monday morning to Friday afternoon) with 5 minute intervals, using a

portable IAQ meter IAQ-CALC (model 7545; TSI, Inc.) (Figure 6). Within each building and corresponding outdoor measurements were recorded concurrently.

Figure 6 - TSI 7545 IAQ-CALC

Regarding CO_2 measurements, the instrument includes an infrared non-dispersive sensor in a range from 0 to 5000 parts per million (ppm) with accuracy of ±3% of reading or ±50 ppm. For T and RH, the equipment includes a thermistor for measuring T in a range from 0 to 60 °C with an accuracy of ±0.6 °C, and a thin-film capacitive sensor for RH (range of 5 to 95% RH; accuracy ±3.0% RH). The equipments used were calibrated annually, according to manufacturer's specifications.

Data were downloaded onto LogDat2™ downloading software and then exported for data management.

4.3. Calculation and Data Analysis

Biological assessment results (quantification of bacteria, fungi and endotoxins as well as fungi identification) were compared to Portuguese and international reference levels and guidelines.

Classical statistical methods were used to estimate means, medians and frequencies (percentages), standard deviation and ranges. Descriptive statistics were described as scatter and bar charts. The indoor to outdoor (I/O) ratio was calculated to determine the impact of outdoor sources on indoor air biological concentrations.

The Shapiro-Wilk test was performed to check for distribution normality. Since non-Gaussian (or non-Normal) distributions were observed for the majority of cases, non-parametric tests were used. Nonetheless, it was decided to use the mean for descriptive purposes.

Mann-Whitney (U) test for independent samples was used to compare indoor biological parameters measurements within categorical variables. Wilcoxon Signed-Rank (Z) test for two paired samples allowed insight on differences between indoor and outdoor percentages of the predominant fungi species detected in the present study. Kruskall-Wallis (H) test for multiple independent samples was applied in order to compare indoor biological concentrations between schools. Spearman's rank correlation coefficient (r_s) was performed to assess possible correlations between some continuous variables in study.

A p-value < 0.05 was considered as statistically significant. Expanded uncertainty was evaluated for 95% confidence interval based on probability distributions propagation of measurements, obtained by multiple samples and considering instrumental uncertainty obtained from traceable calibrations.

All data were analyzed and graphed using International Business Machines (IBM)® Statistical Package for Social Sciences (SPSS)® software (version 23.0.0.0) for Windows and Microsoft Office Excel 2010 (version 14.0.7153.5000).

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5. RESULTS

This section holds the findings of the present investigation, namely: a presentation of building schools and classrooms characteristics through the application of a standardized checklist; the results of biological assessment and indoor/outdoor ratios; a description of fungi genera/species identification; correlation results between chemical, physical and biological parameters; and finally, the outcomes of the explored associations between school and classroom characteristics and the biological indoor concentrations.

5.1. Schools' characteristics: buildings and classrooms

The points of SINPHONIE checklist we chose to register were those related to the analyzed outcomes, namely the ones described in Table 1, in accordance with what was mentioned in 4.2.1.

Table 1 - Main sections of SINPHONIE school and classroom checklists considered in the present study

School checklist	**Classroom checklist**	
Construction characterization	Indoor characterization	Natural ventilation
Ventilation	Visible problems	Mechanical ventilation
Past occurrences or visible problems	Heating characterization	Classroom use of IAQ sources

Table 2 presents the main buildings characteristics and corresponding values obtained. Most of them (65%) were built between 1951 and 1971 and 15% (n=3) were constructed between 1972 and 1991. Ninety-five percent of the schools were refurbished after 2004: 58% in 2004-2009 and 42% in the period of 2010-2014. Mean total area per school was 1382.40 m^2, with a minimum of 752 m^2 and a maximum of 2500 m^2, to a mean total of 228 occupants per school, with a range of 110 to 417 occupants per school. All schools had massive wall structure with 60% of the buildings including double wall. One school (5%) had flat roof; the others 19 (95%) presented ridge roof. The same was observed for roof structure: 95% had massive structure and 5% lightweight. Eighty-five percent of schools (n=17) had only natural ventilation and 10% (n=2) had natural assisted ventilation by exhaustion. One school (School 19) had mechanical ventilation, with only supply system and local manual control. System intake was located on the schools' roofs and the air exhaustion grids were located at the classrooms ceilings. There was no

air handling units and maintenance was performed twice a year or often, i.e. filters cleaned and/or replaced, as well as cleaning of supply air devices and exhaust air grids.

Regarding water leakage or flooding in the past twelve months, 35% of the schools (n=7) had it at the roof level and 15% (n=3) at windows level. In 45% of the schools (n=9) were observed visible air leaks in the structure.

Table 2 - Buildings' characteristics

Building characteristics (n=20 schools)							
	n	%	Mean	SD	Median	Min.	Max.
Year of Construction							
1930 – 1950	4	20					
1951 – 1971	13	65					
1972 – 1991	3	15					
Building Refurbishment							
No	1	5					
Yes	19	95					
2004 – 2009	11	58					
2010 – 2014	8	42					
Total area (m^2)			1382.40	455.81	1278.50	752	2500
Total number of occupants			228	79	221	110	417
Wall Type							
Single wall	8	40					
Double wall	12	60					
Wall Structure							
Massive	20	100					
Lightweight	0	0					
Roof Type							
Flat Roof	1	5					
Ridge Roof	19	95					
Roof Structure							
Massive	19	95					
Lightweight	1	5					
Ventilation strategy							
Natural	17	85					
Natural assisted (exhaustion)	2	10					
Mechanical	1	5					
Water leakage or flooding [a]							
Roof	7	35					
Windows	3	15					
Façade	0	0					
Basement	0	0					
Water Pipes	0	0					
Visible air leaks [b]							
Yes	9	45					
No	11	55					

SD: Standard deviation; Min.: Minimum; Max.: Maximum. [a] in the last 12 months; [b] in the structure; cracks in the construction.

All evaluated classrooms (n=71) were occupied 5 days per week, Monday until Friday, from 9:00 a.m. to 5:30 p.m. Most of them (59%) were located at 1st floor of the buildings. Classrooms size and ceiling height ranged between 34 m^2 and 95 m^2 (mean=50.65±7.82 m^2) and 2.94 m and 3.92 m (mean=3.39±0.19 m), respectively. On average, windows' area was 13.79±4.80 m^2 and 96% of classrooms had aluminum windows frame and 68% single glazing. The range of occupants per classroom varied from 13 to 28, with a mean of 21±3 occupants. Density of occupation ranged from 1.71 to 4.30m^2/occupant, with mean value of 2.40±0.45m^2/occupant.

Regarding walls, floors and ceiling materials, all classrooms had water-based wall covering with 70% synthetic smooth floor covering and 92% paint ceiling surface. Visible mold growth was noticed in 11 classrooms (15%) on ceilings, walls and their joints; other damp/mold symptoms were registered: noticeable mold odor (n=5) and visible damp spots on walls, ceiling or floor (n=10). Condensation on windows was reported for 21% (n=15) of the studied classrooms.

The majority of classrooms (94%, n=67 classrooms) had heating systems and natural ventilation. The other 4 classrooms, all in School 19, had mechanical ventilation. In 45% of classrooms windows were not usually open during heating season. Nevertheless, windows were reported to be open mainly during breaks (48%) or during teaching hours (47%). None of the 71 evaluated classrooms registered air cleaners, humidifiers and/or dehumidifiers, as well as rugs or cushions. The number of space heaters per classroom ranged from 0 to 3; in most of the classrooms (55%, n=39) none was registered and 32% (n=23) had one space heater. In Tables 3a and 3b all these aforementioned classrooms' characteristics were described.

Table 3a - Classrooms' characteristics

Classroom characteristics (n=71 classrooms)					
	Mean	SD	Median	Min.	Max.
Floor area (m^2)	50.65	7.82	48.65	33.97	94.52
Ceiling height (m)	3.39	0.19	3.42	2.94	3.92
Windows area (m^2)	13.79	4.80	12.65	2.80	25.82
Occupants per class (number)	21	3	21	13	28
Density of occupation (m^2/occupant)	2.40	0.45	2.36	1.71	4.30

SD: Standard deviation; Min.: Minimum; Max.: Maximum.

Table 3b - Classrooms' characteristics

Classroom characteristics (n=71 classrooms)		
	n	%
Floor number		
0	25	35
1	42	59
2	4	6
Window frame		
Metal	3	4
Aluminum	68	96

Table 3b - Classrooms' characteristics - conclusion

Classroom characteristics (n=71 classrooms)		
	n	%
Type of glazing		
Single	48	68
Double	23	32
Ceiling surface		
Paint	65	92
Wood	6	8
Wall covering		
Water-based	71	100
Floor covering		
Synthetic smooth	50	70
Wood/Cork	21	30
Visible mold growth		
No	60	85
Yes	11	15
Ceiling	4	40
Ceiling and wall(s)	2	20
Joint of ceiling with wall	4	40
Other damp/mold symptoms		
Noticeable mold odor	5	7
Visible damp spots on walls, ceiling or floor	10	14
Bubbles or yellow discoloration of plastic floors	0	0
Blackened wood floor	0	0
Condensation on windows		
No	56	79
Yes	15	21
Inside the frame	16	23
Heating system		
Heating only	67	94
Heating + domestic hot water	4	6
Ventilation system		
Openable windows	67	94
Hybrid/mixed model (natural + mechanical)	4	6
Number of windows opened in heating season		
0	32	45
1	30	42
2 / 3	9	13
When are windows opened?		
Before school time	4	6
During breaks	34	48
During teaching hours	33	47
After school time	4	6
During night	4	6
During cleaning time	13	18
Number of space heaters		
0	39	55
1	23	32
2	7	10
3	2	3

5.2. Biological Assessment

Biological assessment took place during heating season, with outdoor mean air T in Porto of 16°C [10–30°C], and mean RH of 63% [20-79%]. Figures 7 and 8 show pictures of air samples collected for bacteria and fungi determination, respectively, and after the respective periods of incubation.

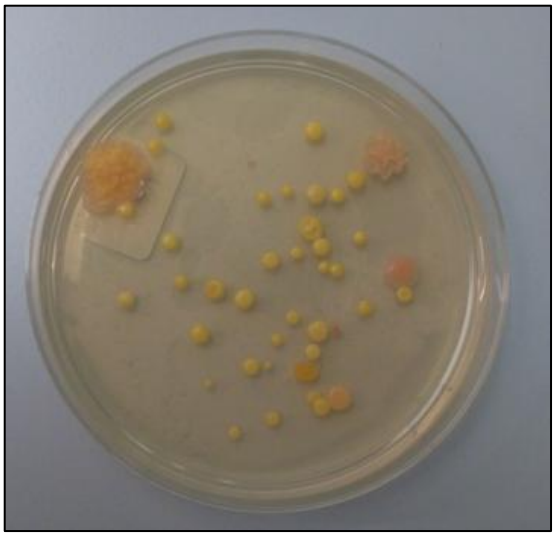

Figure 7 - Bacteria plate after incubation

Figure 8 - Fungi plate after incubation

Table 4 shows the results for bacteria and fungi concentration and descriptive statistics by primary school, along with descriptive statistics, and reference values for both biological agents established by Portuguese legislation (Ordinance no 353-A/2013 from 4th of December 2013).

Mean bacteria concentrations were higher than the reference value (outdoor + 350 CFU/m^3) in all schools (100%); of the 71 classrooms evaluated, 93% (n=66) were above the reference value. Mean fungi concentrations exceeded outdoors concentrations (reference value) in 15 of the 20 studied schools (75%); at the classroom level this was observed in 63% (n=45). It is important to notice that bacteria concentrations indoor are 2 to 9 times higher than the established limit value. Figure 9 illustrates how bacteria indoor levels are much higher than what was found outdoors along all schools evaluated.

Table 4 - Bacteria and fungi assessment results: descriptive statistics by primary school (**red mean values** - higher than the reference value; green mean values - lower than the reference value)

Primary School	Bacteria								Primary School	Fungi							
	Indoor Mean±SD	Outdoor	Reference Value [a][b]	Median	P25	P75	Min.	Max.		Indoor Mean±SD	Outdoor	Reference Value [a][c]	Median	P25	P75	Min.	Max.
	CFU/m³									CFU/m³							
01	**2466**±984	398	**748**	2466	1770	3162	1770	3162	01	323±58	394	**394**	323	282	364	282	364
02	**1182**±407	98	**448**	1268	931	1432	612	1578	02	**242**±138	170	**170**	200	145	338	130	436
03	**1442**±308	44	**394**	1497	1202	1681	1044	1728	03	**542**±313	122	**122**	592	292	792	150	834
04	**4080**±3003	96	**446**	2931	2271	5889	1946	8512	04	**6393**±2830	160	**160**	5407	4575	8211	4246	10512
05	**2836**±1782	174	**524**	2540	1552	4119	1044	5218	05	**363**±80	192	**192**	331	312	414	310	480
06	**2819**±848	4	**354**	2917	2098	3540	1898	3544	06	**253**±131	118	**118**	220	150	356	150	422
07	**1333**±1107	44	**394**	1314	383	2282	268	2434	07	**1198**±1084	108	**108**	988	331	2065	250	2566
08	**1522**±747	36	**386**	1522	994	2050	994	2050	08	**589**±24	90	**90**	589	572	606	572	606
09	**1552**±742	688	**1038**	1364	922	2370	922	2370	09	537±250	592	**592**	468	328	814	328	814
10	**2802**±1660	1520	**1870**	2620	1611	3992	1008	4958	10	**338**±89	252	**252**	325	281	395	244	458
11	**3573**±3170	250	**600**	2914	1025	6120	902	7560	11	740±200	1724	**1724**	754	568	911	538	912
12	**1539**±1416	382	**732**	977	633	2445	590	3612	12	674±156	796	**796**	675	544	804	508	838
13	**2367**±167	940	**1290**	2432	2178	2492	2178	2492	13	**293**±164	144	**144**	304	124	452	124	452
14	**2563**±1062	124	**474**	2856	1752	3373	1138	3400	14	**333**±146	236	**236**	337	217	449	162	496
15	**3996**±362	148	**498**	4027	3717	4274	3546	4382	15	**844**±400	580	**580**	967	612	1075	262	1178
16	**3011**±565	114	**464**	2872	2528	3632	2528	3632	16	**7363**±5455	112	**112**	10512	1064	10512	1064	10512
17	**3195**±349	202	**552**	3195	2948	3442	2948	3442	17	**508**±221	136	**136**	508	352	664	352	664
18	**1849**±301	192	**542**	1822	1637	2060	1514	2236	18	**225**±89	178	**178**	215	152	297	138	330
19	**1250**±293	84	**434**	1217	1040	1460	934	1632	19	**88**±39	88	**88**	82	62	114	48	140
20	**2066**±448	56	**406**	1952	1755	2376	1666	2692	20	199±23	298	**298**	193	180	217	180	228

SD: Standard Deviation. P25: 25th Percentile. P75: 75th Percentile. Min.: Minimum. Max.: Maximum. [a] Ordinance no 353-A/2013 of 4th December 2013. [b] **Bacteria**: < outdoor + 350 CFU/m³. [c] **Fungi**: < outdoor.

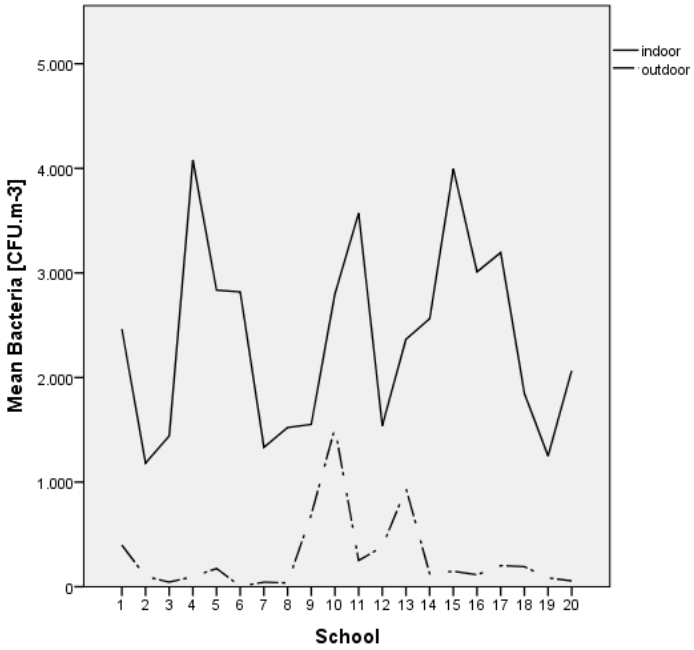

Figure 9 - Indoor and Outdoor Mean Bacteria Concentrations (CFU/m³) by primary school

Regarding fungi concentrations, although in most schools its limit value was exceeded, indoor fungi levels found at Schools 04 and 16 were worrying: ranges from [4246–10512] CFU/m³ and [1064–10512] CFU/m³ corresponding to outdoor levels of 160 and 112 CFU/m³, respectively. This fact means that indoor levels were 40 and 66 times higher than outdoor concentrations, respectively. Both peaks are well perceived on Figure 10. In the majority of schools (75%, n=15), indoor fungi levels did not meet the Portuguese recommendation.

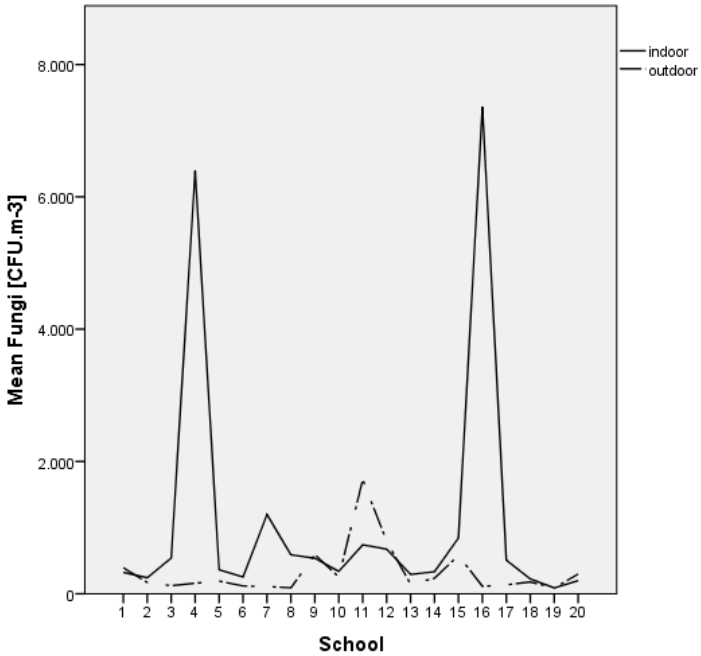

Figure 10 - Indoor and Outdoor Mean Fungi Concentrations (CFU/m³) by primary school

Besides the aforementioned national legislation, another reference value is established for indoor air fungi concentrations: 500 CFU/m³, by WHO (2009). Taking into account this limit value, it was only exceeded in 10 of the evaluated schools (50%). With this comparison it is possible to conclude that a higher number of schools were considered above the reference value when compared with national legislation (75%, n=15 schools) rather than international recommendations (50%, n=10 schools).

Table 5 presents endotoxins' concentrations global results. The considered limit of 50 EU/m³ was not exceeded in any of the schools. In fact, in 18 out of the 20 schools (90%) the levels were below 10 EU/m³, as demonstrated in Figure 11. School 17 had the higher endotoxin level, with a mean value of 22.44 EU/m³ and a range of 14.26–30.62 EU/m³. Nevertheless, none of the maximum values observed exceeded the limit value.

Table 5 - Endotoxins assessment results: descriptive statistics by primary school

Primary School	Endotoxins						
	Indoor Mean±SD	**Reference Value** [d]	**Median**	**P25**	**P75**	**Min.**	**Max.**
	EU/m³						
01	1.32±0.62	**50**	1.32	0.88	1.76	0.88	1.76
02	1.37±0.95		1.15	0.69	2.05	0.50	2.68
03	1.88±1.56		1.79	0.55	3.22	0.51	3.44
04	3.10±0.99		2.86	2.40	3.80	2.20	4.47
05	1.24±0.82		1.24	0.57	1.91	0.32	2.15
06	0.69±0.69		0.56	0.26	1.12	<0.025 [e]	1.64
07	0.86±0.86		0.76	0.17	1.55	<0.025 [e]	1.91
08	0.71±0.66		0.71	0.24	1.18	0.24	1.18
09	4.88±1.01		5.22	3.74	5.68	3.74	5.68
10	1.85±0.78		1.77	1.21	2.49	1.08	2.77
11	1.53±0.25		1.56	1.33	1.73	1.23	1.76
12	4.17±3.06		4.57	2.23	6.10	0.06	7.46
13	7.40±1.42		8.09	5.77	8.35	5.77	8.35
14	3.82±3.04		3.17	1.77	5.87	0.89	8.03
15	3.95±2.98		2.92	1.97	5.92	1.68	8.26
16	10.91±3.71		11.29	7.03	14.42	7.03	14.42
17	22.44±11.57		22.44	14.26	30.62	14.26	30.62
18	1.98±0.89		1.99	1.22	2.73	1.04	2.88
19	4.96±2.74		4.25	3.00	6.92	2.57	8.77
20	5.33±1.61		5.18	4.00	6.66	3.75	7.20

SD: Standard Deviation. P25: 25th Percentile. P75: 75th Percentile. Min.: Minimum. Max.: Maximum. [d] Heederik and Douwes (1997).
[e] **<LOQ** (Limit of Quantification)=0.005 EU/mL; 0.025 EU/m³.

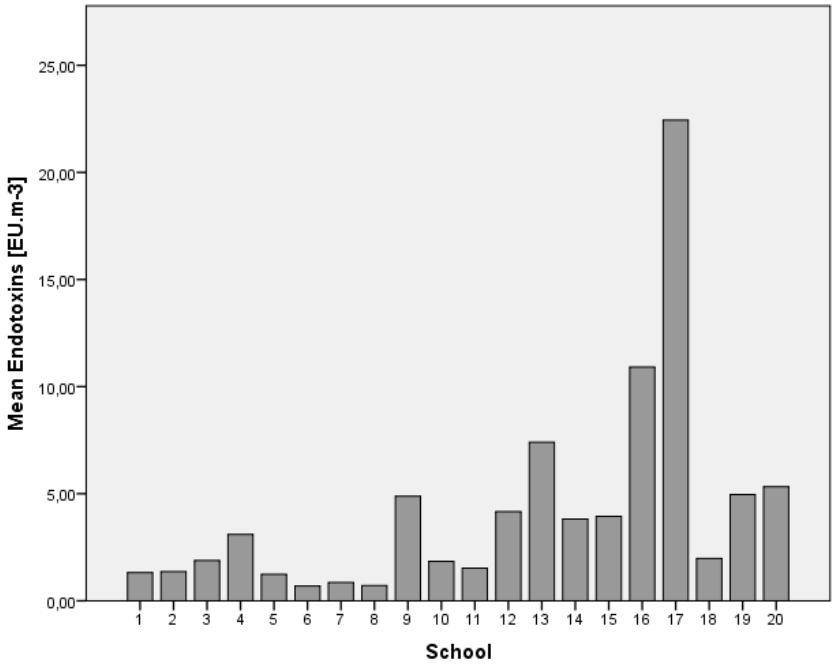

Figure 11 - Mean endotoxins concentrations (EU/m³) by primary school

Mean values of bacteria, fungi and endotoxins concentrations of all 71 classrooms evaluated are summarized in Table 6. The 20 outdoor measurements correspond to each school where the study took place. Overall concentrations of bacteria and fungi were both higher than the reference values, established by the mean outdoor concentrations in all schools. For bacteria, mean indoor concentrations were 4 times higher than the outdoor and for fungi a 3-fold increase was found. Noteworthy the maximum levels found of both agents indoors and outdoors: bacteria with a maximum indoor value of 8512 CFU/m³ against a maximum outdoor value of 1520CFU/m³ (6 times higher). Concerning fungi, values of 10512 CFU/m³ indoors were found compared to the 1724 CFU/m³ outdoors (once again, 6 times higher). Results regarding the impact of outdoor bacteria and fungi concentrations on indoor air will be analyzed in detail in the next subsection (5.3).

Concerning endotoxins, with a range of <0.025–30.62 EU/m³ overall concentrations found in all classrooms were within the reference value.

Table 6 - Overall results of biological assessment

Biological Parameter	Units	Indoor				Outdoor				Reference Value
		n	Mean±SD	Min.	Max.	n	Mean±SD	Min.	Max.	
Bacteria	CFU/m³	71	**2373**±1489	268	8512	20	280±374	4	1520	**630** [f] [g]
Fungi		71	**1086**±2234	48	10512	20	325±383	88	1724	**325** [f] [h]
Endotoxins	EU/m³	71	3.74±4.52	<0.025 [i]	30.62		-			**50** [i]

SD: Standard Deviation. Min.: Minimum. Max: Maximum. [f] Ordinance no 353-A/2013 of 4th December 2013. [g] **Bacteria**: < outdoor + 350 CFU/m³. [h] **Fungi**: < outdoor. [i] Heederik and Douwes (1997). [i] **<LOQ** (Limit of Quantification)=0.005 EU/mL; 0.025 EU/m³.

To understand the variability of indoor biological concentrations between the 20 studied schools was applied a Kruskall-Wallis (*H*) test. Results showed significant statistical differences between schools for bacteria ($H(19)=32.588$; $p<0.05$), fungi ($H(19)=50.597$; $p<0.001$) and endotoxins ($H(19)=47.839$; $p<0.001$) concentrations, just as expected through the observation of concentrations showed on Figures 9, 10 and 11. The sampling campaign took place in two different heating periods, with ten schools assessed in each period (first sampling period from January to April 2014: School 01 to School 10; second sampling period from October 2014 to January 2015: School 11 to School 20). In this sense, it was considered important to investigate if there were differences between these ten schools of each sampling period. For bacteria concentrations, significant statistical differences were only found between the ten schools assessed in the second sampling period (first: $H(9)=14.528$; $p>0.05$; second: $H(9)=16.983$; $p<0.05$). Based in Figure 9, is easy to identify that in each sampling campaigns, one school standout from the others, with higher bacteria concentrations. School 04, in the first sampling campaign, and School 15, in the second sampling campaign. Nevertheless, the concentration variability in these two schools was in opposite poles. In School 15, mean (3996 CFU/m³) and median (4027 CFU/m³) values were very similar, with low standard deviation (362 CFU/m³), while in School 04 mean (4080 CFU/m³) and median (2931 CFU/m³) values were very different, and with a high standard deviation (3003 CFU/m³). Contrarily, for fungi concentrations were found significant statistical differences in both sampling periods (first: $H(9)=19.357$; $p<0.05$; second: $H(9)=28.342$; $p<0.01$), between the ten schools assessed in each. It is important to refer that in each sampling period one school had pronounced higher mean values than the others, as showed in Figure 10. Equivalent *H* test results for endotoxins showed significant statistical differences between endotoxin concentrations between the ten schools assessed, in each sampling period (first: $H(9)=18.149$; $p<0.05$; second: $H(9)=21.210$; $p<0.05$). Also for endotoxins were observed higher concentrations in one school, in each sampling campaign.

5.3. Outdoor *vs.* Indoor air biological concentrations

In this study, indoor and outdoor levels of bacteria and fungi concentration were very distinct. Figure 12 shows the significant difference found between indoor and outdoor bacteria concentrations (U=37.000; p<0.001) as well as between indoor and outdoor fungi concentrations (U=393.500; p<0.01), with indoor levels clearly higher than outdoors for both biological agents.

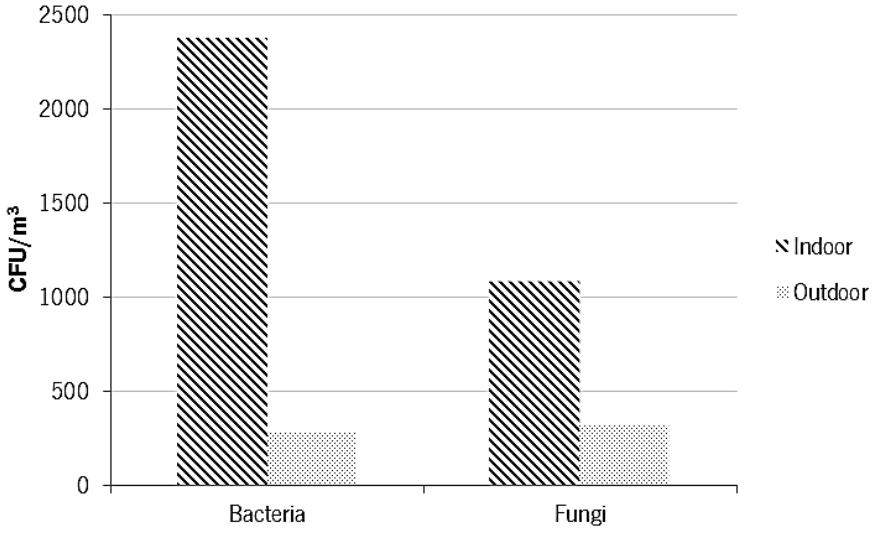

Figure 12 - Mean indoor and outdoor bacteria and fungi concentrations (CFU/m³)

In order to obtain a comparable measure for the difference between indoor and outdoor levels of bacteria and fungi, I/O ratios were calculated taking into account the average of all classrooms at each school and presented at Table 7. For bacteria, we obtained an I/O ratio higher than 1 in all evaluated schools. In fact, the minimum I/O ratio observed was 1.8 at School 10 and the maximum 704.8 at School 06. In the overall evaluation of I/O ratio, indoor bacteria concentrations were 9 times higher than outdoors. Schools 01, 09, 11, 12 and 20 had an I/O ratio for fungi below 1, meaning that in these schools outdoor concentrations of fungi were higher comparing to what was found indoors. Nevertheless, as mentioned before, Schools 14 and 16 had indoor fungi concentrations of 40 and 66 times higher than outdoors. Taking into consideration the mean values of all 71 classrooms and all 20 outdoors measurements, indoor fungi levels were 3 times higher than the levels found outdoors.

It is known that in indoor air, particularly in areas naturally ventilated, that one of the major sources of fungi is outdoor air (Levetin *et al.*, 1995; Tang, 2009). To investigate if high outdoor fungi concentrations had influence on indoor fungi levels, Spearman's rank correlation coefficient (r_s) was applied and there was no correlation between these two variables (r_s=0.048; p>0.05).

The same hypothesis of investigation was considered regarding bacteria concentrations and, once more, no correlation was found (r_s=0.259; p>0.05).

Table 7 - Indoor/Outdoor ratios for bacteria and fungi concentrations

Primary School	Bacteria			Primary School	Fungi		
	Indoor Mean	Outdoor	I/O Ratio		Indoor Mean	Outdoor	I/O Ratio
	CFU/m³				CFU/m³		
01	2466	398	**6.2**	01	323	394	0.8
02	1182	98	**12.1**	02	242	170	**1.4**
03	1442	44	**32.8**	03	542	122	**4.4**
04	4080	96	**42.5**	04	6393	160	**40.0**
05	2836	174	**16.3**	05	363	192	**1.9**
06	2819	4	**704.8**	06	253	118	**2.1**
07	1333	44	**30.3**	07	1198	108	**11.1**
08	1522	36	**42.3**	08	589	90	**6.5**
09	1552	688	**2.3**	09	537	592	0.9
10	2802	1520	**1.8**	10	338	252	**1.3**
11	3573	250	**14.3**	11	740	1724	0.4
12	1539	382	**4.0**	12	674	796	0.8
13	2367	940	**2.5**	13	293	144	**2.0**
14	2563	124	**20.7**	14	333	236	**1.4**
15	3996	148	**27.0**	15	844	580	**1.5**
16	3011	114	**26.4**	16	7363	112	**65.7**
17	3195	202	**15.8**	17	508	136	**3.7**
18	1849	192	**9.6**	18	225	178	**1.3**
19	1250	84	**14.9**	19	88	88	1.0
20	2066	56	**36.9**	20	199	298	0.7
Overall Status	2373	280	**8.5**	**Overall Status**	1086	325	**3.3**

5.4. Fungi identification

Table 8 presents all fungi genera/species identified in the study (71 classrooms and 20 outdoors) and mean percentages of each genera/species are also described.

Table 8 - Indoor and outdoor mean percentage of fungi genera/species

Fungi genera/species	**Indoor (%)** (n=71)	**Outdoor (%)** (n=20)
Acremonium sp.	0.1	0.1
Alternaria sp.	0.7	3.0
Aspergillus sp.	2.6	1.4
Aspergillus fumigatus	5.7	5.1
Aspergillus flavus	0.1	0.2
Aspergillus niger	0.5	0.6
Aureobasidium pullulans	0.9	0.6
Beauveria sp.	0.1	0.0
Botrytis sp.	<0.1	0.0
Chaetomium sp.	0.2	0.3
Chrysonilia sp.	0.0	0.2
Cladosporium sp.	28.2	42.5
Fusarium sp.	0.5	2.6
Geotrichum sp.	0.8	2.5
Mucor sp.	<0.1	0.0
Paecilomyces sp.	0.7	0.9
Penicillium sp.	43.1	30.0
Phoma sp.	0.2	1.0
Rhizomucor sp.	0.0	0.2
Rhizopus sp.	0.7	0.1
Rhodotorula sp.	9.1	2.0
Scedosporium sp.	0.1	0.3
Sterile mycelium	0.3	2.4
Trichoderma viridae	<0.1	0.5
Verticillium sp.	0.1	0.8
Yeast	5.5	3.2

Concerning outdoor air, *Cladosporium sp.* (42%) was the most prevalent fungi species for 11 of the 20 schools, and *Penicillium sp.* (30%) the other main species in 7 schools. *Aspergillus fumigatus* was identified as the outdoor most prevalent fungi in 2 schools at a mean percentage of 5%.

Figure 13 illustrates the distribution of fungi genera/species identified indoors by mean percentage of all 71 classrooms. Through the analysis of Table 8 and Figure 13, it is clear that the most predominant fungi species found indoors were *Penicillium sp.* (43%) at 38 classrooms and *Cladosporium sp.* (28%) at 25 classrooms. As it is shown in Figure 14, these two predominant fungi genera were identified in all schools, and between these two predominant fungi genera, it is also possible to identify in each school which one was predominant. Important to notice that in Schools 04 and 16 *Penicillium sp.* was the only fungi genera found in all samples of all classrooms with a percentage of 100%. Figures 15 and 16 show microscopic and culture plate views of *Penicillium sp.* and *Cladosporium sp.*, respectively. *Rhodotorula sp.* (9%), *Aspergillus fumigatus* (6%) and Yeast (5%) were also identified in most indoor environments but

with an inferior percentage. A microscopic view of *Aspergillus fumigatus* is presented at Figure 17, as well as its phenotypical characteristics after a 3 week incubation period.

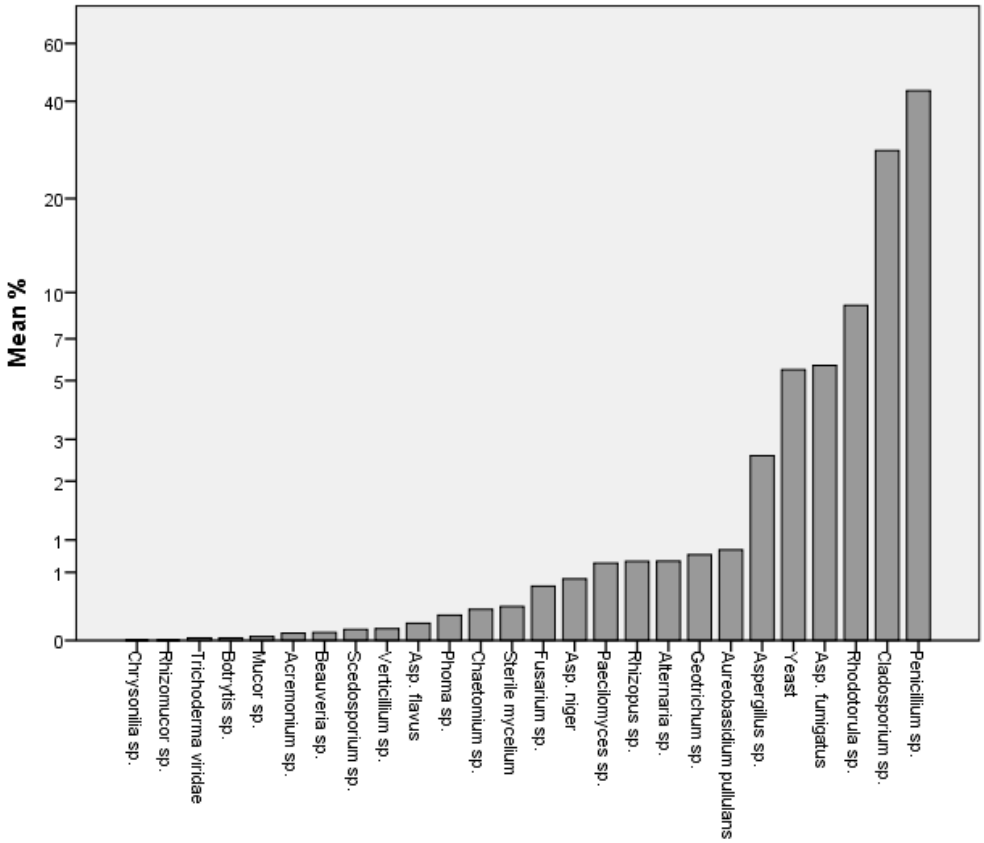

Figure 13 - Mean percentage of fungi genera/species identified indoors

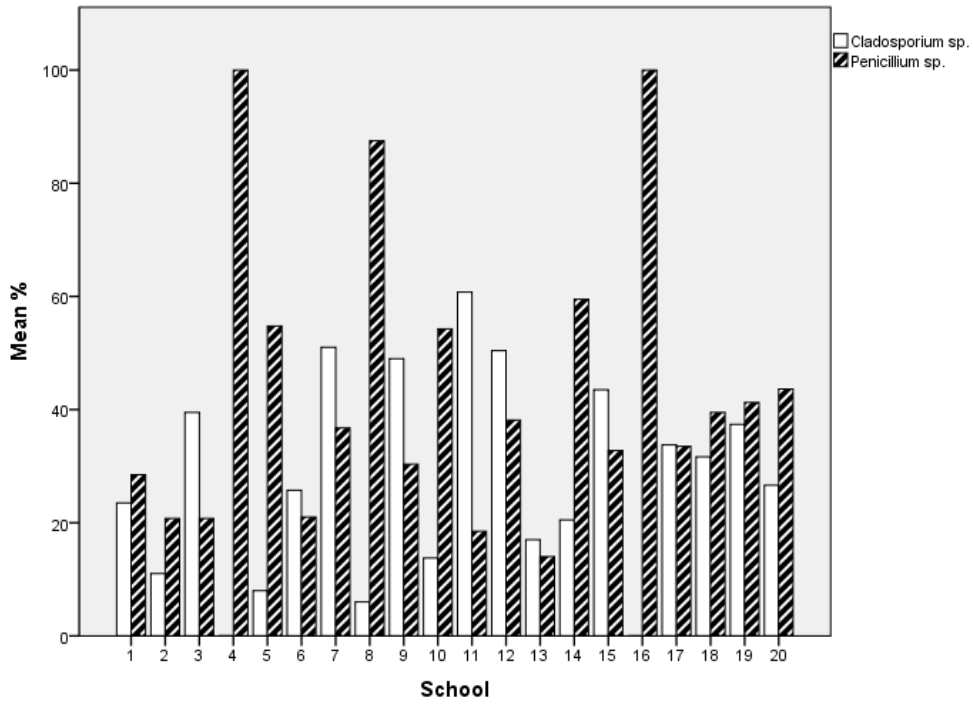

Figure 14 - *Penicillium sp. vs. Cladosporium sp.* indoors: main species by primary school

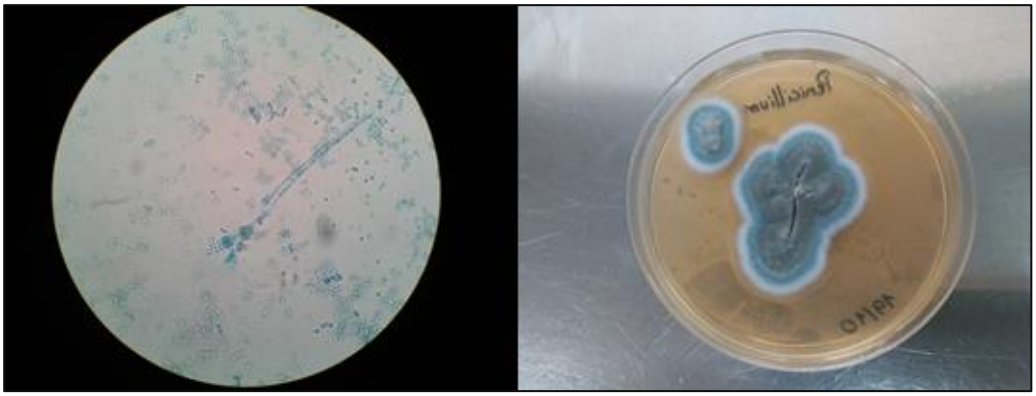

Figure 15 - Microscopic (400 x magnification) and macroscopic (plate of MEA, 25±3°C, 10 days) observation of *Penicillium sp.*

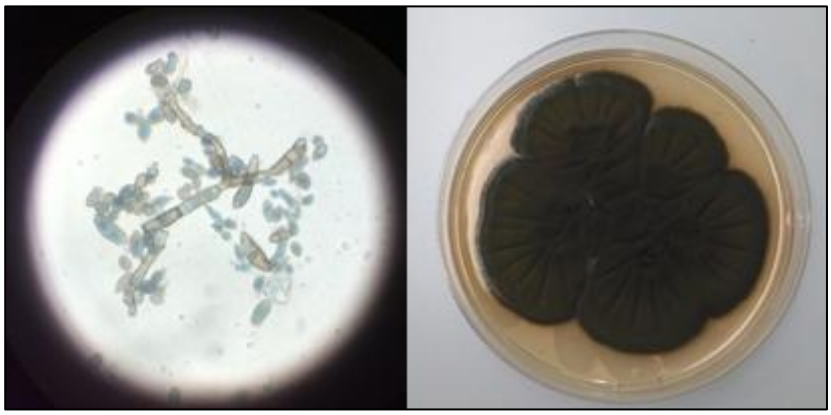

Figure 16 - Microscopic (400 x magnification) and macroscopic (plate of MEA, 25±3°C, 10 days) observation of *Cladosporium sp.*

Figure 17 - Microscopic (400 x magnification) and macroscopic (plate of MEA, 25±3°C, 10 days) observation of *Aspergillus fumigatus*

Both *Penicillium sp.* and *Cladosporium sp.* are considered to be common in indoor environments, according with the guidelines presented at Portuguese national legislation, Ordinance no 353-A/2013. Other prevalent species identified in 4 of the 71 evaluated classrooms, *Aspergillus fumigatus*, is also referred in the legislation and considered to be toxigenic in concentrations above 12 CFU/m^3.

Figures 18 to 21 present the main fungi genera/species identified in each classroom in comparison with the main fungi genera/species found in each school's outdoor. In six out of twenty schools (30%), the main fungi genus identified indoors in all classrooms was the same main fungi genus identified outdoors: *Cladosporium sp.* in Schools 09 and 11, and *Penicillium sp.* in Schools 05, 08, 10 and 20. School 1 (Figure 18) was the only School with *Penicillium sp.* as main fungi genus indoors (Classroom 1 – 29% and Classroom 2 – 28%) and *Cladosporium sp.* as main fungi genus outdoors (33%). In seven schools (35%), namely Schools 07, 12, 14, 15, 17, 18 and 19, the most frequent fungi genera identified per schools' classroom were both *Penicillium sp.* and *Cladosporium sp.* considered common in indoor air, and also found as the most frequent outdoors.

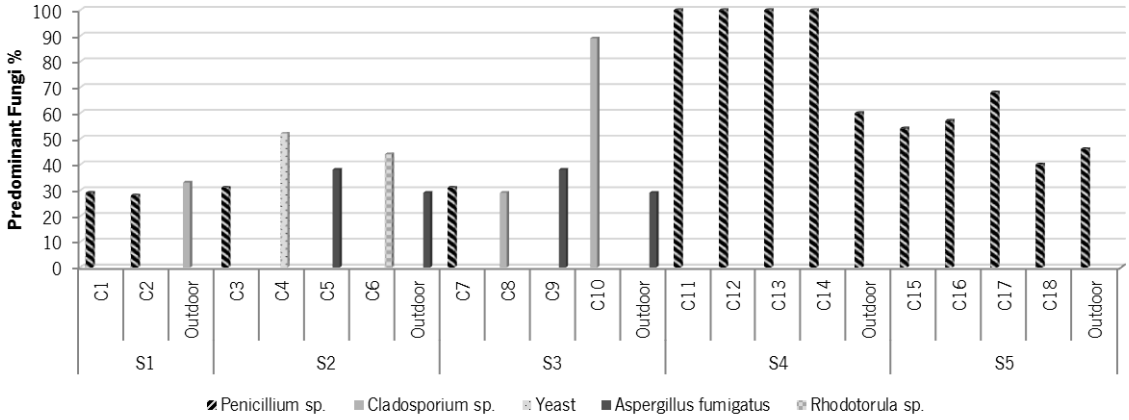

Figure 18 - Predominant fungi genera/species by each school classroom and outdoor - Schools 01 to 05 (S1-S5), Classrooms 01 to 18 (C1-C18)

Schools 02 and 03 (Figure 18) have *Aspergillus fumigatus* as predominant fungi species outdoors with a percentage of 29% in both. However, from the four classrooms evaluated in each school, only in one classroom of each (Classroom 05 from School 02 and Classroom 09 from School 03) *Aspergillus fumigatus* was also the predominant fungi species, both with a percentage of 38%.

In fact, the other three classrooms of School 03 had *Cladosporium sp.* (Classroom 08 – 29% and Classroom 10 – 89%) and *Penicillium sp.* (Classroom 07 – 31%) as main fungi genera. School 02 had different predominant fungi genera/species in all four evaluated classrooms: *Penicillium sp.* at classroom 3 (31%), Yeast at classroom 4 (52%), *Aspergillus fumigatus* at classroom 5 (38%) and *Rhodotorula sp*. at classroom 6 (44%).

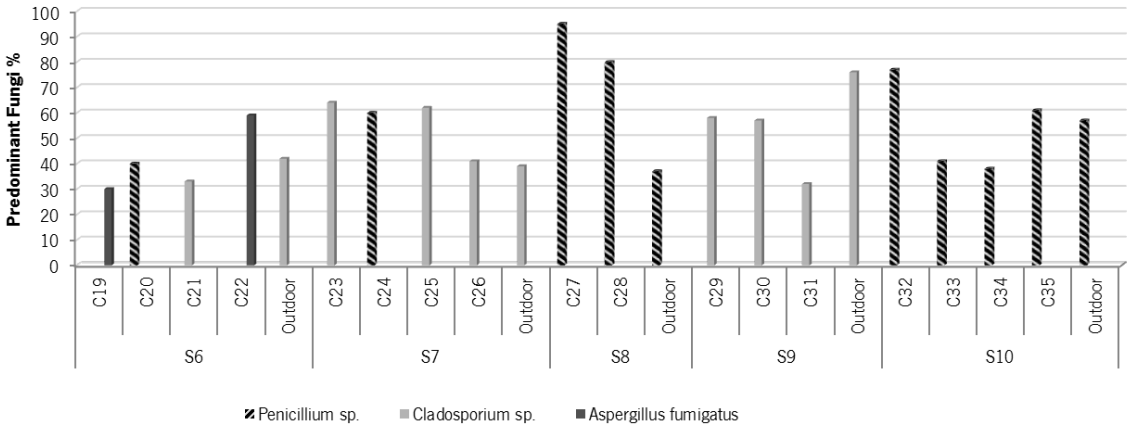

Figure 19 - Predominant fungi genera/species by each school classroom and outdoor - Schools 06 to 10 (S6-S10), Classrooms 19 to 35 (C19-C35)

School 06 (Figure 19) had two classrooms, 19 and 22, where *Aspergillus fumigatus* was the main fungi species identified, with 30% and 59% respectively, and outdoor, *Cladosporium sp.* was the main fungi genus identified (42%). In the other two classrooms of this school, the main fungi genera identified were *Penicillium sp.* (Classroom 20 – 40%) and *Cladosporium sp.* (Classroom 21 – 33%). Another similar case happened at School 13 (Figure 20): two of the three evaluated classrooms had *Rhodotorula sp.* identified as main fungi genus, Classroom 44 with 43% and Classroom 45 with 39%, and *Cladosporium sp.* again as main fungi genus outdoors (32%). The other classroom, Classroom 46, had *Penicillium sp.* (32%) as main fungi genus identified.

Fungal samples from School 04 classrooms (Figure 18) and three classrooms of School 16 (Figure 21) had 100% of *Penicillium sp*. In both schools, the main fungi genera outdoor was also *Penicillium sp.* but at percentages of 60% and 40%, respectively.

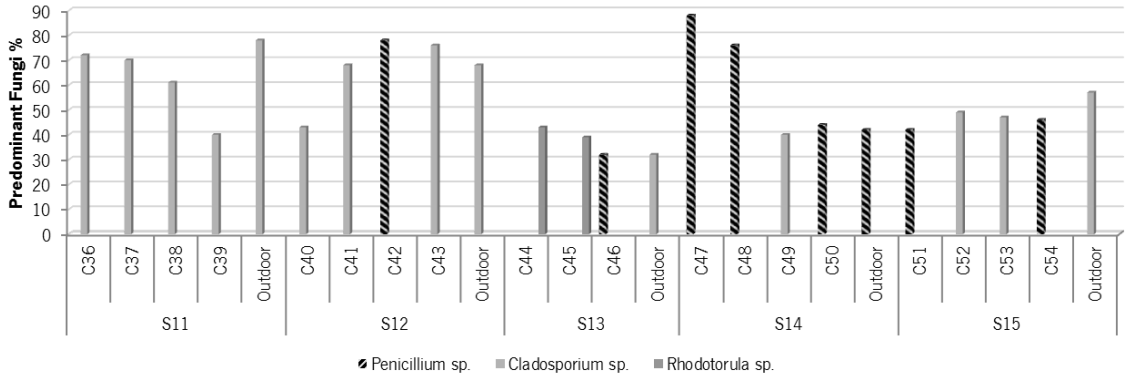

Figure 20 - Predominant fungi genera/species by each school classroom and outdoor - Schools 11 to 15 (S11-S15), Classrooms 36 to 54 (C36-C54)

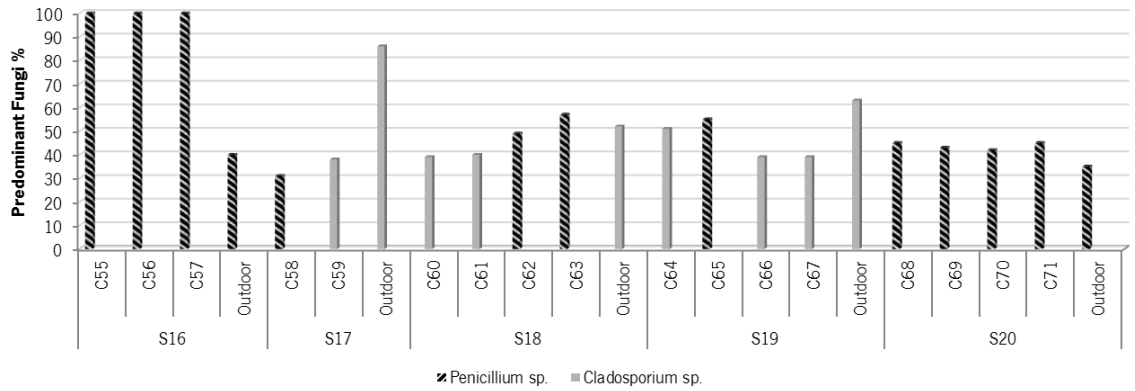

Figure 21 - Predominant fungi genera/species by each school classroom and outdoor - Schools 16 to 20 (S16-S20), Classrooms 55 to 71 (C55-C71)

Figures 22 to 29 present the main identified fungi genera/species (*Cladosporium sp.*, *Penicillium sp., Rhodotorula sp.*, Yeast and *Aspergillus fumigatus*) in order to understand its presence or absence, and levels both indoors and outdoors. It was considered also important to present the differences between indoor/outdoor concentrations of other fungi genera/species considered as potentially toxigenic, such as *Aspergillus flavus* and *Aspergillus niger*. *Fusarium* is a fungi genus that comprises two species considered toxigenic by Portuguese legislation, Ordinance no 353-A/2013, (*Fusarium moniliforme* and *Fusarium culmorum*). By this fact it was also included in this analysis.

The distribution of *Penicillium sp.* indoors and outdoors (Figure 22) indicates that it was present in all classrooms and in all schools outdoors, with a minimum of 3% indoors, at School 13, and 8% outdoors at School 17, and maximum values of 100% indoors (Schools 04 and 16) and 60% outdoors (School 04). *Cladosporium sp.* (Figure 23) was also present in almost all classrooms, with exception for School 04 where it was not found. Outdoor results showed a mean percentage of 57±19% and a common presence at all schools. *Aspergillus fumigatus* (Figure 24) was identified in 50% of the schools indoor environments, at a range of 2–59%, with a maximum value in Classroom 22 of School 06. In seven schools this species was recognized outdoors; nevertheless in four out of ten schools where *Aspergillus fumigatus* was identified indoors, it was not present outdoors. Figure 25 shows *Rhodotorula sp.* in almost every indoor environment: in eighteen out of the twenty schools, with maximum values in Schools 02 (44% at Classroom 06) and 13 (43% at Classroom 44). Also, in these both schools, *Rhodotorula sp.* was identified in all classrooms. Moreover, comparing to outdoor levels, *Rhodotorula sp.* was only identified in eight outdoor evaluations and at levels much lower than indoors: from 2% to 11%. Yeasts (Figure 26) were found in 80% of schools indoor air, corresponding to sixteen schools, while its presence

outdoors was only registered in half (eight schools). In these eight schools where Yeasts were detected outdoors, it was also identified indoors.

Aspergillus flavus (Figure 27) was only identified in indoor air out of a total of four classrooms from three schools (02, 03 and 13) and at low levels: about 2%. 2% was also the percentage attributed to *Aspergillus flavus* outdoors, identified at Schools 02 and 03. *Aspergillus niger* (Figure 28) was identified in eleven schools' indoors and five schools' outdoors. However, at low levels: maximum of 5% indoors and 4% outdoors. *Fusarium sp.* (Figure 29) was more common outdoors than indoors. This species was identified at nine schools' indoors, with maximum values of 9% in one classroom of School 09 where it was not identified outdoors. Other scenario is where *Fusarium sp.* was not detected indoors in any classroom but show positive results in twelve schools' outdoors, with a maximum value of 20% at School 16.

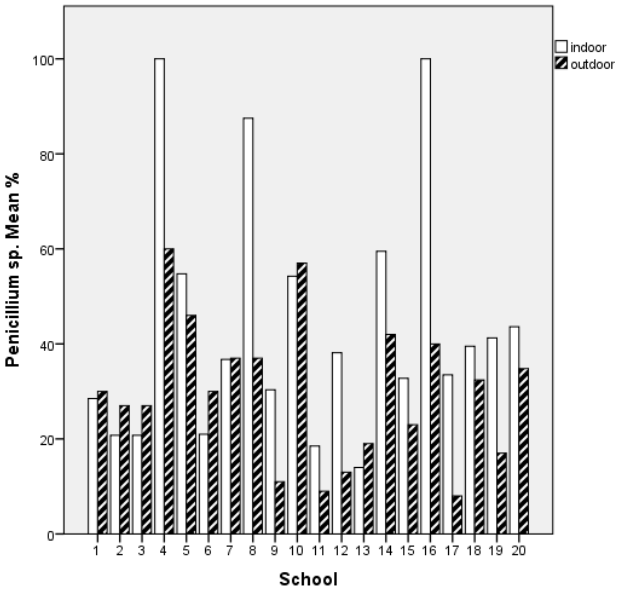

Figure 22 - Indoor *vs.* Outdoor comparison: *Penicillium sp.*

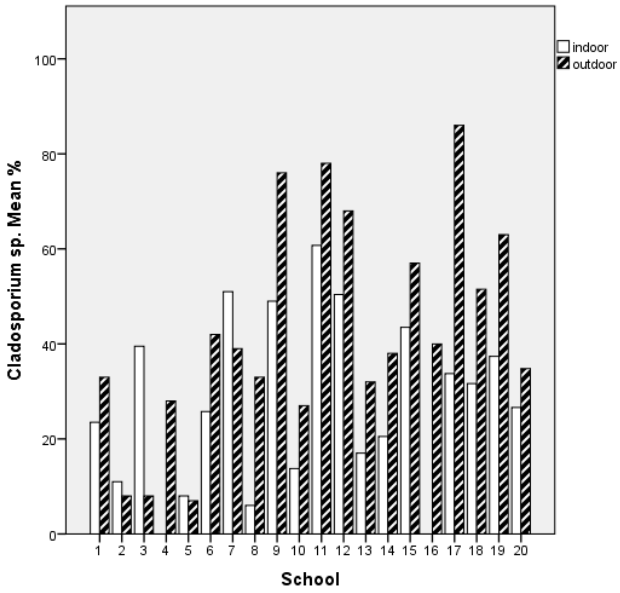

Figure 23 - Indoor *vs.* Outdoor comparison: *Cladosporium sp.*

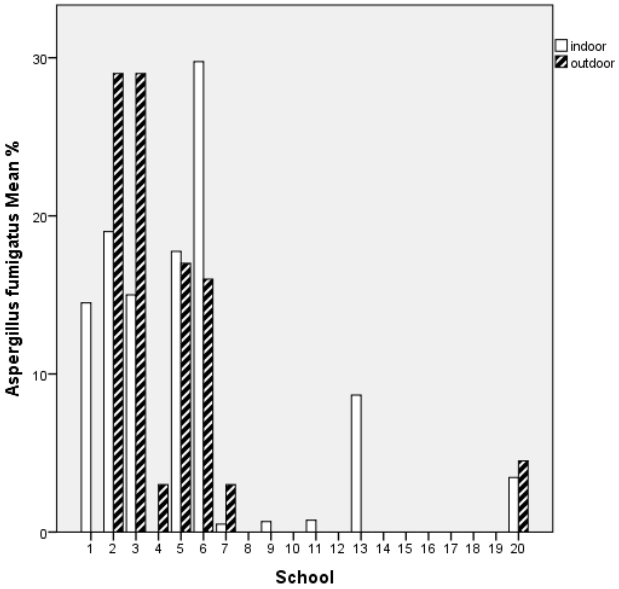

Figure 24 - Indoor *vs*. Outdoor comparison: *Aspergillus fumigatus*

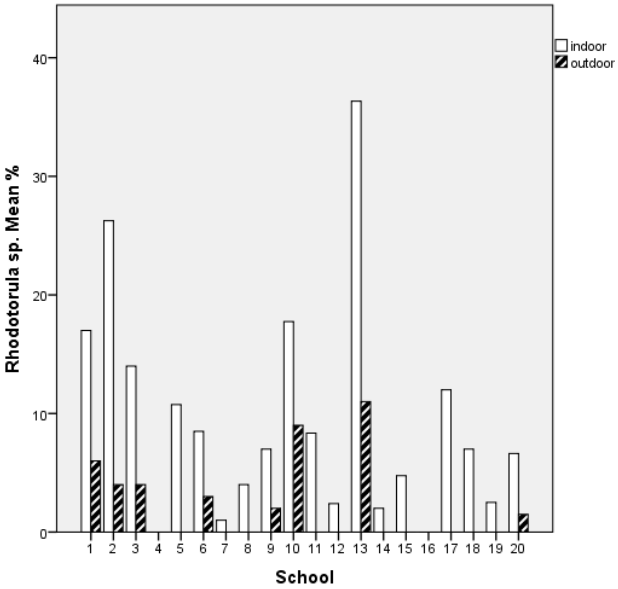

Figure 25 - Indoor *vs*. Outdoor comparison: *Rhodotorula sp*.

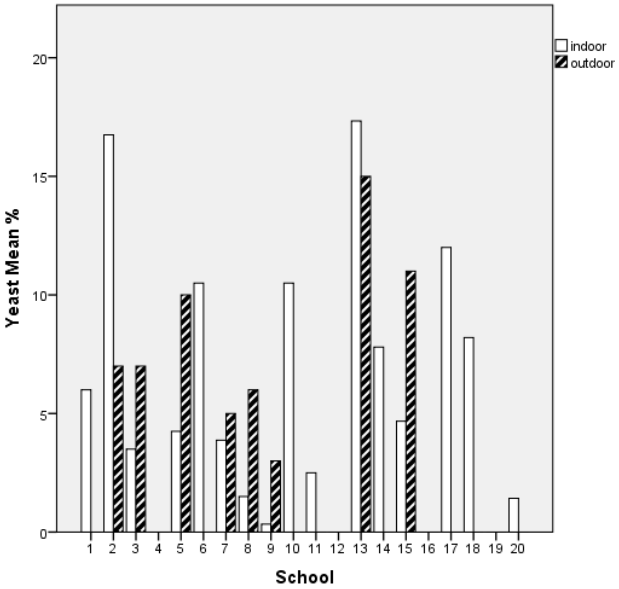

Figure 26 - Indoor *vs*. Outdoor comparison: Yeast

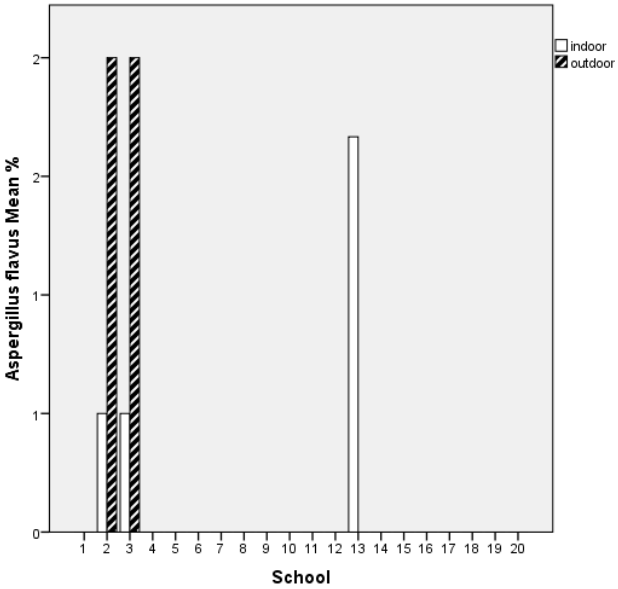

Figure 27 - Indoor *vs*. Outdoor comparison: *Aspergillus flavus*

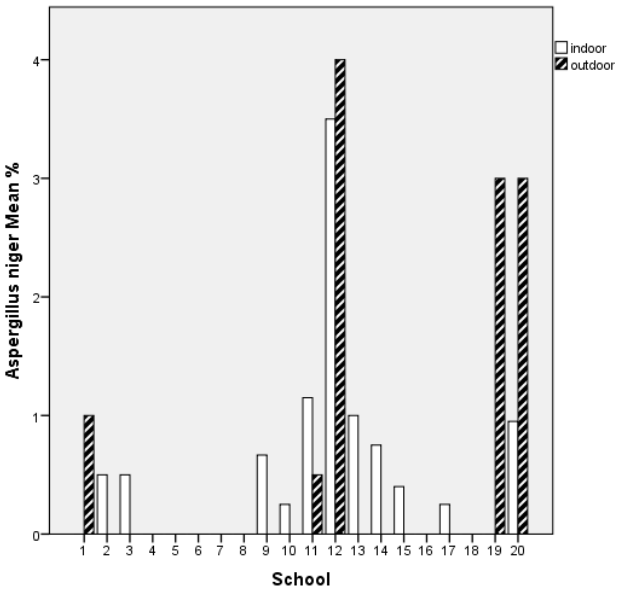

Figure 28 - Indoor *vs.* Outdoor comparison: *Aspergillus niger*

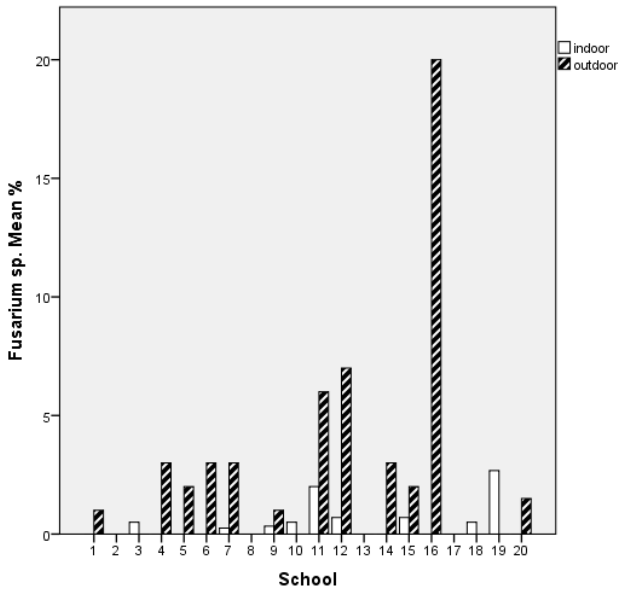

Figure 29 - Indoor *vs.* Outdoor comparison: *Fusarium sp.*

Wilcoxon signed-rank test was applied in order to investigate statistical differences between indoor and outdoor levels for each of the predominant fungi species of the present study, per school. Only for *Aspergillus fumigatus* were not found significant statistical differences between indoor and outdoor levels (Z=0.846; p>0.05; r=0.134). For *Cladosporium sp.*, outdoor levels were statistically different from indoor percentages (Z=2.950; p<0.01; r=0.466), with outdoor values higher than indoors. For Yeast (Z=2.189; p<0.05; r=0.346), *Penicillium sp.* (Z=2.215; p<0.05; r=0.350) and *Rhodotorula sp.* (Z=3.727; p<0.001; r=0.589), significant statistical differences were also found between indoor and outdoor values, and Wilcoxon test results showed that all had indoor percentages above outdoor levels.

5.5. Correlation between chemical, physics and biological parameters

Table 9 presents the descriptive statistics results of T, RH and CO_2, by indoor and outdoor values, and also the respective reference values established by the Portuguese legislation: Decree-Law no. 243/86 for T and RH and Ordinance no. 353-A/2013 for CO_2. According to these guidelines, mean and median values of T and RH were within the recommended range; CO_2 mean and median levels exceeded the limit value and recorded maximum peak values almost 3 times higher than reference value.

Table 9 - Descriptive statistics of Temperature, Relative Humidity and Carbon Dioxide in Schools

Parameters	Temperature (T)		Relative Humidity (RH)		Carbon Dioxide (CO_2)	
	Indoor [a]	**Outdoor** [b]	**Indoor** [a]	**Outdoor** [b]	**Indoor** [a]	**Outdoor** [b]
Units	°C		%		mg/m³	
Mean±SD	20.4±2.9	16.0±5.7	57.4±8.2	62.9±14.2	2553±1129	609±115
Median	19.4	14.4	58.7	67.0	2541	585
[P25-P75]	[18.7–21.6]	[12.6–16.7]	[51.9–63.6]	[55.7–72.3]	[1564–3378]	[541–717]
Range	14.7–29.5	9.9–30.2	35.0–72.4	20.2–78.6	1056–5981	396–781
Amplitude	14.8	20.3	37.4	58.4	4925	385
Reference Values	**[18–22]** [c]	**-**	**[50–70]** [c]	**-**	**2250** [d]	**-**

[a] n=71. [b] n=20. [c] Decree-Law no. 243/86 of 20th August. [d] Ordinance no. 353-A/2013 of December 4th. SD: Standard Deviation. P25: 25th Percentile. P75: 75th Percentile.

Table 10 shows Spearman's rank correlation coefficients results and levels of statistical significance between chemical, physical and biological parameters. Bacteria concentrations were moderate positively correlated with RH (r_S=0.519) and CO_2 (r_S=0.490) levels, with high statistical significance (p<0.001). Figure 30 expresses the positive correlation found between mean indoor CO_2 levels and mean indoor bacteria concentrations along all 20 studied schools.

Table 10 - Spearman's rank correlation coefficients between chemical, physical and biological parameters

	Bacteria	Fungi	Endotoxins
Temperature (T)	-0.029	0.215	0.148
Relative Humidity (RH)	**0.519** [a]	0.074	**0.301** [c]
Carbon Dioxide (CO_2)	**0.490** [a]	0.089	**0.395** [b]
Bacteria	–	**0.331** [b]	0.155
Fungi	–	–	-0.003

Note: Significant correlations are marked in **bold** for [a] p<0.001; [b] p<0.01; [c] p<0.05.

Additionally, correlations found between fungi concentrations and other chemical or physical parameters in study were very weak (r_S=0.215 for T; r_S=0.074 for RH; r_S=0.089 for CO_2), and with low statistical significance (p>0.05).

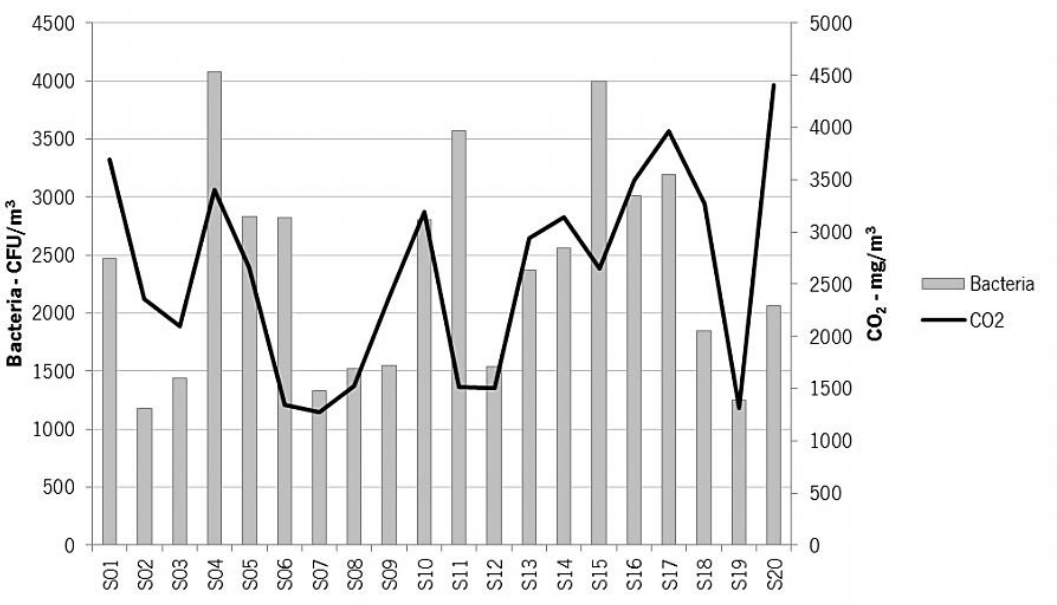

Figure 30 - Indoor concentrations of Bacteria and CO_2, by School (S01-S20)

Figure 31 presents the distribution of fungi concentration and RH levels showing the non-correlation between these two variables.

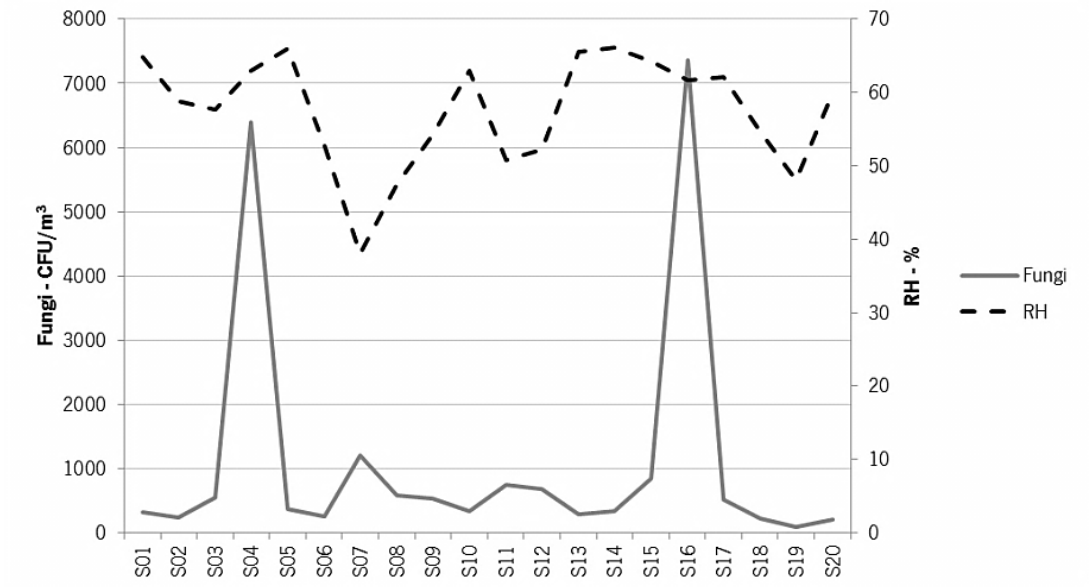

Figure 31 - Indoor concentrations of Fungi and RH levels, by School (S01-S20)

Similarly to bacteria, endotoxin concentrations have moderate positive correlation with CO_2 level (r_S=0.395) and a weak positive correlation between endotoxins and RH levels (r_S=0.301) was also found. Both correlations have statistical significance (p<0.01 and p<0.05, respectively).

Spearman's rank correlation coefficient was also applied to investigate the relationship between the three biological variables in study (Table 10). The only correlation found with statistical significance was between bacteria and fungi concentrations; however it was a weak positive

correlation (r_S=0.331; p<0.01). The correlation found between the two biological parameters is graphically expressed in Figure 32.

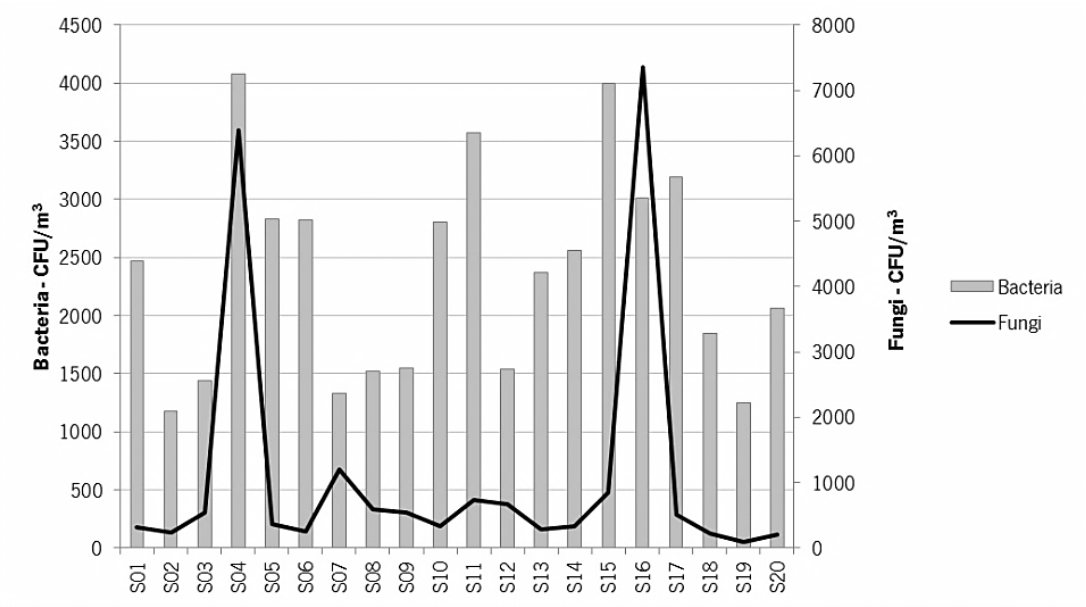

Figure 32 - Indoor concentrations of Bacteria and Fungi, by School (S01-S20)

Regarding endotoxins concentrations, there was a very weak positive correlation with bacteria levels (r_S=0.155) and also a very weak but negative correlation with fungi concentrations (r_S=-0.003); both correlations had low statistical significance (p>0.05). Figures 33 and 34 present the concentrations of bacteria & endotoxins and fungi & endotoxins, respectively, in all 20 schools. For bacteria & endotoxins (Figure 33), although these variable concentrations fluctuate very similarly when compared to CO_2 and RH levels, the variation between them is not related. Regarding fungi and endotoxin levels, Figure 34 shows the poor relation between these two variables; in schools with similar low levels of endotoxins, fungi concentrations vary independently.

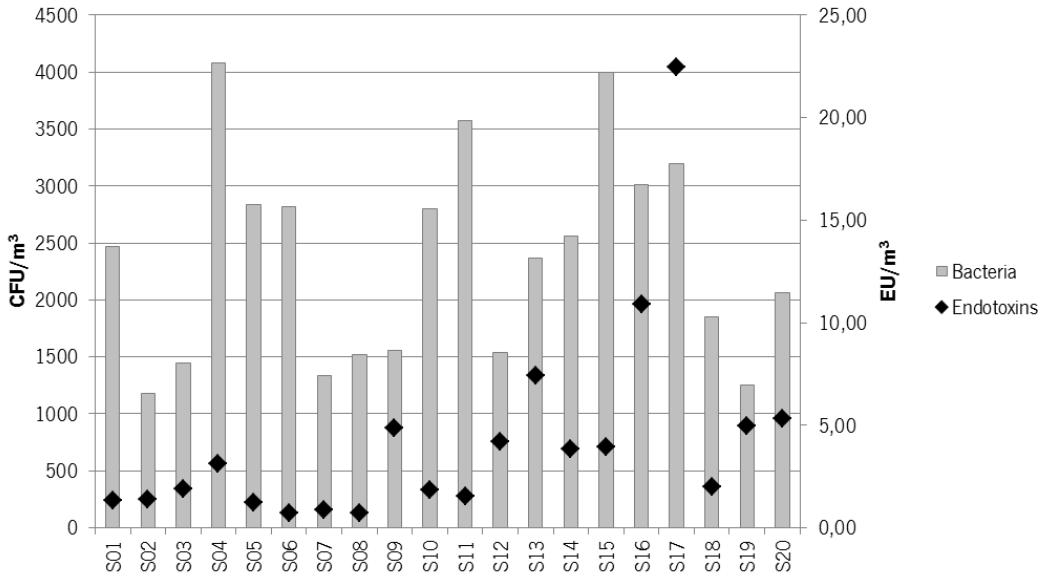

Figure 33 - Indoor concentrations of Bacteria and Endotoxins, by School (S01-S20)

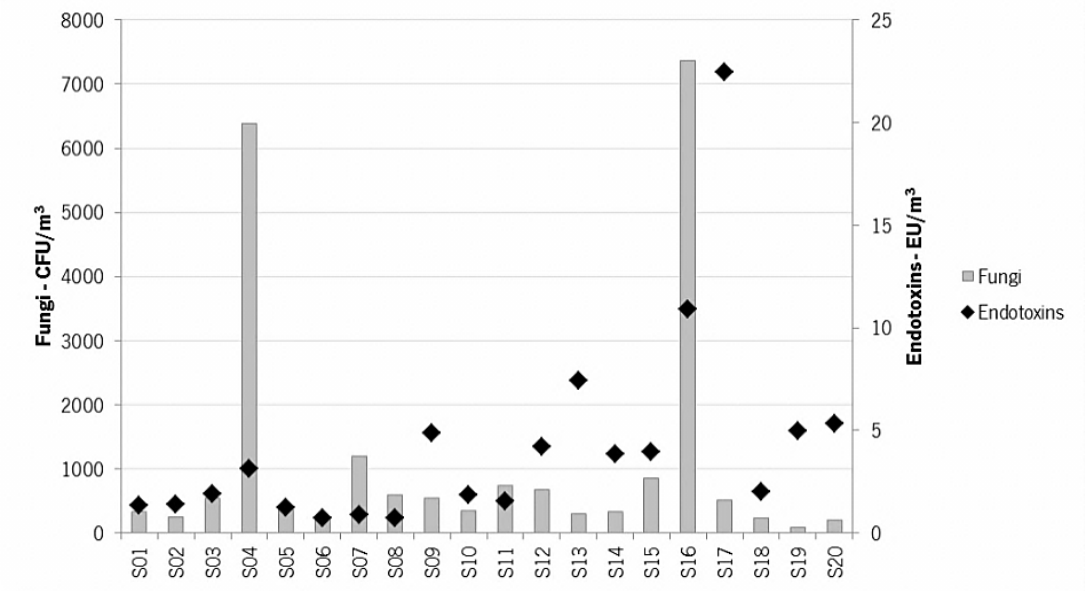

Figure 34 - Indoor concentrations of Fungi and Endotoxins, by School (S01-S20)

Table 11 presents Spearman's rank correlation coefficients found between the predominant fungi species mentioned in the subsection 5.4 and physical (T and RH) and chemical (CO_2) parameters.

Table 11 - Spearman's rank correlation coefficients between predominant fungi species, chemical and physical parameters

Fungi Species	Temperature (T)	Relative Humidity (RH)	Carbon Dioxide (CO_2)
Aspergillus fumigatus	**-0.341** [b]	0.109	-0.102
Cladosporium sp.	**0.391** [b]	**-0.437** [a]	**-0.305** [c]
Penicillium sp.	-0.093	0.219	**0.265** [c]
Rhodotorula sp.	**-0.259** [c]	0.210	0.090
Yeast	-0.169	0.216	0.182

Note: Significant correlations are marked in **bold** for [a] $p<0.001$; [b] $p<0.01$; [c] $p<0.05$.

Cladosporium sp. had a weak negative correlation with CO_2 (r_S=-0.305; $p<0.05$), whereas with T and RH were found positive (r_S=0.391; $p<0.01$) and negative (r_S=-0.437; $p<0.001$) moderate correlations, respectively, with all three correlations having statistical significance. On the other hand, Yeast had only weak correlations with the chemical (r_S=0.182) and physical parameters (T: r_S=-0.169; RH: r_S=0.216) in analysis and none with statistical significance ($p>0.05$). Regarding the other three predominant fungi species, to notice the weak negative correlations found between T and *Rhodotorula sp.* (r_S=-0.259; $p<0.05$) and *Aspergillus fumigatus* (r_S=-0.341; $p<0.01$), both with statistical significance, and *Penicillium sp.* was found to be positively correlated with CO_2 (r_S=0.265; $p<0.05$). Nevertheless, although statistical significant, this last correlation was considered weak.

5.6. Building and classroom characteristics *vs.* Biological assessment

This section presents a summary view of the results for associations between school and classrooms' characteristics, and the biological levels found (Tables 12 and 13).

Table 12 - Influence of school buildings characteristics on indoor air biological concentrations

School Building variable	Biological Parameter			Statistical Test
	Bacteria	Fungi	Endotoxins	
Year of Construction	0.179	0.405	-0.081	Spearman's rank correlation coefficient (r_S)
Year of Building Refurbishment	0.208	-0.208	**0.463** [a]	
Wall Type	33.000	42.000	**21.000** [a]	Mann-Whitney (U)
Roof leaking	36.000	39.000	**19.000** [a]	
Windows leaking	18.000	20.000	11.000	
Visible air leaks	34.000	34.000	47.000	

Note: Significant correlations are marked in **bold** for [a] $p<0.05$.

Analyzing Spearman's rank correlation coefficient (r_S) and Mann-Whitney (U) tests results, expressed in Table 11, only endotoxin concentrations were influenced by the following two schools buildings characteristics: wall type (U=21.000; p<0.05) and the presence of roof leaking in the last 12 months (U=19.000; p<0.05). Endotoxin concentrations were higher when buildings had single walls and were also higher in buildings without roof leaking. Nevertheless, a positive moderate correlation was found between endotoxin concentrations and the year of building refurbishment (r_S=0.463; p<0.05), meaning that, the more recently the intervention was made, the higher were concentrations of endotoxins in indoor air.

Table 13 - Influence of classrooms characteristics on indoor air biological concentrations

Classroom characteristic	Biological Parameter			Statistical Test
	Bacteria	Fungi	Endotoxins	
Number of occupants	0.080	-0.002	**0.330** [b]	Spearman's rank correlation coefficient (r_s)
Density of Occupation (m²/occupant)	0.068	0.191	**-0.300** [c]	
Number of windows opened in heating season	0.111	-0.011	0.008	
Number of space heaters	**0.268** [c]	0.198	**0.452** [a]	
Ventilation system	**55.000** [c]	**3.000** [a]	75.000	Mann-Whitney (U)
Windows frame	39.000	57.000	37.500	
Type of glazing	430.000	**347.000** [c]	418.500	
Ceiling surface	140.000	140.000	**87.500** [c]	
Floor covering	454.000	411.000	**285.000** [b]	
Visible mold growth	270.500	284.000	212.000	
Noticeable mold odor	**73.000** [c]	120.000	80.000	
Visible damp spots	258.500	239.000	212.000	
Condensation on windows	411.000	411.000	**249.500** [c]	
Heating system	**55.000** [c]	**3.000** [c]	75.000	
Windows open:				
Before school time	**31.000** [b]	107.000	129.000	
During breaks	509.500	602.500	562.500	
During teaching hours	501.500	500.500	548.500	
After school time	110.000	106.000	113.000	
During night	110.000	106.000	113.000	
During cleaning time	**199.000** [b]	280.000	**216.000** [c]	

Note: Significant correlations for r_s and differences for U are marked in **bold** for [a] p<0.001; [b] p<0.01; [c] p<0.05.

Correlations with statistical significance were found between endotoxins levels and the classrooms variables: number of occupants (r_S=0.330; p<0.01) and density of occupation (r_S=-0.300; p<0.05) (Table 13). For the first, a positive correlation was obtained (higher

concentrations of endotoxins when classrooms had more students) and for the second, a negative correlation (endotoxin levels decreases as density of occupation rises). However, both correlations were considered weak. There seems also to be no influence from windows opening during heating season on indoor air biological concentrations (bacteria: r_S=0.111, p>0.05; fungi: r_S=-0.011, p>0.05; endotoxins: r_S=0.008, p>0.05). Number of space heaters in classrooms was another variable where positive correlations with statistical significance were found regarding bacteria and endotoxins concentrations. The correlation between number of space heaters and bacteria concentrations was weak (r_S=0.268; p<0.05), but for endotoxin concentrations the correlation was considered moderate (r_S=0.452, p<0.001). This finding indicates that endotoxin levels in classrooms were higher when they had more space heaters.

From Mann-Whitney's (U) test application, statistical differences were found between indoor air biological concentrations and the following classroom characteristics: ventilation system, type of glazing, ceiling surface, floor covering, noticeable mold odor, condensation on windows, heating system, and opening windows before school time and during cleaning time. Fungi concentrations were higher when classrooms were ventilated by windows opening, without mechanical ventilation system (U=3.000; p<0.001), when windows had single glazing (U=347.000; p<0.05), and when heating system did not include domestic hot water (U=3.000; p<0.001). Ventilation (U=55.000; p<0.05) and heating systems (U=55.000; p<0.05) had the same influence on bacteria concentrations has described for fungi. It was also found that when windows were closed before the beginning of teaching hours (U=31.000; p<0.01) and during cleaning procedures (U=199.000; p<0.01) bacteria concentrations were higher than when windows were opened in both times. Regarding the time of the day when windows were opened, these two were the only presenting influence on bacteria indoor levels. Other classrooms' characteristic that had influence on bacteria concentrations was the "noticeable mold odor" (U=73.000; p<0.05). When this condition was not present bacteria concentrations were higher.

Endotoxins concentrations were also influenced by "window opening during cleaning time" (U=216.000; p<0.05), as bacteria concentrations. Concentrations were lower when this task was done with the windows opened. Floor (U=285.00; p<0.05) and ceiling (U=87.500; p<0.05) coverings were also correlated with endotoxin levels. Higher values were found in classrooms with paint ceiling and synthetic floor than in classrooms with wood ceiling and wood floor. Additionally, higher levels of endotoxins were related with classrooms without windows condensation (U=249.500; p<0.05). No statistical differences were found between the biological

parameters concentrations and the presence of mold growth (bacteria: U=270.500; p>0.05; fungi: U=284.000; p>0.05; endotoxins: U=212.000; p>0.05) and/or damp spots (bacteria: U=258.500; p>0.05; fungi: U=239.000; p>0.05; endotoxins: U=212.000; p>0.05) in classrooms.

Table 14 presents the correlations found between the predominant fungi species in this study and classroom characteristics, through the application of Spearman's rank correlation coefficient.

Table 14 - Influence of classrooms characteristics on predominant fungi species identified [Spearman's rank correlation coefficient]

Classroom characteristic	Predominant Fungi Species				
	Aspergillus fumigatus	***Cladosporium sp.***	***Penicillium sp.***	***Rhodotorula sp.***	**Yeast**
Floor area (m²)	-0.096	0.023	0.183	-0.232	0.162
Number of occupants	-0.220	-0.095	0.179	-0.167	-0.100
Density of Occupation (m²/occupant)	0.224	-0.011	0.007	0.006	0.142
Number of space heaters	**-0.465** [a]	**0.274** [c]	-0.137	-0.060	0.059
Ventilation system	-0.165	0.113	0.094	-0.163	**-0.239** [c]
Type of glazing	0.148	-0.104	0.123	0.106	0.091
Ceiling surface	-0.131	**0.260** [c]	-0.105	-0.019	0.031
Floor covering	0.001	-0.040	-0.098	**0.246** [c]	0.187
Visible mold growth	0.026	0.130	-0.010	-0.160	0.018
Noticeable mold odor	-0.084	0.094	-0.109	0.041	0.091
Visible damp spots	0.055	0.082	0.037	-0.119	0.058
Condensation on windows	**0.379** [b]	**-0.384** [b]	0.189	0.023	0.133
Heating system	-0.165	0.113	0.094	-0.163	**-0.239** [c]
Number of windows opened in heating season	-0.123	-0.155	0.086	-0.006	-0.003

Note: Significant correlations for r_s are marked in **bold** for [a] p<0.001; [b] p<0.01; [c] p<0.05.

Moderate correlations were found between classroom characteristic "tendency for condensation on windows" and *Aspergillus fumigatus* (r_s=0.379; p<0.01) and *Cladosporium sp.* (r_s=-0.384; p<0.01); the first a positive correlation and the second a negative correlation. Also, a negative moderate correlation was found for *Aspergillus fumigatus* and the "number of space heaters" (r_s=-0.465; p<0.001). This classroom characteristic had also a weak, but positive, correlation with *Cladosporium sp.* (r_s=0.274; p<0.05). Another weak positive correlation was found between *Cladosporium sp.* and the "type of ceiling surface" of the evaluated classrooms (r_s=0.260; p<0.05). All these five correlations had statistical significance. From all the above Spearman's rank correlation coefficient tests, there were only more three correlations with statistical significance, all of them considered weak between: (i) Yeast and "heating system" (r_s=-0.239; p<0.05); (ii) Yeast and "ventilation system" (r_s=-0.239; p<0.05); (iii) *Rhodorotula sp.* and "type of floor covering" (r_s=0.246; p<0.05). None of the selected classrooms characteristics showed

influence in the presence or absence of *Penicillium sp*. In fact, the results showed that indoor *Cladosporium sp.* was the only fungi species which was influenced by three of the selected classroom characteristics. None of the classrooms' characteristics that are considered to be related with fungi presence, such as, "visible mold growth", "noticeable mold odor" and "visible damp spots on walls, ceilings or floors", were correlated with any of the fungi species considered predominant in this study.

6. Discussion

This section presents the result analysis and discussion for the biological assessment, indoor *vs.* outdoor air, fungi identification, correlations between chemical, physical and biological parameters and building and classroom characteristics. In this section are also suggested some strategies thought to improve IAQ of primary schools. And finally, the last sub-section holds study strengths and limitations.

6.1. Biological assessment

The biological parameters studied were selected based on the existing knowledge on adverse health effects of compounds and/or their link to indoor conditions of moisture damage and dampness (Csobod *et al.*, 2014), and often linked to health impairment of children attending primary schools. Due to their ubiquitous presence in nature, the presence of biological agents is almost inevitable in most enclosed environments (Madureira *et al.*, 2015a), and also endotoxins are considered as one of the more powerful microbial agents (Jacobs *et al.*, 2013).

Mean bacteria concentrations were higher than the reference value in 66 out of a total of 71 (93%) evaluated classrooms and all schools had mean levels above the national legislation threshold, from 2 to 9 times higher. Consequently, overall concentrations of bacteria (mean value of all twenty evaluated schools) were above the reference value (average of bacteria concentrations found in all twenty schools' outdoors), being 4 times higher and registering a maximum indoor level of 8512 CFU/m^3. It is also important to notice that at Table 4 only in three schools minimum levels were below the reference value. Regarding fungi concentrations, levels were above the reference value in 45 classrooms (63%), and mean concentrations of 15 schools (75%) exceeded the outdoor concentration guideline. Overall levels of fungi were higher than the reference value, with indoor levels 3 times above outdoors and a maximum value of 10512CFU/m^3.

These high concentrations can be caused by the high number of students per classroom (average density of occupation: 2.4 m^2/occupant), lack of ventilation caused by closed doors and windows, a common practice during heating season when weather is mainly rain and cold, taking into consideration that most of the schools are naturally ventilated, and also by active behavioral pattern/activity level of children in relatively small spaces (Madureira *et al.*, 2015d). According to WHO (2009) and Rao *et al.* (1996), the main sources of bacteria in the indoor environment are

people and the types of activities occurring in the areas, indoor bacterial growth, fungal content and outdoor air. Higher indoor bacteria concentrations are probably due to inadequate ventilation rates, movement causing particle suspension, human oral and respiratory fluid emitted via coughing, sneezing, talking, and breathing, or the direct shedding of skin associated microbiota, taking into account that all indoor samplings were performed during occupied periods (Madureira, 2014). In the study conducted by Kembel *et al.* (2012), findings suggested that humans can be important dispersal vectors for microbes that colonize the built environment, as the indoor air communities were dominated by a small number of bacterial taxa from clades that are commonly associated with humans as commensals or pathogens. Macedo *et al*. (2013) and Pegas (2012) also referred that respiratory morbidity among children - mainly in winter time, could also contribute to airborne spread of bioaerosols and higher levels of indoor bacteria. Indoor fungal growth is mainly affected by factors such as T, RH, and the type of building/furnishing materials. These factors may influence the variation reported between different geographic locations, seasons, and building particular characteristics (Madureira *et al*., 2014; Meklin *et al*., 2002b; Pegas, 2012).

This study results are in accordance with previous works developed by other authors who studied IAQ in primary schools under heating season (e.g. Madureira (2014); Madureira *et al*. (2015a); Madureira *et al*. (2015b); Madureira *et al*. (2015c); Madureira *et al*. (2015d)). There are other studies developed in Autumn, Spring, and Winter that documented indoor bacteria and fungi levels above the national legislated limit in the majority of the studied schools' classrooms (e.g. Pegas *et al*. (2010); Pegas *et al*. (2011b)). To notice though that at the time these studies were performed the national legislation preconized 500 CFU/m^3 as the reference value both for indoor bacteria and fungi concentrations. Comparing their results with the actual legislation, the conclusions remain the same: indoor levels were above the recommended values (bacteria: indoors > outdoors + 350 CFU/m^3; fungi: indoors > outdoors). Similarly, Jo and Seo (2005) reported in their study conducted during winter season at 11 elementary schools in Korea, for both bacteria and fungi, higher indoor concentrations compared to the outdoor environment; however with fungi levels ranging from 281 to 501 CFU/m^3, much lower than obtained in this study (48–10512 CFU/m^3). Another study with lower indoor fungi levels when compared with the present study was the one performed by Meklin *et al*. (2003), which conducted a comparison between concrete and wooden schools: viable airborne fungi concentrations varied from below 5 to 507 CFU/m^3 in concrete schools, and from 5 to 948 CFU/m^3 in wooden schools. Mandal and

Brandl (2011), in a literature review, found indoor bacteria counts high as 1696 CFU/m^3 and fungal counts ranged from 70 to 6370 CFU/m^3; their study included sampling performed by air impact technique and cultivation methods, as the present study, and mean values of 782 CFU/m^3 and 1002 CFU/m^3, ranges from 65–425 CFU/m^3 for bacteria concentrations were found, as well as for fungi, mean values of 811 CFU/m^3 and 415 CFU/m^3 were registered. Nevertheless, all these values are considered low when compared to the results obtained in the present study. In Madureira *et al*. (2015a) a comparison of indoor biological levels between children day care centers, primary schools, elderly care centers and homes was performed and revealed that median indoor concentrations of bacteria at primary schools were approximately 14% higher than elderly care centers; regarding fungi, and comparing the four building types, the highest median fungal concentrations were measured in child daycare centers and the lowest in elderly care centers. In Madureira *et al.* (2014), fungi concentrations registered mean indoor values of 332±274 CFU/m^3, in a range from 16 to 1686 CFU/m^3. In Levetin *et al*. (1995), fungi concentrations were assessed in indoor air of schools from four different cities of United States of America demonstrating different results between them: levels of 136 to 4969 CFU/m^3 in Kansas City, while in Spokane maximum values of 531 CFU/m^3 were registered, in Santa Fe overall indoor concentrations were relatively low, although all indoor sites had concentrations higher than outdoors, and in Orlando indoor concentrations reflected the outdoor levels. Opposite results from the present study was also found: Shelton *et al.* (2002) found indoor fungi levels lower than outdoors, and Robertson (1998) discovered lower indoors fungi concentrations (range=0 to 6077 CFU/m^3) when compared to outdoors (range=0 to 12668 CFU/m^3). In the literature review conducted by Annesi-Maesano *et al*. (2013), schools' bacteria concentrations varied according to different studies: from a minimum mean concentration of 250 CFU/m^3 to maximum of 17000 CFU/m^3. Regarding fungi concentrations, it was also mentioned a considerable variation between the analyzed results, but worthy to mention that mean values of 18000 CFU/m^3 and 14800 CFU/m^3 were reported among the reviewed articles.

Although in the present study bacteria identification was not performed, it is important to mention that studies of indoor air from Europe have demonstrated that Gram-positive cocci (*Micrococcus*, *Staphylococcus* species) are the most commonly found bacteria in indoor air environments, though some Gram-negative bacteria (*Pseudomonadaceae* family, *Aeromonas* species) are also often present (Tang, 2009).

Regarding endotoxins assessment, according to the actual literature, no regulatory value is currently available. A dose-effect relationship between endotoxin exposures and observed symptoms is not yet available. However, several recommendations have been proposed in order to enable endotoxins concentrations results to be interpreted and analyzed. According to available literature and particularly to the review done by Duquenne *et al.* (2012), the lowest considered limit for endotoxins was 50 EU/m^3.

All classrooms had endotoxin levels below 50 EU/m^3 and in 18 out of the 20 schools (90%) concentrations were below 10 EU/m^3. The mean value found among all twenty evaluated schools was 3.74±4.52 EU/m^3, in a range of 0.025–30.62 EU/m^3. One explanation for the low levels of endotoxin in indoor air could be the size of the endotoxin-carrying particles and the fact that they do not remain in the air in the absence of disturbance, explaining the low airborne concentrations (Barnig *et al.*, 2013).

In the actual literature, it was not found any study that has performed endotoxin assessment in schools indoor air with the same methodology as in the present study. The most similar was the investigation conducted by Delfino *et al.* (2011), which characterized personal endotoxin exposure in school children with asthma. The sampling procedure was different from the present study. Nevertheless, an active sampling was also performed and results were expressed in EU/m^3. Their indoor endotoxins concentrations ranged from 0.063 to 7.5 EU/m^3, with mean values of 0.58±0.42 EU/m^3 in one region and 1.49±1.29 EU/m^3 in the other. Their results demonstrate similar low levels as found in the present study, being endotoxins concentrations of the present study slightly higher.

Another study of endotoxins levels in schools was performed by Jacobs, in the scope of HITEA Project, and the results were described in different articles (Borras-Santos *et al.*, 2013; Jacobs, 2013; Jacobs *et al.*, 2013; Jacobs *et al.*, 2014a; Jacobs *et al.*, 2014b). However, the collection was performed by EDC and results were expressed in EU/m^2. Therefore the results of these studies and the results of the present study cannot be simply compared between them. Nevertheless, it is important to mention a conclusion from one of its studies: endotoxin levels differed significantly between countries, with Dutch schools having the highest levels, while Finnish schools the lowest (Jacobs *et al.*, 2014a).

Because of the low endotoxin levels found in indoor air samples, the sampling procedure and extraction method have to be assessed carefully (Barnig *et al.*, 2013). However, for this biological agent, it is difficult to compare different studies because of the different methods used for

sampling, processing and analysis of samples (Annesi-Maesano *et al.*, 2013; Barnig *et al.*, 2013; Duquenne *et al.*, 2012; Paba *et al.*, 2013; Rullo *et al.*, 2002; Salo *et al.*, 2009). Assessment of exposure to airborne endotoxins has been studied for several years, especially in occupational environments. This lack of sampling and analysis standardization makes it very difficult to compare results and set internationally accepted threshold limit values (TLVs) or OELs for endotoxin exposure. The factor that mainly affects endotoxin measurements is the extraction method (Paba *et al.*, 2013).

Although reference methods recommend expressing measurement results in EU/m^3 (Duquenne *et al.*, 2012), in the current available literature on endotoxins studies, endotoxin units are expressed heterogeneously (Barnig *et al.*, 2013; Paba *et al.*, 2013). Some examples of results expression besides EU/m^3 are ng inhalable endotoxins/m^3 (Gioffre *et al.*, 2012; Su *et al.*, 2002), EU/mg (Bouillard *et al.*, 2005; Park *et al.*, 2000; Rullo *et al.*, 2002; Sheehan *et al.*, 2012), EU/g (Oldfield *et al.*, 2007), EU/m^2 (Csobod *et al.*, 2014; Jacobs *et al.*, 2013; Jacobs *et al.*, 2014a; Jacobs *et al.*, 2014b), and EU/mL (Cyprowski *et al.*, 2007). Notwithstanding, the majority of the studies are conducted in industrial and occupational settings, where endotoxins levels are completely different from indoor areas, like schools, offices or residential buildings: airborne endotoxin concentrations in indoor environments are usually very low (Barnig *et al.*, 2013). Taking into consideration only studies where results were expressed in EU/m^3 as in the present study, in homes was found, for airborne endotoxin concentrations, median values of 0.11 EU/m^3 (Singh *et al.*, 2011); mean values of 0.43±2.6 EU/m^3 (0.05–3.99 EU/m^3) (Hyvarinen *et al.*, 2006); 0.77±2.3 EU/m^3 (0.01–30.23 EU/m^3) (Park *et al.*, 2001); 4.2 EU/m^3 (Reponen *et al.*, 2010); and 0.36±2.33 EU/m^3 (0.07–2.00 EU/m^3) in non-farm homes and 1.04±2.84 EU/m^3 (0.15 - 6.14 EU/m^3) in farming homes (Noss *et al.*, 2008); and ranges from 0.9–1.9 EU/m^3 in Barnig *et al.* (2013); in child day care centers the annual geometric mean was 1.78±1.68 EU/m^3 (Roda *et al.*, 2011); and in office buildings and residences, indoor endotoxin levels ranged from 0.16 to 19.97 EU/m^3 (Tsai *et al.*, 2001). Regarding industrial and occupational settings, in the study of Spaan *et al.* (2008) inhalable dust and endotoxin exposure levels were summarized for 46 industries and 4 broadly defined sectors: (i) waste treatment & management; (ii) grains, seeds & vegetables processing; (iii) horticulture; and (iv) animal production. Endotoxins concentrations ranged from 0.6 EU/m^3 (in waste treatment & management sector) until 191400 EU/m^3 (horticulture sector), and geometric mean of all four sectors was 160±8.6 EU/m^3. The higher mean was found in animal production sector with levels of 681±5.2 EU/m^3. In fact, the

study performed by Dungan (2011), in which air sampling procedure comprised the button aerosol sampler (the same as in the present study), the highest endotoxin concentrations, ranging from 2841 to 49066 EU/m^3, were found inside swine barns, with outdoor mean levels of 11.8±1.0 EU/m^3. In greenhouses, stationary inhalable endotoxins samples were collected using also button aerosol samplers and endotoxins results ranged from 100–10000 EU/m^3 (Adhikari *et al.*, 2011). Su *et al.* (2002) performed a study of airborne endotoxins levels in 3 textile factories and values of 1567 EU/m^3, 1111 EU/m^3 and 1536 EU/m^3 were registered.

Studies revealed that endotoxin levels are higher in schools than at homes, which could be explained by overcrowded classrooms (Annesi-Maesano *et al.*, 2013), and it also indicates that exposure at school can contribute considerably to environmental endotoxin exposure of children and teachers (Annesi-Maesano *et al.*, 2013; Jacobs *et al.*, 2013; Sheehan *et al.*, 2012). In the study performed by Rullo *et al.* (2002) mean levels of endotoxin in children schools were 3 times higher than those previously detected in homes; in Roda *et al.* (2011) airborne endotoxin levels in child day care centers were also higher than those found in homes. Shedding from human skin or dirt brought in by shoes are probably important sources of endotoxin in classrooms (Jacobs *et al.*, 2013).

Among several types of endotoxins assessment, the most common in the actual literature consists in collecting endotoxins through active sampling, in airborne or floor dust samples, or passive sampling, by using an EDC. Several studies reported weak or even none correlations between airborne and dust levels of endotoxins (Barnig *et al.*, 2013; Hyvarinen *et al.*, 2006; Noss *et al.*, 2008; Park *et al.*, 2000; Park *et al.*, 2001; Singh *et al.*, 2011), indicating different types or durations of potential microbial exposures from dust *vs.* air (Reponen *et al.*, 2010). In the study conducted by Noss *et al.* (2008), EDC endotoxin correlated from moderately to strongly with endotoxin measured by active airborne sampling. Airborne endotoxin measurements are expected to reveal stronger associations between inhalable endotoxin exposures and respiratory outcomes, such as asthma and allergies, than settled dust measurements of endotoxin in "reservoir" dust from floors or mattresses (Annesi-Maesano *et al.*, 2013; Delfino *et al.*, 2011; Noss *et al.*, 2008), due to the fact that they may be more representative of true exposure as endotoxins could penetrate into the body through the lungs (Barnig *et al.*, 2013).

In the same way, for bacteria and fungi assessment no standard sampling or analytical procedures have been established (Annesi-Maesano *et al.*, 2013; Rao *et al.*, 1996). Results may vary depending on the sampling procedure and methodology analysis (equipment, number of

samples, volume of air, culture medium, incubation periods, counting and identification procedures), season of year and region of the assessment and climate (Madureira *et al.*, 2014; Rao *et al.*, 1996). The existence and use of a standard protocol would allow the accumulation of broadly relevant baseline data by investigators across the world (Rao *et al.*, 1996).

Seasonal variations must be taken into consideration when interpreting indoor/outdoor fungi concentration ratios, since outdoor fungal levels are strongly influenced by climate and weather (Rao *et al.*, 1996). As the measurements were taken in heating season, the weather conditions such as low temperatures and precipitation levels could explain lower outdoor fungi concentrations. Thus, it is an ideal period for indoor air sampling due to the absence of contribution of outdoor air fungi and, as during heating season occupants spend more time in indoor environments, to more often closed windows, heating systems turned on, possibly leading to insufficient ventilation. Therefore, suitable conditions are gathered for indoor factors that enhance the growth of biological agents, such as indoor T and RH (Madureira, 2014; Meklin *et al.*, 2003). Notwithstanding, according with the results obtained and/or the statements by Barnig *et al.* (2013), Duquenne *et al.* (2012), Park *et al.* (2000), Park *et al.* (2001), and Roda *et al.* (2011), there is no consensus regarding seasonal variations in airborne indoor endotoxin concentrations, with the assumption made by Barnig *et al.* (2013) that this biological agent appears to be little influenced by seasonal variations. The lack of a seasonal pattern indoors in the presence of significant seasonal variation in outdoor airborne endotoxin level, may suggest that indoor sources are important and that indoor airborne endotoxin level is driven by time invariant indoor factors (Park *et al.*, 2001).

Existing quantitative standards/guidelines for biological agents in indoor air issued by governmental agencies are based primarily on baseline data (rather than health effects data), and are either absolute (numerical) or relative (indoor/outdoor comparisons) or a combination of the two (Rao *et al.*, 1996). In national legislation a relative guideline was expressed for bacteria and fungi at Ordinance no 353-A/2013: indoor bacteria concentrations should be below the outdoor concentrations + 350 CFU/m^3, accounting for the natural contribution of occupants, and fungi levels should be inferior of what is found outdoors. The principle is that lower indoor than outdoor levels indicate an acceptable indoor environment and its major advantage is that, within limits, different sampling and analysis methods can be used by different investigators as long as methods used indoors and outdoors are identical (Rao *et al.*, 1996). Nevertheless, WHO (2009), states that "as the relations between dampness, microbial exposure and health effects cannot be

quantified precisely, no quantitative health-based guideline values or thresholds can be recommended for acceptable levels of contamination with microorganisms. Instead, it is recommended that dampness and mold-related problems be prevented. When they occur, they should be remediated because they increase the risk of hazardous exposure to microbes and chemicals". Health-based guidelines for microorganisms are unlikely to be established due to the huge diversity and variation of microbial spores, cells, fragments and metabolites and to differences in responses among individuals (Csobod *et al.*, 2014).

Results showed significant statistical differences between schools for bacteria, fungi and endotoxins concentrations. These results were in accordance with other studies that assessed biological agents levels in schools: Jacobs *et al.* (2014a) found differences in endotoxin levels between schools of each country where the study took place (Spain, Finland and The Netherlands); and Levetin *et al.* (1995) also stated that indoor fungi concentrations varied considerably between American schools within the same city.

6.2. Indoor *vs.* Outdoor air

Indoor biological concentrations should be evaluated in relation to the outdoor levels (Levetin *et al.*, 1995). I/O ratios are used to assess the probability of indoor sources (Madureira *et al.*, 2015a; Rao *et al.*, 1996): if the I/O ratio is greater than 1, the source is more likely in the indoor environment (Madureira *et al.*, 2015a; Spengler *et al.*, 2000). In healthy buildings, the ratio of I/O fungi concentrations is lower than or slightly higher than 1, as the majority of fungi present indoors originate from outdoor sources. Conversely, troubled buildings display ratios higher than 1 (Cabral, 2010). Therefore, the specific comparison of individual indoor and outdoor biological agents concentrations may be of some benefit in evaluating the potential risk for exposure (Robertson, 1998).

In the present study, significant differences were found between indoor and outdoor bacteria and fungi concentrations: globally, indoor levels were 9 and 3 times higher than outdoor, respectively. This fact may indicate the presence of internal/indoor sources for both biological contaminants. Their major sources might be any activities carried out inside classrooms; density of occupation, and/or ventilation (windows are closed more often in winter and ventilation might be insufficient) (Madureira *et al.*, 2015a). According to Franklin (2007), there are two reasons why indoor concentrations may exceed levels outdoors: first, the large number of emission sources inside buildings and, second, the "tightness" of buildings built since the mid-1970s.

For bacteria, all studied schools had I/O ratios higher than 1, with a maximum of 705 at School 06. For fungi, the most worrying obtained values were the maximum ratios of 40 and 66 at Schools 14 and 16, respectively.

Correlations between indoor and outdoor bacteria and fungi levels also demonstrated that no relationship exists between these variables, demonstrating that indoor concentrations of both agents are not originated from outdoors and enhancing the internal sources possible contribution on indoor air biological contamination. As the assessment was performed during the heating season (January to April 2014 and October 2014 to January 2015), weather conditions such as low temperatures (mean=16°C) and heavier precipitation levels might explain I/O ratios higher than 1. In addition, as already mentioned in the subsection 6.1, during heating season occupants spend more time indoors, windows are more often closed, and ventilation, in particular natural ventilation, tends to be poor; thus, higher indoor T and RH become suitable for bacteria and fungi growth indoors (Madureira *et al.*, 2014). So, the outdoor air affects less the indoor environments in heating season allowing indoor sources (like human sources) to be better detected (Rintala *et al.*, 2008).

Similar findings were observed in other studies available in current literature. In the context of the SINPHONIE project, indoor air pollutants have been found in school classrooms often in concentrations higher than outdoors (Csobod *et al.*, 2014). In the study conducted by Pegas *et al.* (2010), from the three evaluated schools, only one had I/O fungal ratios higher than 1, and for bacteria, I/O ratios ranged from 0.62 and 1.95. These I/O ratios have lower values than what was found in the present study. Macedo *et al.* (2013) reported a value of 6 for bioaerosol I/O ratio. In two out of four American cities where the investigation conducted by Levetin *et al.* (1995) was performed, and although indoor fungi levels were low, these were higher than fungi concentration found outdoors.

I/O ratios results have also been analyzed in several studies: Madureira (2014) and Madureira *et al.* (2015a) showed I/O ratio ranges from 0.82 until 119.4 for bacteria (median=8.82) and from 0.50 until 4.23 for fungi (median=1.26). These results are very similar to the ones obtained in this work for both biological agents. In Madureira *et al.* (2015a), where child-day care centers, primary schools, elderly care centers and homes were compared regarding their indoor biological levels, the highest I/O ratios for bacteria concentrations were observed in child day-care centers and primary schools. In Madureira *et al.* (2014), indoor fungi concentrations were higher than outdoors (I/O=1.5), but no significant differences were observed. In Madureira *et al.* (2015b)

significant differences between indoor and outdoor levels of bacteria were found, with indoors being higher, while for fungi there were no significant differences.

Nevertheless, opposite findings to the present study should also be discussed. In Madureira *et al.* (2015a), for fungi indoor and outdoor concentrations, the median I/O ratio was around 1 and indoor levels were relatively low, pointing out that outdoor air was one of the main sources for fungi, suggesting that in that study the overall impact of outdoor sources on fungi indoor concentrations seemed more relevant than the contribution of indoor sources. The investigation executed by Cooley *et al.* (1998) demonstrated that indoor fungi concentrations were 50–90% less than the outdoor air, with statistical significant differences. In Roda *et al.* (2011), outdoor fungi levels were also significantly higher than indoor levels, contrarily to what was found in the present study. Finally, data obtained by Robertson (1998) indicated indoor fungi levels 5.5 times less than outdoor concentrations.

6.3. Fungi identification

In outdoor air, *Cladosporium sp.* and *Penicillium sp.* were the most prevalent fungi species, with *Aspergillus fumigatus* identified as the outdoor most prevalent fungi in 2 schools. Indoors, the most predominant fungi species were *Penicillium sp*. and *Cladosporium sp.*, both considered to be common in indoor environments, according with the guidelines presented at Portuguese national legislation, Ordinance no 353-A/2013. *Rhodotorula sp.*, *Aspergillus fumigatus* and Yeast were also identified in most indoor environments but at an inferior percentage.

The main fungi species found in the present study are similar from those identified in other studies that included indoor air fungi identification in primary schools, both indoors (Annesi-Maesano *et al.*, 2013; Diette *et al.*, 2008; Ejdys, 2007; Haliki-Uztan *et al.*, 2010; Jo & Seo, 2005; Levetin *et al.*, 1995; Madureira *et al.*, 2014; Madureira *et al.*, 2015a; Meklin *et al.*, 2002a; Meklin *et al.*, 2003; Qian *et al.*, 2012; Roda *et al.*, 2011; Santos, 2010; Scheff *et al.*, 2000; Spengler *et al.*, 2000) and outdoors (Cabral, 2010; Cooley *et al.*, 1998; Levetin *et al.*, 1995; Madureira *et al.*, 2014; Madureira *et al.*, 2015a). In all these studies, the aforementioned prevalent fungi species were considered common in nature and the most predominant.

Important to notice that in two of the twenty evaluated schools, *Penicillium sp.* was the only fungi genera found in all samples of all classrooms at a percentage of 100%, meaning that this fungi genus had an opportunistic growth above the other fungi genera/species of these environments. Higher concentrations of *Penicillium sp.* have been observed in buildings with moisture damage

or with visible mold growth (Cabral, 2010; Fischer & Dott, 2003; Madureira *et al.*, 2014). However in classrooms of Schools 04 and 16 it was not observed any visible mold growth on any surface, wall or ceiling. In the study performed by Cooley *et al.* (1998), levels of *Penicillium sp.* were significantly higher in air samples from complaint areas than outdoor air samples and indoor air samples from noncomplaint areas. According to Cabral (2010), in healthy buildings indoor fungi are dominated by *Cladosporium sp.* and *Penicillium sp.*, in similar concentrations, with indoor levels lower than outdoor. In these types of buildings there is no appreciable indoor fungal growth in walls, ceilings, and furniture and indoor fungi is originated essentially from outdoors, and therefore *Cladosporium sp.* is the dominant, or at least, a major fungus in the atmosphere. On the other side, sick buildings have indoor *Penicillium sp.* concentrations 5–6 times higher than outdoor, that can be caused by the fact that fungi have the ability to grow on the surface of the building materials, furniture, and dust, originated from the existence of a certain amount of indoor humidity, either in the air and/or in the walls and ceilings. The degree of indoor humidity is enough to allow xerophilic fungi, such as *Penicillium sp.* and *Aspergillus sp.*, to grow and sporulate, releasing conidia and fragments to the atmosphere (Cabral, 2010).

Aspergillus fumigatus was identified in 50% and in 35% of schools' indoor and outdoor environments, respectively. *Rhodotorula sp.* was detected in almost every indoor environment, with low outdoor levels. Yeasts were found in 80% of schools indoor air, while in 40% of schools outdoor air. Moreover, statistical differences were found between indoor and outdoor levels of Yeasts and *Rhodotorula sp.*, with higher levels indoors. These facts indicate that its sources are mainly indoors. Similar findings were obtained by Levetin *et al.* (1995). Yeast can be found on the surface of the skin and in the intestinal tract of humans, where they may live symbiotically or as parasites. In damp schools, its presence is reported in second place after *Penicillium sp.* (Ejdys, 2007). *Rhodotorula sp.* are ubiquitous saprophytic yeasts that can be recovered from many environmental sources.

Nevertheless, the most prevalent fungi species found indoors and outdoors were the same: the distribution of *Penicillium sp.* and *Cladosporium sp.* indoors and outdoors indicates their presence in almost all classrooms and in all schools outdoors. Hence, in the present study the influence of outdoor air in fungi composition of indoor air is also proved, as stated in other studies (Cabral, 2010; Madureira *et al.*, 2014; Madureira *et al.*, 2015a; Roda *et al.*, 2011). Important also to notice that biological assessment was performed in heating season, factor that might reduce the impact of outdoor air on indoor air fungi levels.

The differences in fungi genera prevalence between studies and indoor environments could be attributed to geographic location, season, differences in culture procedures, different experimental and sampling approaches, relative humidity and building characteristics (Madureira *et al.*, 2014; Madureira *et al.*, 2015a; Meklin *et al.*, 2003).

Significant statistical differences were found between indoor and outdoor percentages of *Cladosporium sp.*, Yeast, *Penicillium sp.* and *Rhodotorula sp*. The differences found between the most abundant fungal genera identified indoors and outdoors may also be related with the individual ability to grow inside buildings or to persist indoors for a longer time after penetration into the building (Madureira *et al.*, 2014). Spore release from mature surface fungal colonies has been shown to vary with air speed, with many fungi species requiring more active disturbance for spore release, and between different taxa. An example is *Cladosporium sp.*, with its conidia release requiring about twice the air speed as spores of *Penicillium sp.* or *Aspergillus sp.*, possible explanation for the fact that *Penicillium sp.* or *Aspergillus sp.* normally dominates air samples (Spengler *et al.*, 2000). Another important factor that certainly favors the dominance of *Cladosporium sp.*, *Penicillium sp*. and *Aspergillus sp*. in indoor environments is that they are able to produce high numbers of small and light spores (Cabral, 2010). *Aspergillus sp.* and *Penicillium sp.* can be hazardous to humans in high concentrations owing to their abilities to produce mycotoxins (Cabral, 2010; Tang, 2009). At high concentrations, mycotoxins induce severe and acute intoxications, and the effects are relatively straightforward to examine and quantify. At lower concentrations, the negative effects of mycotoxins are more difficult to study and evaluate (Cabral, 2010).

Identification and quantification of fungi in schools assume particular importance considering that some of identified airborne fungi are associated with health problems, particularly asthma and respiratory problems (Madureira *et al.*, 2014). Thus it is of extreme importance to characterize the indoor air biological composition as different health effects may result from exposure to different fungal profiles (Madureira *et al.*, 2015a). Fungi species of *Penicillium sp.*, *Aspergillus sp.* and *Cladosporium sp.* have been the most frequently indoor allergens associated with allergy, asthma and respiratory problems (Cooley *et al.*, 1998; Fischer & Dott, 2003; Gaffin & Phipatanakul, 2009; Madureira, 2014; Madureira *et al.*, 2014; Madureira *et al.*, 2015a; Madureira *et al.*, 2015d; Mandal & Brandl, 2011). As referred by Madureira *et al.* (2014), a study that has similar and comparable results to the ones obtained in the present study,

concurrent exposure to multiple fungal genera found in primary schools of both studies might play a role in increased asthma risk due its ability to cause respiratory sensitization.

Aspergillus fumigatus is considered an indicator of moisture damage in buildings (Meklin *et al.*, 2002) and is the best known of the opportunistic human pathogenic fungi (Fischer & Dott, 2003; Gniadek, 2012; Spengler *et al.*, 2000). Even though it can grow at room temperature (25°C), it may lose competition with other, more rapid-growing species characteristic of this temperature (Ejdys, 2007). This fungal species may cause acute and chronic inhalatory respiratory tract infections (aspergillosis, aspergilloma) (Gniadek, 2012). Nevertheless, it is important to remember that intense exposure to *Aspergillus fumigatus* is common and although such exposure may lead to acute or even chronic allergic respiratory disease, it almost never leads to infection unless the exposed person is seriously immunocompromised (Spengler *et al.*, 2000).

Aspergillus flavus was only identified in indoor air of a total of four classrooms from three schools and at very low levels (2%). This species causes infections of the respiratory system, allergic aspergillosis, chronic invasive sinusitis as well as deep fungal infections (Gniadek, 2012).

Aspergillus niger was identified in eleven schools' indoors and five schools' outdoors, also at very low levels (5% indoors). It may cause infections of the inner and outer ear (otomycosis) as well as pulmonary aspergillosis. Nevertheless, it was established that the *Aspergillus niger* species was considerably less cytotoxic than *Aspergillus fumigatus* (Gniadek, 2012).

6.4. Correlations between chemical, physical and biological parameters

Air T and RH can influence the prosperity and transmission of microorganisms indoors, with significant relationship found between indoor environmental conditions and airborne bacterial community structure (Kembel *et al.*, 2012). Since the majority of bacteria and fungi need specific environmental conditions to grow and propagate, their levels are strongly affected by T and RH (Mandal & Brandl, 2011). For instance, it is assumed that RH in the air is a good indicator for the likely presence of microorganisms indoors (Spengler *et al.*, 2000). Gniadek (2012) referred that humidity in rooms where human occupancy is assumed should be between 30-70%. Portuguese national legislation, Decree-Law no. 243/86, establishes the range of RH in 50-70%. Excessively humid air encourages multiplication of microorganisms, like bacteria and fungi, decomposition and water condensation, which increases microbiological contamination of the air (Gniadek, 2012).

Bacteria and endotoxins concentrations were both positively correlated with RH and CO_2 levels, all with statistical significance. This shows that values of bacteria and endotoxins concentrations increase when higher values of CO_2 and RH are present. These positive correlations could possibly be related with low ventilation rates inside classrooms along with high number of occupants, leading to higher values of CO_2, RH, bacteria and endotoxins. This information could be used as an indicator of an excess of occupants or insufficient ventilation of the classrooms (Madureira *et al.*, 2015a). Contrarily, both parameters showed negative correlations and low statistical significance with T levels. But still, the same variations between these two biological contaminants are worth noticing.

Similar findings were reported by Madureira *et al.* (2015a) and Madureira *et al.* (2015b) concerning positive and significant correlations between bacteria and CO_2 levels, and by Dales *et al.* (2008) and Park *et al.* (2000) in homes, as well as Jacobs *et al.* (2014a), in Spain primary schools, found analogous results regarding endotoxin and RH levels being positively and statistically significantly correlated. T and bacteria were also not correlated in the study conducted by Madureira *et al.* (2015a), whereas in Popescu *et al.* (2013) T and bacteria had a significant and negative correlation, but at an industrial setting. A negative and with low statistical significance correlation was also found between T and endotoxins in the study performed by Park *et al.* (2000), in homes; on the other hand, Jacobs *et al.* (2014a) found that increased endotoxin levels were registered in Spanish primary schools with higher T. Madureira *et al.* (2015a) did not find a similar correlation for RH and bacteria: these two variables had no correlation between them. Contrarily, in the study performed by Lawniczek-Walczyk *et al.* (2013) at an industrial setting, a strong and significant statistical correlation was found between bacteria and RH, and a similar correlation was found in the investigation of Popescu *et al.* (2013), also at an industrial setting. In Kembel *et al.* (2012), indoor levels of bacteria were higher with lower RH, an opposite finding from the present study. Available data on the effects of T and RH on the survival of airborne bacteria are far inconsistent and even bacteria within the same structural classification (e.g., Gram-negative) may vary in how they respond to different changes in T and RH (Fernstrom & Goldblatt, 2013; Tang, 2009).

Correlations found between fungi concentrations and other chemical or physical parameters in study were very weak, and with low statistical significance. Nevertheless, the correlation with higher value was found between T and fungi levels. This fact may indicate that optimal T ranges for fungal growth may have been achieved for some fungi genera or species (Madureira *et al.*,

2014; Madureira *et al.*, 2015b). Fungi grow mainly in the environment where air humidity exceeds 45%, T is within the range of 5-35°C (optimum 18-27°C), and water activity (a_w) exceeds 0.8 (Gniadek, 2012). With the values of T (15-30°C) and RH (35-72%) obtained in the present study, in fact, optimal indoor environmental conditions were gathered in order to enhance fungi proliferation. Madureira *et al.* (2015a) and Madureira *et al.* (2015b) obtained similar results, while Madureira *et al.* (2014) had a significant positive correlation between indoor fungi concentrations and T levels, and Popescu *et al.* (2013) had also a statistically significant but negative correlation between these two variables, at an industrial setting, with RH levels also positively correlated with fungi concentrations. According to Tang (2009), most studies confirm a positive correlation between fungi spore levels and higher T indoors, and also indicate that fungi spore concentrations are usually higher with higher RH levels, a connection that was not found in the present study.

Between the three biological parameters in study, a weak positive correlation with statistical significance was found between bacteria and fungi concentrations. Endotoxin concentrations had weak correlations with bacteria and fungi, being the first a positive correlation (higher values of endotoxin match higher values of bacteria) and the second a negative correlation (lower levels of endotoxin corresponds to higher fungi concentrations). Nonetheless, in addition to weak, these correlations had low statistical significance. Thus, although bacteria and endotoxins concentrations fluctuate very similarly when compared to CO_2 and RH levels, the variation between them is not related. Opposite results were found by Su *et al.* (2002) regarding endotoxin and bacteria relationship; in their study, the Spearman rank correlation between these two variables was statistically significant and assumed a moderate value (r_S=0.463). A high correlation between endotoxin and bacteria levels is expected, in theory, due to the fact that endotoxin is a component of the outer membrane of gram-negative bacteria. Hence, total bacterial counts may be an acceptable surrogate for the estimation of endotoxin exposures. Notwithstanding, further investigation is necessary to identify the major genera of bacteria contributing to endotoxin (Su *et al.*, 2002). On the other hand, similar results with the present study were found by Tsai *et al.* (2001) regarding fungi and endotoxin correlation: these two parameters were not significantly connected.

Cladosporium sp. had a weak negative correlation with CO_2, whereas with T and RH were found positive and negative moderate correlations, respectively. A study available in current literature with similar analysis, performed by Madureira *et al.* (2014), had the same tendency correlations,

but with no statistical significance. According to Jacob *et al.* (2002), in homes, high RH levels have been associated with several specific genera, including *Cladosporium sp.* and *Penicillium sp.*, but no positive or negative correlation is mentioned.

In this study, Yeast did not have any correlation with the chemical and physical parameters in analysis. Contrarily, in Madureira *et al.* (2014), Yeast had statistically significant and positive correlations with CO_2 and RH.

Negative weak correlations were found between T and *Rhodotorula sp.*, like in Madureira *et al.* (2014), and also between T and *Aspergillus fumigatus*, a fungi species that was not analyzed regarding the influence of chemical and physical parameters in its presence indoors in Madureira *et al.* (2014). *Rhodotorula sp.* had a positive and significant correlation with CO_2 in Madureira *et al.* (2014), finding that was not verified in the present study.

Regarding *Penicillium sp.*, a positive weak correlation was found with CO_2 in the present study. In Madureira *et al.* (2014) this fungi species had no statistical significant correlations, but the tendency was similar to the present study: positive correlations for CO_2 and RH, and negative correlation for T. To mention that Ramachandran *et al.* (2005) found a positive significant effect of CO_2 on culturable airborne fungi and on the increase of fungi concentrations.

As stated in Madureira *et al.* (2014) and likewise in the present study, the lack of consistent correlations between RH and some reported fungi species might be due to fluctuations in the determinants of fungi growth related to RH or the cross-sectional nature of RH measurements, which may not reflect the proper conditions for mold growth.

It is noteworthy that statistically significant associations do not necessarily mean causal relationships, although they help to generate hypotheses or direct attention to possible background associations (Csobod *et al.*, 2014).

6.5. Building and classroom characteristics

The pollution load in a school building depends to a large extent on the interaction between the building and its outdoor environment, as well as on the way the building is constructed, furnished and used, the type of ventilation system, and the activities of its occupants (Kephalopoulos *et al.*, 2014).

The age of the building is an important factor encouraging indoor biological contamination (Ejdys, 2007). In the present study, although the majority of the schools have refurbished in the last ten years, most of the studied schools were built between the 50's and 70's. A positive moderate

correlation was found only between endotoxins concentrations and the year of building refurbishment, meaning that as more recently the intervention was made, the higher were concentrations of endotoxins in indoor air. Contrary results were found by Jacobs *et al*. (2014a) which referred lower endotoxin levels in school buildings that were recently built. In Sheehan *et al.* (2012) study, the age of the school was not correlated with indoor concentration of endotoxin. Once more, in the investigation of Meklin *et al*. (2003), the effect of the age of buildings on fungal aerosol levels did not show any clear trend, as well as in the study performed by Madureira *et al.* (2014). According to Csobod *et al.* (2014) and Annesi-Maesano *et al*. (2013), building age is suspected to have an impact on occupants' health, with a tendency towards more symptoms in recently built schools compared to older buildings, due to the modern type of building construction that crave to create enclosed environments in order to accomplish energy requirements and neglects its effects on IAQ.

Building frame should be taken into account when matching buildings for exposure assessment in epidemiological studies (Meklin *et al*., 2003), as materials used inside for floors, walls or ceilings also have an impact on indoors biological agents (Csobod *et al*., 2014; Madureira *et al*., 2014), and on health (Annesi-Maesano *et al*., 2013). All of the evaluated schools had massive wall structure and water-based wall covering, with 60% of the buildings including double wall. Endotoxin concentrations were higher when buildings had single walls. Bacteria and fungi levels were not influenced by schools' type of building frame.

Endotoxin concentrations were the only biological parameter influenced by the presence of roof leaking in the last 12 months, which occurred in 7 of the 20 evaluated schools, with higher levels in buildings without roof leaking. Windows leaking in the last 12 months, which happened in 3 schools, did not show any statistical significant variation in the evaluated biological parameters, as well as visible air leaks, that were observed in 45% of the Porto schools studied. Jacobs *et al*. (2014a) found that higher endotoxin levels were associated with major water intrusion in the school building during the last 5 years. Although it is well established that there is a positive correlation between water infiltrations, indoor dampness, and proliferation of indoor fungi (Cabral, 2010), in the present study roof or windows leaking was not found to be related with higher fungi levels in indoor air of primary schools.

Regarding to density of occupation, it ranged between 1.71 until 4.30 m^2/occupant, with a mean value of 2.4±0.5 m^2/occupant. This value is very similar from what was found in the scope of SINHPONIE study, which reported a mean occupation density of 2.44 m^2/occupant, ranging from

0.83 m^2/occupant in an Albanian school, until 6.15 m^2/occupant in Italia (Csobod *et al.*, 2014). Taking into consideration American Society of Heating and Air-Conditioning Engineers (ASHRAE) 62-2001 criterion (ASHRAE, 2004), which establishes a minimum of 2.0 m^2/occupant, the mean value obtained in the present study is according with this guideline. Nevertheless, 18% of the classrooms were below the recommended value. Once again, a similar result between the present study and SINPHONIE project: about 20% of all schools evaluated in SINPHONIE scope was operating with occupation densities lower than 2 m^2/occupant (Csobod *et al.*, 2014). Madureira *et al.* (2014) had also an analogous result: 19% of classrooms had density of occupation lower than 2 m^2/student.

Correlations with statistical significance were found between the variables number of occupants and density of occupation and endotoxins levels. The first, a weak positive correlation, meaning that endotoxin concentrations increase with the number of students in the classrooms. This may indicate that endotoxin may have been brought in by the occupants or that skin sheds or other sources of the occupants influence endotoxin levels (Jacobs *et al.*, 2014a). The second, a weak negative correlation, indicating that endotoxin levels decreases as density of occupation rises, which is in agreement with the previous correlation result. Jacobs *et al.* (2014a) found similar results: higher classroom occupancy was positively and significantly associated with endotoxin levels. It was expected that bacteria concentrations were higher when classrooms had more children, but in the present study this correlation was not verified. A similar result was found by Madureira *et al.* (2015b): no significant correlation was found between indoor bacteria concentrations and the density of occupation. In the study conducted by Qian *et al.* (2012) that involved classrooms biological assessment, bacteria and fungi indoor concentrations were higher when the room was occupied. In the present study, the correlation between fungi concentration and number of occupants had a result that demonstrates nearly an absence of relationship between them, and a comparable result was found in Madureira *et al.* (2014).

It is expected that the heating season is prone to have bad IAQ due to poor ventilation. Indeed, in the present study, 45% (n=32) and 42% (n=30) of the classrooms had no window or only 1 window usually opened in heating season. Although this lack of natural ventilation, Spearman's rank correlation coefficient showed no connection between "window opening" or not with the concentration of biological parameters indoors.

Heating may exert an adverse impact on pollutant concentration and health (Annesi-Maesano *et al.*, 2013). Space heater number varied between classrooms and 32% of the classrooms had 1

space heater, while 10% and 3% had 2 or 3, respectively. This variable was weak and moderate positively correlated with bacteria and endotoxins concentrations, respectively, both with statistical significance. This finding indicates that endotoxins levels in classrooms were higher when they had more space heaters. 94% of the classrooms evaluated in this study had heating system and only 6% had heating system providing also domestic hot water. SINPHONIE study results were a little different from the present study: in 59% of schools the system was exclusively for heating, and in 33% of schools it provided heating plus hot water (Csobod *et al.*, 2014).

Regarding the windows materials, only 3 classrooms had metal frames, against 68 classrooms with aluminum frame, and in the majority of the classrooms (68%) windows had single glazing. Windows frame had no influence on indoor biological levels, whereas when windows had single glazing fungi concentrations were higher.

Classrooms' floor covering varied, with 70% with synthetic smooth floor and 30% with wood or cork floor, as well as for ceiling surfaces, 92% of the classrooms had paint ceiling while only 8% had wood ceiling. These two variables, floor and ceiling coverings were correlated with endotoxins levels: higher values were found in classrooms with paint ceiling and synthetic floor than in classrooms with wood ceiling and wood floor. Instead, in the study performed by Jacobs *et al.* (2014a), floor material of classrooms was not consistently associated with endotoxin levels. The type of floor covering was not related with bacteria and fungi concentrations in the present study, and similar results were found regarding fungi levels in the study executed by Madureira *et al.* (2014), also in primary schools. It is also important to mention that, according to Csobod *et al.* (2014), it was found a link between plastic flooring and doctor-diagnosed allergies.

Mold odor is a good predictor of presence of active microbial growth and is also an indicator of hidden damage in the construction (Borras-Santos *et al.*, 2013). In the present study this was a characteristic present in 5 classrooms (7%). Similar prevalence of mold odor in 8% of the SINPHONIE classrooms was found (Csobod *et al.*, 2014). Statistical significant differences were found between bacteria concentrations when noticeable mold odor in classrooms was not detected, with higher bacteria values in the absence of this characteristic. Endotoxins and fungi concentrations were not related with noticeable mold odor in the evaluated classrooms. Similar finding was reported by Jacobs *et al.* (2014a), regarding endotoxins levels and mold odor.

Visible damp spots on walls, ceilings or surfaces were detected in 14% of the evaluated classrooms (n=10). In the study performed by Madureira *et al.* (2014) interior damp stains were observed in 20% of the classrooms. Visible mold growth was observed in 11 classrooms, mostly

in ceiling and in the joint of ceiling with wall. However, no statistical differences were found between the biological parameters concentrations and the presence of mold growth and/or damp spots in classrooms, finding that indicates that other sources are probably more relevant for indoor biological contamination and that not all subjective indicators of dampness can serve as indicators for the presence of indoor fungi. As stated by Spengler *et al.* (2000), observation of growth is not proof of exposure to hazardous agents, but probably would prompt recommendations for remediation. Comparable results with the present study were found in Madureira *et al.* (2014), which affirmed that the correlation between visible mold with indoor fungi concentration was not significant. On the other hand, and contrarily to the present study, in the same referred study, statistical significant correlation was found between damp stains and indoor fungi levels. Nevertheless, these signs suggest potential exposure to fungi, as these factors may produce high levels of air contamination by fungal fragments, which are not detected by culturable methods but still have the ability to penetrate the respiratory system and exert respiratory symptoms (Madureira *et al.*, 2014). Meklin *et al.* (2005) affirms that excess of moisture could be associated with higher levels of bacteria. Nevertheless, similar outcomes to the present study were referred by Jacobs *et al.* (2014a) in the scope of HITEA study: moisture explained little endotoxin variability between schools and findings did not necessarily reflect endotoxin levels in schools that were specifically affected by dampness. Only in the Netherlands a clear association was observed between school dampness and microbial levels (Jacobs *et al.*, 2014b). Notwithstanding, in HITEA study it was concluded that moisture damage in schools may have adverse respiratory health effects in pupils, but a clear association between moisture damage in schools and respiratory infections was not found (Borras-Santos *et al.*, 2013). In fact, contradictory statements can be found in current literature regarding health effects of dampness and moisture in buildings. According to *Green Schools: Attributes for Health and Learning* (2006), there is sufficient scientific evidence to establish an association between excess moisture, dampness, and mold in buildings and adverse health outcomes, particularly asthma and respiratory symptoms, among children and adults. For Clausen *et al.* (2011), although much research has focused on dampness in buildings and how exposure to dampness in buildings affects humans, causal links between dampness indicators, including microbial pollutants, and health remain elusive.

In 15 classrooms, corresponding to 21%, there was a tendency for condensation on windows, inside the frame. In the study done by Madureira *et al.* (2014), this classroom characteristic was

observed in almost 50% of the evaluated classrooms. In the present study, higher levels of endotoxin were related with classrooms without windows condensation, while this characteristic had no influence on bacteria and fungi concentrations.

The preferred window opening period reported was during breaks (48%) and during teaching hours (47%), with 16% of the classrooms having the windows opened during cleaning time. These prevalent periods of window opening are in accordance with the practices throughout Europe as reported in SINPHONIE study (Csobod *et al.*, 2014): in 88% of the schools the window opening period was during breaks, and in 70% it was during teaching hours. As noted in Csobod *et al.* (2014), it should be pointed out that natural ventilation by window opening may interfere with other environmental quality parameters such as thermal comfort, noise, drafts, outdoor pollution, with the impact on classroom occupants being greater if the windows are opened during teaching hours. It was found that when windows were not opened before school and during cleaning times' bacteria concentrations were higher than when these two practices were taken. Regarding the time of the day when windows were opened, these two were the only presenting influence on bacteria indoor levels. This means that although windows are mainly open during breaks and during teaching hours, it does not affect indoor air bacteria, fungi or endotoxin concentrations. Endotoxin concentrations were also influenced by "window opening during cleaning time", as bacteria concentrations. Concentrations were lower when this task was done with the windows opened. In the study conducted by Singh *et al.* (2011), in homes, airborne endotoxin levels were not significantly associated with any of the considered home characteristics. In the present study, fungi levels did not suffer any variation regarding the different times of the day that windows were opened.

The source of ventilation air influences the diversity and composition of the built environment microbiome (Kembel *et al.*, 2012), and therefore, ventilation system should be taken into account in comparisons of airborne microflora in buildings (Meklin *et al.*, 2003). Regarding ventilation system in the present study, similar percentages as referred in heating systems were found: 94% of the evaluated classrooms, corresponding to n=67, had only natural ventilation, while 6% (n=4, all from the same school) had a hybrid/mixed mode of ventilation. SINPHONIE results demonstrate that along European schools, natural ventilation is the predominant ventilation system: 92% of the classrooms had ventilation assured by natural mechanism and only 7% had mechanical ventilation systems (Csobod *et al.*, 2014). In the study performed by Macedo *et al.* (2013) all classrooms depended only on natural ventilation through existing vents,

windows, and doors. Research comparing the effects of natural versus mechanical ventilation on human health is inconclusive (Spengler *et al.*, 2000). Nevertheless, in the study performed by Simoni *et al.* (2010), poor ventilation rates was significantly more frequent in naturally ventilated classrooms than in those with mechanical ventilation, due to the fact that natural ventilation depends on building characteristics, occupant activities and number, and weather conditions.

In the present study fungi and bacteria concentrations had higher values when classrooms were ventilated through the windows opening, without mechanical ventilation system, and when heating system did not include domestic hot water. Bacteria originate mainly from humans and high concentrations of viable airborne bacteria usually indicate insufficient ventilation of the building (Meklin *et al.*, 2002a). Endotoxins concentrations were not influenced by classrooms' type of ventilation and heating systems. Ventilation is an essential element of IAQ because it promotes the dilution of the pollution load and can therefore have a positive impact on children's health. Different types of ventilation, for example mechanical or natural, can have a different effect on health (Csobod *et al.*, 2014). Zuraimi *et al.* (2007) observed the effects on health of different ventilation systems, noting more symptoms where there was hybrid ventilation, while the combination of air conditioning and mechanical ventilation seemed to have a negative effect on rhinitis compared with natural ventilation.

Regarding the influence of school building and classroom characteristics on the main fungi species identified in the present study, the results show that the only moderate correlations found were between the fungi species *Aspergillus fumigatus* and *Cladosporium sp.* and the classroom characteristic of tendency for condensation on windows: percentages of *Aspergillus fumigatus* were higher in classrooms where condensation was present in windows, and the opposite happened for *Cladosporium sp.*; in other words, where windows condensation was observed, lower levels of *Cladosporium sp*. were found. The presence of these two species can be related with moisture damage and excess water in structures, which lead to growth of both common fungal species, like *Cladosporium sp.*, and less frequently found fungi, such as *Aspergillus fumigatus*, due to the available conditions of nutrient and moisture (Meklin *et al.*, 2003).

Number of space heaters was another classroom characteristic where significant correlations were found with *Aspergillus fumigatus* and *Cladosporium sp.*: the first, a negative moderate correlation and the second a weak, but positive, correlation, meaning that the more space heaters present in classrooms, the lower were the percentages of *Aspergillus fumigatus* and the higher were the percentages of *Cladosporium sp.*

Cladosporium sp. had another weak positive correlation with the type of ceiling surface of the evaluated classrooms, where higher values were found in wood ceilings. In the study performed by Madureira *et al.* (2014), *Cladosporium sp.* was not significantly correlated with any of the selected school characteristics.

Yeast was weak and negatively correlated with heating system and ventilation system, meaning that higher values were found in classrooms with natural ventilation and with only heating system. Although the presence of Yeasts is correlated more with living organisms occupying it (Ejdys, 2007), in the present study this fungi species was not correlated with the number of occupants and in Madureira *et al.* (2014) this correlation was also not observed. In this same study, visible mold was significantly correlated with Yeast, whereas in the present study this relation was not observed.

Rhodorotula sp. was found to be weakly and positively correlated with the type of floor covering, with higher values when classrooms' floor was lined with wood. Results found by Madureira *et al.* (2014) showed that indoor prevalence of *Rhodotorula sp.* registered higher values when schools' floor had PVC floor material.

None of the selected classrooms characteristics showed influence in the presence or absence of *Penicillium sp*. in the evaluated primary schools, like in the study of Madureira *et al*. (2014).

None of the classrooms' characteristics that are considered to be probably related with fungi presence such as visible mold growth, noticeable mold odor and visible damp spots on walls, ceilings or floors, were correlated with any of the fungi species considered predominant in this study. No relation was also found between the predominant fungi species and the number of classrooms occupants or the density of occupation.

Kembel *et al*. (2012) affirm that, in their study, the observed relationship between building design and airborne bacterial diversity suggests that if we manage indoor environments, through building design and operation, the community of microbial species that potentially colonize the human microbiome during our time indoors can be changed. In the present study, the assumption made by Kembel *et al.* (2012) cannot be made due to the few statistical correlations and statistical differences found in the present study between indoor biological concentrations and building/classroom characteristics.

6.6. Strategy for good IAQ in Primary Schools

Facing the present study results it is important to stress that improvement and/or intervention measures should be considered in an attempt to reduce biological contaminants from indoor air in Porto primary schools.

US EPA has provided the "IAQ Tools for Schools" action kit to school officials, staff members of various school facilities, teachers, healthcare professionals, as well as students and their parents, which provides best practices, guidelines and a sample IAQ management plan to improve school air at low or no cost, available on: http://www.epa.gov/iaq/schools (Kephalopoulos *et al.*, 2014).

In 2014, a pragmatic set of guidelines has been recently published in the scope of SINPHONIE project entitled "Guidelines for healthy environments within European schools" available at www.sinphonie.eu. It intends to provide advice that can be regarded as generally applicable in most school environments in Europe. However, as each school environment is unique (in terms of design, climatic conditions, operational modes, etc.), the guidance needs to be adapted as appropriate at national or local level. SINPHONIE guidelines are not therefore intended to replace, but rather to enrich and reinforce, existing national and local guidance that should continue to be the first point of reference (Csobod *et al.*, 2014).

Good engineering practices recommend that preventive measures, or in other words, measures focused on strategies of source control, should be the first line of action. In fact, EnVIE Project pointed out to source control as the first priority since preventing exposure is the most effective way of protecting human health from environmental threats (Madureira, 2014). In this perspective, the following aspects should be taken into consideration, according with SINPHONIE guidelines (Csobod *et al.*, 2014; Kephalopoulos *et al.*, 2014) and Madureira (2014):

- To carefully study the location of future buildings, in particular when dealing with susceptible populations like children, and its relationship with the outdoor environment;
- Proper design and construction of school buildings and selection of clean materials;
- Proper level of continuous thermal insulation of the envelope avoiding solutions of continuity that may cause condensations and dampness indoors;
- Decoupling, as far as possible, of heating/cooling functions from the ventilation function;
- Definition and enforcement of limits for maximum permitted occupation densities in classrooms, as well as definition of classes and breaks durations, establish routines of cleaning and maintenance operations;

- Periodical monitoring of IAQ and health parameters in schools.

An important factor regarding biological contamination of school buildings is related with dampness and moisture. Future green school guidelines and source control measures should emphasize the control of excess moisture, dampness, and mold, through the use of dehumidifiers for instance, as well as prevent or eliminate water leaks whenever possible, to protect the health of children and adults in schools and to protect the building's structural integrity (Annesi-Maesano *et al.*, 2013; Diette *et al.*, 2008; Mendell *et al.*, 2011; Tsai *et al.*, 2001). These measures are likely to significantly reduce the current global burden of children's respiratory and allergic disease (Mendell *et al.*, 2011).

Control of biological contamination and preventative maintenance should always be the first line of defense against the airborne microbiological exposure. However, even under ideal preventative maintenance conditions, opportunities for microorganism growth and airborne exposure often do occur (Rao *et al.*, 1996).

After source control, ventilation measures, an intervention action that should only be considered after strategies of source control, plays also an important role in contributing to the required air quality level (Madureira, 2014). The following aspects, referred in SINPHONIE guidelines (Csobod *et al.*, 2014; Kephalopoulos *et al.*, 2014) and Madureira (2014), should be considered when adoption of ventilation measures is imperative:

- Ventilation rate should be health-based and defined and expressed as liters/second per person (L/s.person). According with Turunen *et al.* (2014), National Building Code of Finland currently requires a minimum of 6 L/s per student. Portuguese guidelines are established at Ordinance no 353-A/2013, that suggests a ventilation rate of 24 m^3/(hour.person) for classrooms (minimum flow of fresh air determined according to the pollutant load due to occupation);
- The method of ventilation in the classrooms (natural ventilation, cross ventilation, mechanical ventilation) depends on how airtight the building is, the climatic zone, the season, the quality of the outdoor air and noise levels surrounding the school building, and the reserve capacity of the heating system that should allow for the rapid reheating of the classrooms after ventilation;
- It should be explored the opportunities for the admission of outside air, unless confirmed outdoor sources or pollution are located nearby school building;

- A good ventilation practice could be, at the very least, to ventilate classrooms before the school day starts, during each classroom break and also after cleaning procedures, in all seasons;
- Two simple and effective measures that can enhance classrooms natural ventilation are the implementation of tilting windows and the introduction of ventilation grills in windows frames;
- When natural ventilation is not sufficient to reach the desired ventilation rate, a mechanical system should be implemented, preferably with intake of fresh air, and attention must be paid to its regular inspection and maintenance to guarantee that the filtered air is always clean.

A crucial and not less important measure, that should not be forgotten, is related with the appropriate training of students, teachers and school staff who are responsible for the management, maintenance and cleaning of school buildings (Csobod *et al.*, 2014).

All the aforementioned measures, either interventional, preventive or improvement, should be submitted to a thoughtful study of technical and economic feasibility for each school building, and should be adjusted to each school's reality and state of IAQ.

Moreover, the implementation of energy-efficiency requirements in Europe will result in the gradual movement towards a more energy-efficient building stock, as regards new and existing buildings, including school buildings. When coping with school buildings' energy efficiency requirements, it is recommended that attention should also be paid to preserving good IAQ, to avoid it negatively affecting the health, comfort and productivity of its occupants. The challenge is to rationalize and optimize energy expenditure while adequately meeting the health and comfort requirements of school building occupants (Kephalopoulos *et al.*, 2014).

6.7. Study strengths and limitations

Biological assessment of bacteria and fungi was performed through active air sampling on culture media (TSA for bacteria and MEA for fungi) – cultivated-based method - for the enumeration of viable microorganisms in short-term air samples. The number of visible colonies can be counted by visual inspection after incubation resulting in a direct quantitative estimate of the number of culturable microorganisms in the sampled air (Mandal & Brandl, 2011).

Several features of the present study are remarkable as strengths: (i) it provides an indoor air biological investigation in a considerable number of classrooms of public primary schools located

in Porto; (ii) sampling and analysis were performed using standardized and accredited procedures; and (iii) the objective measurement of specific biological agents in classrooms allowed a better appraisal of individual exposure compared to indirect methods such as the use of questionnaires or checklists.

Active air sampling is considered to be the most suitable sampling method for an exact assessment of human exposure, as the health effects of biological parameters are mainly respiratory. However, bioaerosol have been found to exhibit varying patterns in spore release into the air depending on several environmental factors (Madureira *et al.*, 2015a; Niemeier *et al.*, 2006).

The most important advantages of culture methods have in consideration that this is a reliable, with high sensitivity technique, and with potential to identify cultivable organisms (Eduard & Halstensen, 2009; Sebastian & Larsson, 2003), allowing the identification of many different colonies to the species level (Douwes *et al.*, 2003; Madureira *et al.*, 2014) and a large reference database is available for proper identification of colonies (Niemeier *et al.*, 2006). These features are important in studies of fungal contamination of indoor environments where fungal concentrations are considered low (Eduard & Halstensen, 2009), in comparison with fungi levels of industrial settings (Sabino *et al.*, 2012).

There are also by now very well-known limitations in culture-based sampling methods for characterizing health-related bioaerosol composition and concentrations indoors (Madureira *et al.*, 2015a). The culture method has been criticized because of the short-term nature of the measurement, which could not be representative of long-term exposure (Eduard & Halstensen, 2009; Madureira, 2014; Madureira *et al.*, 2015a; Rintala *et al.*, 2008; Viegi *et al.*, 2004). Spore release from fungal colonies is sporadic, and short-term air sampling might not accurately represent airborne levels (Niemeier *et al.*, 2006). This problem could be overcome by increasing the sampling time and the number of samples (Madureira, 2014); but these were considered to be impractical due to cost and time limitations.

It is widely agreed that only a small fraction (0.1 to 10%) of the total microbial flora in an indoor environment is currently culturable (Sebastian & Larsson, 2003), and therefore culture methods are limited by the lack of detection of non-viable components that are also immunologically active (Douwes *et al.*, 2003; Jacobs, 2013; Rao *et al.*, 1996; Rintala *et al.*, 2008). In fact, it has been suggested that allergic reaction to fungi is generally independent of culturability of spores (Niemeier *et al.*, 2006). Results based on cultivation are, therefore, at best semi-quantitative

(Douwes *et al.*, 2003; Eduard & Halstensen, 2009; Sebastian & Larsson, 2003). Another study limitation is the short sampling periods which may introduce important variations between measurements, resulting in poor reproducibility and precision (Douwes *et al.*, 2003; Eduard & Halstensen, 2009; Jacobs, 2013; Madureira *et al.*, 2015a; Viegi *et al.*, 2004) and weak consistency in comparisons (Madureira *et al.*, 2015a). Moreover, results may not be representative of other Portuguese or European school buildings for several reasons, e.g. climatic zones and ambient air quality, as well as building characteristics (Madureira *et al.*, 2015b).

It is also important to mention that Porto primary schools were assessed during heating season. Monitoring during warming season would be of extreme importance in order to explore seasonal differences (Madureira *et al.*, 2015a; Madureira *et al.*, 2015b; Rintala *et al.*, 2008).

In the present study, bacteria identification was not performed and some fungi species could not be identified at the species level. This fact may contribute to the lack of association between microbiological agents' exposure and health outcomes (Madureira, 2014). Fungal genus-only identification can result in inaccurate characterization of indoor air fungal contamination, since indoor sources of a specific species may be overlooked and not all members of a fungal genus have the same potential to cause human disease (Rao *et al.*, 1996).

Multiple sampling techniques are suggested when attempting to assess indoor biological contamination and selecting appropriate methods for sampling microbiological agents in indoor environments is crucial in order to link the human exposure and disease caused by microorganisms. It has been argued that the lack of standardized and definitive methods for biological sampling is a primary cause for the poorly understood relationship between biological agents exposure and health outcome (Niemeier *et al.*, 2006). Newer methods, such as quantitative polymerase chain reaction (qPCR) assays, allow the assessment of large groups of viable and non-viable microbial markers and are promising in quantifying DNA (Eduard & Halstensen, 2009; Jacobs, 2013).

Endotoxin assessment was performed through active air sampling and quantitative analysis was performed through LAL method, which is considered to have high sensitivity (Eduard & Halstensen, 2009; Sebastian & Larsson, 2003). Although this method can detect glucans, it measures bioactivity rather than absolute amounts and its reproducibility and specificity has been questioned (Sebastian & Larsson, 2003). Another disadvantage of this method is that it is semi-quantitative, highly variable, and can sometimes produce false negative results (Matković *et al.*, 2012).

In this study, outdoor endotoxins concentrations were not assessed. This was an important study limitation insofar as it was not possible to compare indoor concentrations with background levels, in order to investigate which source (indoor or outdoor) was most significant for the presence and proliferation of endotoxin in indoor air of primary schools.

A cross-sectional study, like the described investigation in twenty selected primary schools of Porto, provide only a snap shot of the actual exposure conditions and do not characterize the long-term exposure conditions (Carrer *et al.*, 2015). Notwithstanding, the culture methods used provide important qualitative information, although they have proven to be of limited use in population-based studies (Douwes *et al.*, 2003).

When comparing the results obtained in the present study with others in current literature, it should be also kept in mind that between different studies, indoor biological characterization is done with instruments, indicators, averaging times and analysis methods that may differ from each other (Madureira *et al.*, 2015b).

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7. CONCLUSIONS

This final chapter includes a systematization of the main findings, according to the research questions and specific objectives established in the subsection 2.2, and, lastly, future research proposals on this study subject are highlighted.

7.1. Main findings

Insight on the biological composition of primary schools indoor air is extremely important from a public health perspective since children spend a large time inside classrooms, with high occupancy densities, contributing considerably to the burden of diseases like asthma, allergies and respiratory symptoms, which are getting very common in early ages. The state of knowledge regarding biological parameters in indoor air of school buildings is still very limited and nonconsensual. The development of indoor biological characterization has progressed significantly in the last years, but more research is needed to establish more and reliable causal relationships between indoor exposure to biological agents and its health effects.

In the present study, mean indoor bacteria concentrations were above national limit values in all of the evaluated Porto primary schools, from 2 to 9 times higher. Regarding fungi concentrations, indoor levels were above the reference value in 75% of the schools and overall indoors levels registered a 3-fold increase comparing with outdoor values. These high bacteria and fungi concentrations can be caused by the combination of several factors: (i) high number of students per classroom; (ii) lack of ventilation caused by closed doors and windows, common practice in Portuguese schools in heating season; (iii) active behavioral pattern/activity level of children; (iv) type and preservation of the school building and classrooms particular characteristics. These biological concentrations can lead to an inadequate IAQ in classrooms full of young children and even more important can cause a negative impact on their health and well-being. Endotoxins concentrations were below the recommended value of 50 EU/m^3 in all primary schools evaluated in the present study. Similar results were found in current literature in indoor environments, although few studies have been conducted in school buildings and at airborne level. Nevertheless, studies reported higher endotoxins levels in schools environments rather than in homes indoor air, possible due to overcrowded classrooms. Harmonization and standardization in sampling and analysis procedures is crucial for the results comparison between studies.

No correlations were found between indoor and outdoor levels of bacteria and fungi concentrations, with higher levels found indoors. Overall I/O ratios of 9 and 3 for bacteria and fungi concentrations, respectively, demonstrated that indoor sources are the major influence on biological levels at schools. With only natural ventilation in 19 of the 20 schools targeted in this study, it was also stated that windows are kept mainly closed in this period, in order to keep classrooms warmer, enhancing indoor biological concentrations and preventing the renovation of indoor air with fresh air from outdoor.

Penicillium sp. and *Cladosporium sp.* were the prevalent fungi species found indoors and outdoors of primary schools. Both genera are considered common in indoor environments according with national legislation and several studies available in current literature. *Rhodotorula sp.*, Yeast and *Aspergillus fumigatus* were also considered predominant in this study, but at inferior percentages. Important to refer the known toxigenic properties of *Aspergillus fumigatus* and that caution should be addressed when this fungi species is found indoors above 12CFU/$m^{3,}$ according with national guidelines. Statistical significant differences were found between indoor and outdoor levels of both *Penicillium sp.* and *Cladosporium sp.*, as well as of *Rhodotorula sp.* and Yeast.

The present study results showed that bacteria and endotoxins concentrations were higher with higher levels of RH and CO_2, once again, possibly related with low ventilation rates and high number of occupants inside classrooms. Fungi concentrations did not fluctuate with T, RH or CO_2, but did positively relate with bacteria concentrations: higher values of fungi corresponded to higher levels of bacteria. Contrarily to what was expected, bacteria and endotoxins concentrations showed no relation between them. Regarding the predominant fungi species found indoors, results showed that *Cladosporium sp.* percentages were influenced by T, RH and CO_2, *Penicillium sp.* had higher values with higher CO_2 levels, and as T decreases, *Aspergillus fumigatus* and *Rhodotorula sp*. percentages rises.

Endotoxins were the studied biological parameter that showed to be more affected by school building and classrooms characteristics: “year of building refurbishment”, “wall type”, “roof leaking”, “ceiling surface”, “floor covering”, “number of occupants”, “density of occupation”, “number of space heaters”, “condensation on windows” and “windows opened during cleaning time” were the characteristics that showed influence on indoor levels of endotoxins, with statistical significance. Different fungi concentrations were found according the “ventilation system”, the “heating system” and the “type of glazing” existent in classrooms. Bacteria

concentrations did not relate with "number of occupants" or "density of occupation" but were also influenced by "ventilation system" and "heating system" like fungi; by "number of space heaters" and "windows opened during cleaning time" like endotoxins; and by other characteristics like "noticeable mold odor" and "windows opened before school time". No statistical differences were found between the biological parameters concentrations and "visible mold growth" and/or "visible damp spots" in classrooms. While results showed that *Cladosporium sp.* percentages were influenced by "number of space heaters", the type of classrooms' "ceiling surface" and the presence inside of "condensation on windows", *Penicillium sp.* percentages did not fluctuate according with any of the selected school building and/or classroom characteristic.

To improve IAQ of primary schools, regarding indoor air biological parameters, strategies of source control should be adopted as these are the most consistent and efficient for the prevention of adverse health consequences to children in schools, like the development and aggravation of asthma, allergies and respiratory symptoms. These source control measures go from proper design and construction of school buildings and selection of clean material, until the definition and enforcement of limits for maximum permitted occupation densities in classrooms, as well as definition of classes and breaks durations. Periodical monitoring of IAQ and health parameters in schools and control of excess moisture, dampness, and mold, are also other preventive measures extremely important. Ventilation practices like (i) opening the windows before the school day starts, during each classroom break and also after cleaning procedures; (ii) introduce tilting windows in classrooms and/or ventilation grills in windows frames; (iii) assuring ventilation rates of 24 m^3/(hour.person) in classrooms; and (iv) when natural ventilation is not sufficient to reach the desired referred ventilation rate, and when economically and technically viable, implement a mechanical system, preferably with intake of fresh air. Proper training of students, teachers and school staff who are responsible for the management, maintenance and cleaning of school buildings is also another crucial measure.

The present study results are intended to provide quantitative and qualitative information on indoor biological parameters in primary schools, which are known inducers of allergic and respiratory diseases, in a perspective of using the present data for assessing health effects of biological exposure and enable more knowledge on the subject. This study results also intend to allow the development of recommended limit values of exposure as well as the promotion and implementation of public health prevention programs. The articulation of recommendations that

aims to provide healthier indoor school environments is hopefully another important contribution of this thesis.

7.2. Future Research Studies

A set of different and multidisciplinary future research studies are exposed and suggested:

1. Assess indoor environment in a larger sample of primary schools, in an integrated IAQ audit, which should comprehend chemical (particulate matter, TVOC's, formaldehyde, carbon monoxide and carbon dioxide), physical (T and RH) and biological (bacteria, fungi, endotoxins) parameters, along with thermal comfort, noise and illuminance in these classrooms, in order to have a full comprehension of IAQ status in Portuguese schools. Important not to forget the extremely key characterization of school building and classrooms characteristics, as these may have a crucial influence on IAQ. Studying a larger sample of schools would also enable to have more insight and bring more comprehension on how the year of buildings refurbishment can influence endotoxins indoor concentrations. These IAQ studies should use standardized protocols and methodology in order to provide comparable data (Annesi-Maesano *et al.*, 2013);
2. In biological assessment, include the evaluation of allergens, as this is a well-known inducer of asthma and allergic reactions in children and adults, and also Gram-negative bacteria, in order to be able to investigate the presence of this biological agent and endotoxin indoors, as endotoxin is a constituent of the outer cell membrane of Gram-negative bacteria;
3. Analyze fungi genera/species that have visible growth on buildings and classrooms surfaces and compare the results with the fungi genera/species that are found in the air of the same areas;
4. Perform endotoxin assessment in rural and urban environments, with and without farming activities, in order to be representative, to study the differences stated in the available literature and investigate if a Portuguese study in this area has the same results of other European studies;
5. Extend this IAQ audit to the indoor environment of children's homes, to more fully understand children's total exposure, since school and home are the two settings where children spend the most part of their time;

6. Perform IAQ audits in the same classrooms of primary schools along all school year, in order to evaluate seasonal changes and concentrations fluctuations;
7. Calculate air exchange rates in an attempt to also develop studies in ventilation area, which should aim to improve knowledge on the connection between sources, indoor air concentrations and exposures, ventilation rates and ventilation systems and health outcomes (Carrer *et al.*, 2015);
8. Develop more and new studies related with the impact of IAQ pollutants exposure on children's health, including standardized questionnaires, medical visits and objective clinical tests, in order to enable a continuous study on the prevalence of allergy, asthma and respiratory problems in Portuguese children and allowing the establishment of unequivocal causal relationships. These studies will also help to develop more knowledge on the potential protective effect of microbial exposures on atopy and atopic diseases;
9. Apply more modern analysis methods, such as molecular approaches in microorganisms' analysis, for instance quantitative PCR, which allow the quantification of microbial species, genera or larger taxonomic groups by the detection and enumeration of specific DNA targets (Csobod *et al.*, 2014). It would also enable to study the diversity of *Aspergillus* species found in indoor environments to bring more knowledge on this specific fungi species distribution and presence indoors. the Studies where molecular methods were applied have improved knowledge on indoor microbial diversity (Douwes *et al.*, 2003; Eduard & Halstensen, 2009; Kelley & Gilbert, 2013; Rintala *et al.*, 2008) and therefore, they are sensitive and reproducible methods that allow the detection of a particular species in an indoor environment (Cabral, 2010). PCR techniques have been developed for the identification and quantitation of specific species of bacteria and fungi in the air (Douwes *et al.*, 2003). An example of a complex genus that has different allergenic potential according with different species is *Penicillium sp.* (Fischer & Dott, 2003), and should, therefore, be identified to species level. This is a perfect example from the present study results that had *Penicillium sp.* as main fungi genera in several classrooms, but without insight of which species was present inside the evaluated classrooms;
10. If intervention and/or improvement measures suggested in the sub-chapter 6.6 are implemented in the schools where the assessment took place, intervention studies should be carried out in order to investigate the effectiveness of these applied measures;

11. Finally, an interesting future research study would be the evaluation of biological effects resultant from the exposure to environmental pollutants through biomarkers analysis. With the development of molecular epidemiology, this method would allow the health risk evaluation caused by indoor air pollution (Sram *et al.*, 2013). In fact, genetic epidemiology may provide means to detect causal exposures and identify underlying mechanisms (Mendell *et al.*, 2011).

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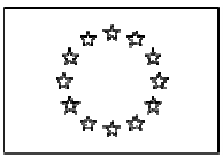
ANNEX I – SINPHONIE CHECKLIST

sinphonie

Schools Indoor Pollution and Health: Observatory Network in Europe

Checklists

Project funded by



European Commission

Health and Consumers Directorate-General

Instructions

The checklists presented next should be fulfilled by the research team of the Institution Partner of SINPHONIE Project. Some parts of the checklist can be completed by the research team in advance (either from documentation already existent about the building school* or by site visit), and some need to be completed with the help of a building manager or equivalent.

* Some buildings may have a book of maintenance, service and operation that will save time and effort. A list of documentation is proposed to be asked to the school that can be an important source of information:

List of Documentation	Notes
Building Energy performance certification	If exists may provide info for the rest of the section
As-built file, if as-built file does not exist, project for execution	If exists may provide info for the rest of the section
Book of operation, service and maintenance	If exists may provide info for the rest of the section
Complaints	
Report of the last regular inspection of the HVAC system	

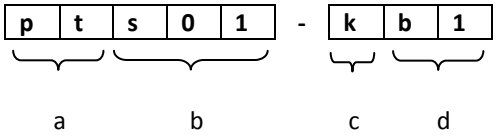
Concerning the questions, please try to obtain answers to all. If this is not possible, please write in the column "additional comments" that you could not reach information about that question.

You can tick all the options you think are applicable to the specific school. If you detect in the visit some important aspect that can help in the interpretation of the results, please write it in additional information section in the final of the checklist.

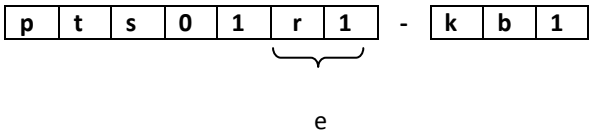
The observations during the measurements should not be recorded in this checklist form: a short questionnaire will be filled during the campaign days.

Checklist codes, example

Building checklist code



Room checklist code



Refers to

a	Country	pt	code for the country (for example pt for Portugal)
b	School	01	from 01 till maximum of schools studied
c	School 'level'	k	k for kindergarten or s for Primary School
d	Building	1	some schools could have more than 1 building; in this case if the classrooms to be studied are located in different buildings a check list has to be fulfilled for each building
e	Room	1	from 1 to 3

Concerning building location please check the category that best characterize the building surrounding area:

- Industrial: Product oriented establishments, such as manufacturing and utilities;
- Commercial: Service oriented establishments, such as retail establishments, restaurants and shopping centers;
- Residential: Area with absence of a dominating industrial or commercial influence.
- Suburban: Essentially an area that is close enough to a major urban center as to be affected by the urban area.

Building checklist code | | | | | | - | | | |

A. Building

Identification

School ☐

Kindergarten ☐

Total number of occupants: _____ Total Area: _____ m^2

Address: _____

_____

_____

GPS coordinates: _____

Contact person: _____

(phone) _____

(e-mail) _____

Building shape, orientation of the building and shading by nearby buildings:
(a sketch of the basic plan of the building and surrounding; an air photo, Google maps picture,...)

Investigator _____ Date _____

1. Outdoor Characterization

1.1. Geographical location

		Additional comments
Interior		
Seacoast		
North of the country		
South of the country		
East of the country		
West of the country		

1.2. Building location

		Additional comments
Industrial area		
Mixed industrial/residential area		
Commercial area		
Mixed commercial/residential area		
City centre, densely packed housing		
Town, with or without small gardens		
Suburban, with larger gardens		
Village in a rural area		
Rural area with no or few other homes nearby		

1.3. Nearby potential sources of outdoor air pollution that might influence the indoor environment

		Additional comments
None		
Car parking		
Attached garage		
Direct access from basement or roof car park		
Busy road (at least part of the day)		
Highway		
Power plant for the building		
Other power plant **(up to 1 km)**		
Gasoline dispensing facilities		
Industry **(up to 10 km)**		
Cooling towers		
Built on a landfill site		
Waste management site (tip or garbage dumpsters) **(up to 3 km)**		
Agricultural sources **(up to 3 km)**		
Other (specify) _____		

1.4. Nearby* noise sources outside the building that might influence the indoor environment

		Additional comments
None		
Car parking close to the building		
Busy road (at least part of the day)		
Highway		
Railway or station		
Subway		
Air traffic **(up to 3 km)**		
Sea, river or canal traffic		
Building, construction etc		
Sports events		
Other entertainment or leisure		
Factories or works		
Commercial premises		
Forestry, farming etc		
Community buildings (halls, churches, etc)		
Other (specify) _____		

*up to 1km

2. Construction Characterization

2.1. Year of construction _____

2.2. Was the school building built originally for being a school?

		Additional comments
No		
Yes		

2.3. Year of conversion and/or refurbishment _____

2.4. Number of storeys

		Additional comments
Occupied above ground		
Unoccupied above ground		
Occupied below ground		
Unoccupied below ground		

2.5. Total number of rooms

		Additional comments
Classrooms		
Dining rooms		
Gymnasiums/Sport hall		
Teachers' rooms/Offices		
Kitchen		
Library		
Bathrooms/Toilets		
Garages		
Other _____		

2.6. External walls construction (massive means made of solid bricks; lightweight means made of wood)

		Additional comments
Single wall		
Double wall		
Mixture of single and double		
Massive structure (high thermal inertia)		
Lightweight structure (low thermal inertia)		
Mixture of massive and lightweight		
Without insulation		
With insulation		
External insulation thickness (mm) _____		
All walls		
Some walls		
Cavity insulation thickness (mm) _____		
All walls		
Some walls		
Internal insulation thickness (mm) _____		
All walls		
Some walls		
Type of insulation		
Mineral wool		
Glass wool		
Fiber glass		
Polystyrene		
Polyurethane		
Cork		
Other (specify) _____		

2.7. Structure of the roof

		Additional comments
Flat roof		
Ridge roof		
Massive structure		
Lightweight structure		
Mixture of massive and lightweight		
Without insulation		
With insulation		
External insulation thickness (mm) _____		
Cavity insulation thickness (mm) _____		
Internal insulation thickness (mm) _____		
Type of insulation		
Mineral wool		
Glass wool		
Fiber glass		
Polystyrene		
Polyurethane		
Cork		
Other (specify) _____		

2.8. Type of foundation/ground floor

		Additional comments
Basement		
Slab on grade		
Crawl space		
Other (specify) _____		

2.9. Has the school got a yard?

		Additional comments
No		
Yes		

2.10. If in a radon-affected zone*, is there proper construction of foundation and ventilation (control of pressure difference), or other measures to control migration of radon?

		Additional comments
Not in a radon affected zone		
Radon zone		
Migration controlled		
Migration not controlled		
Unknown		

* Please contact National Authorities in natural radiation to inquire this

2.11. Who is the maintainer of the building?

		Additional comments
Municipality		
Foundation/Institution		
Church		
Private		
Other (specify) _____		

2.12. Has the building been certified by any program*?

		Additional comments
No		
Yes		
Which _____		

* Legislation, Regulation (Energy Performance, IAQ, Sustainability)...

3. Ventilation

3.1. Type of general ventilation strategy

		Additional comments
Natural		
Natural assisted (exhaustion)		
Mechanical		

If you answered "Natural" please jump to section 4

3.2. Type of mechanical ventilation

		Additional comments
Supply system only		
Both exhaust and supply		
Exhaust system only		
Toilets/other polluted rooms only		
Other rooms		
Permanent		
Non permanent		
Days per week		
Hours per day		

3.3. Air handling units (AHU)

		Additional comments
100% fresh air		
Recirculation _____% of fresh air		
With free cooling system		
Other (specify) _____		

3.4. Type of control

		Additional comments
Manual (on/off) – central		
Manual (on/off) – local		
Automatic		
CO2 controlled		
Other (specify) _____		

3.5. Outdoor air filter type

		Additional comments
Pre filter	_____	
Main filter	_____	

3.6. How often are the filters replaced

		Additional comments
No regular period		
Twice a year or often		
Once a year		
Once every two years		
Less often		
Date of last replacement _____		

3.7. How often are the filters cleaned

		Additional comments
No regular period		
Twice a year or often		
Once a year		
Once every two years		
Less often		
Date of last cleaning _____		

3.8. Heating systems

		Additional comments
No		
Yes		
In the whole building		
In some parts of the building		

3.9. Cooling systems

		Additional comments
No		
Yes		
In the whole building		
In some parts of the building		

3.10. Air duct material

		Additional comments
Asbestos cement		
PVC		
Galvanised steel		
Other (specify) _____		

3.11. Duct insulation

		Additional comments
None		
Internal		
Mineral fibre		
Other (specify) _____		
External		
Mineral fibre		
Other (specify) _____		

3.12. How often are the air ducts cleaned

		Additional comments
No regular period		
Twice a year or often		
Once a year		
Once every two years		
Less often		
Date of last cleaning _____		

4. Past Occurrences or Visible Problems

4.1. Water leakage or flooding in the last 12 months (if yes, specify the date)

		Additional comments
No		
Yes		
Roof	____/___/___	
Windows	____/___/___	
Façade	____/___/___	
Basement	____/___/___	
Water pipes	____/___/___	
Other (specify) _____	____/___/___	

4.2. Fire damage (if yes, specify the date)

		Additional comments
No		
Yes ____/___/___		
Extent of the fire damage		
Building wide		
Limited spaces		
Floors damaged		

4.3. Visible air leaks (cracks in the construction) in the structure?

		Additional comments
No		
Yes		

5. Building Use IAQ Sources

5.1. Use of pesticides in the last 12 months

	Indoors	Outdoors	Additional comments
Rats			
Mice			
Cockroaches			
Ants			
Other (specify) _____			

5.2. Is there a pesticide treatment plan for the building?

		Additional comments
No		
Yes (frequency) _____		

5.3. Is there any storage location inside for the pesticides?

		Additional comments
No		
Yes (where) _____		

5.4. Distance from the building to the outdoor trash storage _____ m

5.5. Is there a cleaning schedule for the communal parts of the building?

		Additional comments
No		
Yes		

5.6. Is there a kitchen inside the building?

		Additional comments
No		
Yes		
With air exhaustion (for ex. hood)		
Without air exhaustion		

5.7. Are there copy machines inside the building?

		Additional comments
No		
Yes		
How many _____		

5.8. Are there bathrooms with hot water showers inside the building?

		Additional comments
No		
Yes		

5.9. Special use spaces

		Additional comments
Laboratory		
Graphic arts		
Computer rooms		
Gymnasium		
Swimming Pool		
Sauna		
Mechanical workshops		
Training kitchen/ Cafeteria		
Trash storage or trash separation room		
'Smoking allowed' rooms		
Other (specify) _____		

6. Building Information for Modelling Purposes

6.1. Is there meteorological information for the outdoor of the building available?

		Additional comments
No		
Yes		
Temperature (acquisition time-step)		
Relative humidity (acquisition time-step)		
Wind speed (acquisition time-step)		
Wind direction (acquisition time-step)		

6.2. Is indoor temperature measured?

		Additional comments
No		
Yes		
Acquisition time-step		

6.3. Is indoor relative humidity measured?

		Additional comments
No		
Yes		
Acquisition time-step		

6.4. Are there traffic counting (and car fleet characterization) available for the main roads nearby?

		Additional comments
No		
Yes		

6.5. Is the buildings volumetry in a small domain around the building available? (GIS file with the buildings 3D coordinates)

		Additional comments
No		
Yes		

6.6. Are there any point sources (industries) in the nearby* of the building?

		Additional comments
No		
Yes (distance) _____		

* The nearby can vary from 1 km to a few km radius, depending on the definition of the case study and the potential impact of dominating winds

6.7. Are emissions for those point sources available?

		Additional comments
No		
Yes (distance) _____		

Classroom checklist code | | | | | | | | - | | | |

B. Classroom

Identification

School ☐

Kindergarten ☐

Location of the classroom in the building:
(a sketch of the basic plan, per floor, with location of the windows, board, desks, trash storage...)

Investigator _____ Date _____

1. Indoor Characterization

1.1. Storey number _____

1.2. Floor area _____ m^2

1.3. Ceiling height _____ m

1.4. Windows area _____ m^2

1.5. Type of classroom

		Additional comments
Normal		
Special use (specify) _____		

1.6. Occupation

		Additional comments
Same class		
Several classes		

1.7. Number of students* (nominal) _____

*If not constant please consider the predominant occupancy

1.8. Days per week the classroom is occupied _____

1.9. Hours per day the classroom is occupied

		Additional comments
Monday		
Tuesday		
Wednesday		
Thursday		
Friday		
Saturday		

1.10. Type of lighting

		Additional comments
Natural		
Artificial		
Mixture		

1.11. Glass % in the different façades

		Additional comments
North		
South		
East		
West		

1.12. Windows frames

		Additional comments
Metal		
Wood		
PVC		
Aluminium		
Other (specify) _____		

1.13. Type of glazing

		Additional comments
Single glazing		
Double glazing		
Double clear glazing with filling (Argon or other)		
Double clear glazing with coating		
Double glazing with tinted internal pane		
Triple glazing		
Other (specify) _____		

1.14. Solar shading devices

		Additional comments
None		
South side only		
Other façades _____		
External		
Internal		

1.15. Solar shading devices hamper the use of windows or decrease the ventilation capacity?

		Additional comments
No		
Yes		

1.16. Control of the shading devices

		Additional comments
No control (fixed)		
Individual		
Central down, individual up		
Automatic		
Other (specify) _____		

1.17. Are the materials used indoors low emitting materials? (classified by any recognized labelling system, the identification of the scheme should be done: GEV, AgBB...)

		Additional comments
Adhesives & sealants		
Paints & coatings		
Ceiling and wall systems		
Flooring systems		
Composite wood & agrifiber products		
Furniture & furnishings		
Other (specify) _____		

1.18. Presence of asbestos

		Additional comments
No		
Yes, exposed		
Yes, but sealed		

1.19. Presence of lead elements

		Additional comments
No		
Yes		
Water pipes		
Paint		
Other (specify) _____		
Not known		

1.20. Is there a suspended ceiling?

		Additional comments
No		
Yes		

1.21. Main ceiling surface

		Additional comments
Concrete		
Paint		
Wallpaper		
Synthetic material		
Mineral fibre tiles		
Wood fibre tiles; cork tiles		
Wood		
Gypsum/plaster		
Other (specify) _____		

1.22. Main type of wall covering

		Additional comments
Concrete		
Water based paint		
Solvent based paint		
Wallpaper		
Porous fabrics including textiles		
Stone/ceramic tiles		
Wood/cork		
Gypsum/plaster		
Other (specify) _____		

1.23. Main type of floor covering

		Additional comments
Concrete		
Carpet		
Synthetic smooth (linoleum, vinyl, ...)		
Laminate parquetry		
Stone/ceramic tiles		
Wood/cork		
Other (specify) _____		

1.24. Modifications in the last 12 months (if yes, specify the date)

		Additional comments
Floor structure	____/___/___	
Insulation	____/___/___	
Walls	____/___/___	
Ceiling/roof	____/___/___	
Heating system	____/___/___	
Ventilation system	____/___/___	
Windows	____/___/___	
New furniture	____/___/___	
Other (specify) _____	____/___/___	

2. Visible Problems

2.1. Visible mould growth in the room

		Additional comments
No		
Yes		
Where _____		
Extent _____		

2.2. Other damp/mould symptoms

	No	Yes	Additional comments
Noticeable mould odour			
Visible damp spots on walls, ceiling or floor			
Bubbles or yellow discoloration of plastic floors			
Blackened wood floor			

2.3. Tendency for formation of condensation on windows

		Additional comments
No		
Yes		
Inside		
On the frame		

3. Heating Characterization

3.1. Heating system

		Additional comments
Heating only		
Heating + domestic hot water		

3.2. Energy used for heating

		Additional comments
Electricity		
Joule effect (electric resistance)		
Heat pump		
Gas		
Natural		
Butane/Propane		
Oil		
Solid fuel		
Wood		
Coal		
Other (specify) _____		

3.3. Heating terminal units

		Additional comments
Hot water radiators or convectors		
Electrical radiators or convectors		
Heating floor		
Warm air flow		
Fireplaces		
Open		
Closed		
Other (specify) _____		

3.4. Are heaters located below windows to prevent draught in winter?

		Additional comments
No		
Yes		

3.5. Temperature set point and deadband range in winter

		Additional comments
Not controlled by the system		
Set point _____ºC		
Range min _____ºC max _____ºC		

4. Natural Ventilation

4.1. How is the classroom ventilated

		Additional comments
Openable windows		
Other natural ventilation (e.g. passive stack)		
Hybrid/mixed model (natural + mechanical)		

4.2. Can the windows be open?

		Additional comments
No		
Yes		
All		
Some (estimate % of openable windows)		
But occupants are not allowed to open them		

4.3. Number of windows usually open

		Additional comments
Heating season		
Cooling season		

4.4. Usually, when are the windows open?

		Additional comments
Before school time		
During breaks		
During teaching hours		
After school time		
During night		
Other (specify) _____		

4.5. Location of grids (passive ventilation)

		Additional comments
Ceiling		
Walls		
Windows		
Other (specify) _____		

4.6. Are the grids open?

		Additional comments
Always		
Often		
Sometimes		
Never		

4.7. In naturally ventilated, or exhaust only ventilated buildings, are there air transfer openings between rooms?

		Additional comments
No		
Yes		

5. Mechanical Ventilation

5.1. Type of control

		Additional comments
Manual (on/off) – central		
Manual (on/off) – local		
Automatic		
CO2 controlled		
Other (specify) _____		

5.2. Position of ventilation system intake

		Additional comments
Roof		
Façade		
Ground		
Other (specify) _____		

5.3. Height of ventilation system intake above ground level _____ m

5.4. Shortest distance of system intake from exhaust outlets

		Additional comments
Vertical	_____ m	
Horizontal	_____ m	

5.5. Shortest distance of system intake from cooling towers

		Additional comments
Vertical	_____ m	
Horizontal	_____ m	

5.6. Other potential sources close to the system intake

	Distance (m)	Additional comments
Car parking		
Garage		
Busy road		
Highway		
Power plant for the building		
Other power plant		
Gasoline dispensing facilities		
Other (specify) _____		

5.7. Outdoor design flow rate

_____ m^3/h _____ ach^{-1} _____ $m^3/(h.person)$

5.8. Designed air distribution principle

		Additional comments
Mixing		
Displacement		
Other (specify) _____		

5.9. Location of air supply devices

		Additional comments
None		
Floor		
Windowsill		
Ceiling		
High on wall		
Low on wall		
Other (specify) _____		

5.10. Location of air exhaust grids

		Additional comments
None		
High		
Low		

5.11. How often are the supply air devices cleaned

		Additional comments
No regular period		
Twice a year or often		
Once a year		
Once every two years		
Less often		
Date of last cleaning _____		

5.12. How often are the exhaust air devices cleaned

		Additional comments
No regular period		
Twice a year or often		
Once a year		
Once every two years		
Less often		
Date of last cleaning _____		

6. Classroom Use IAQ Sources

6.1. Board (tick the two most used)

		Additional comments
Black board with chalk		
White board with markers		
Electronic interactive board		
Flip over chart		
Other (specify) _____		

6.2. Electronic equipment (specify the number)

		Additional comments
Audiotape		
Computers/printers/photocopiers		
Data/video projector/TV/Video conference		
Slide projector		
Servers		
Other (specify) _____		

6.3. Other apparatus (specify the number)

		Additional comments
Air cleaners (specify type) _____		
Space heaters		
Humidifiers		
Dehumidifiers		

6.4. Furniture materials

		Additional comments
Wood		
Wood veneer		
Plywood		
Textiles		
Metal		
Plastic laminate or composite		
Other (specify) _____		

6.5. Are there any curtains?

		Additional comments
No		
Yes (area) _____		
Natural textile		
Synthetic textile		
Other (specify) _____		

6.6. Are there any rugs?

		Additional comments
No		
Yes (area) _____		
Natural textile		
Synthetic textile		
Other (specify) _____		

6.7. Are there any cushions?

		Additional comments
No		
Yes (area) _____		
Natural textile		
Synthetic textile		
Other (specify) _____		

6.8. Closet with medicines

		Additional comments
No		
Yes		

6.9. Closet or shelves with gouaches, inks, etc. for graphic arts

		Additional comments
No		
Yes		

6.10. Are there any special precautions when they are used?

		Additional comments
No		
Yes		
Windows are open		
Used under a hood		

6.11. Air fresheners

		Additional comments
No		
Yes		
Permanent (passive or electric plugged)		
Occasionally (spray or other)		
How often used? _____		

6.12. Is there a sink in the room?

		Additional comments
No		
Yes		

6.13. Animals/Pets

		Additional comments
No		
Yes, stuffed		
Yes, live		
Fish/Turtle (aquariums)		
Birds		
Rodents		
Other (specify) _____		

6.14. Number of plants in pots _____

6.15. Cleaning schedule

		Additional comments
Early in the morning or before school time		
During breaks between the classes		
In the afternoon or after school time		
Other (specify) _____		

6.16. Are the windows open during cleaning of the classroom?

		Additional comments
No		
Yes		

6.17. Cleaning activities frequency

	Frequency*						Date of last cleaning
	a	b	c	d	e	f	
Trash pins emptied							
Floors/carpets swept							
Floors/carpets vacuumed							
Smooth floors washed							
Smooth floors waxed							
Smooth floors polished							
Walls dry wiped/vacuumed							
Walls washed							
Ceilings dry wiped/vacuumed							
Ceilings washed							
Surfaces dusted							
Surfaces polished							
Surfaces cleaned							
Curtains washed							
Windows washed							
Other items dusted (e.g. doors)							
Other items polished							

* a) daily; b)twice a week; c)once a week; d) once a month; e) once a year; f) never

6.18. Deep clean* of the floor

		Additional comments
How often		
No regular period		
Once every three months		
Once every six months		
Once a year		
Less often		
Date of last cleaning _____		

* deep clean - activity different from the everyday cleaning, usually a more detailed one, that can include scrubbing or the use of specific cleaning products as for example disinfectants.

6.19. Type of consumer products used

	Spray	Liquid	Additional comments
For **floor** cleaning or conservation			
Bleach or detergent with bleach			
Detergent without bleach			
Polish			
Other category relevant _____			
For **wall** cleaning or conservation			
Bleach or detergent with bleach			
Detergent without bleach			
Polish			
Other category relevant _____			
For **windows** cleaning			
Detergent with ammonia			
Detergent without ammonia			
Other category relevant _____			
For **furniture** cleaning or conservation			
Detergent			
Polish			
Other category relevant _____			

7. Classroom Additional Useful Information