Microbial characterisation of bioaerosols from indoor environments using molecular techniques

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Author's declaration

This thesis has been written by myself and has not been submitted to any previous application for any degree. Unless otherwise stated, the work in this thesis has been carried out by myself. This thesis is written in accordance with the regulations for the degree of Doctor of Philosophy at the University of Essex.

Abstract

Nowadays, the quality of air in the indoor environment is of increasing concern as it is estimated that the majority of people in developed countries spend about 85% of their time in various indoor micro-environments. The biological fraction of airborne particulate matter has become of increasing interest owing to the potential allergenic and pathogenic nature of airborne micro-organisms. Biological aerosols are ubiquitous and abundant in the air we breathe in the built environment and despite the recognition of the importance of bioaerosol exposure on human health, relatively little is known about the microbial agents in indoor air and there are still critical gaps in our understanding of the airborne microbial communities. Molecular biology methods demonstrate a great potential for improving our comprehension of the significance of the indoor aerosol microbial load in the context of human exposure, providing in-depth characterisation of microbial communities. However, the number of bioaerosol studies which have applied culture-independent techniques is still limited and the data on the airborne microbial particles in indoor environments remain scant and need to be further examined. The aim of the present study is to investigate the microbial abundance and diversity of bioaerosols in various different types of indoor environments using molecular techniques. Several research questions are being addressed by application of high throughput next generation sequencing technology, providing insights into the aerosol microbiome in micro-environments where people spend considerable amount of their time on a daily basis, including educational, residential and transportation settings. The results of this project provide novel information on a previously largely unexplored microbiome which could improve and expand our comprehension of the specific characteristics of bioaerosols in the indoor environment. Moreover, the data generated by this study could make a significant contribution to improving air quality by facilitating the development of methods for bioaerosol exposure monitoring.

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Chapter 1 Bioaerosols in the indoor environment

1.1 Indoor air quality and aerosols

Nowadays, there is a growing public awareness regarding the risk associated with poor indoor air quality (IAQ) due to an increasing amount of evidence revealing the significant impact of the quality of the air in the built environment on occupational and public health (Colbeck & Nasir, 2010; Sundell, 2004; Jones, 1999). The recognition of the importance of the indoor environment due to its association with humans, has led to a rapid increase in the number of studies on air pollution within the built environment (Fig.1.1).

The term 'built environment' refers to 'a human-made space in which people live, work and recreate on a day-to-day basis' (Roof & Oleru, 2008) and includes various types of indoor microenvironments (residential, occupational, public transportation and recreational). It is estimated that the majority of people in developed countries spend about 85 - 90% of their time in the built environment (residences, offices, schools, transport systems, health care facilities, public buildings), which underlies the importance of assessing the risks associated with air quality in enclosed spaces (Schweizer et al., 2007; Brasche & Bischof, 2005; Klepeis et al., 2001; Jantunen et al., 1998).

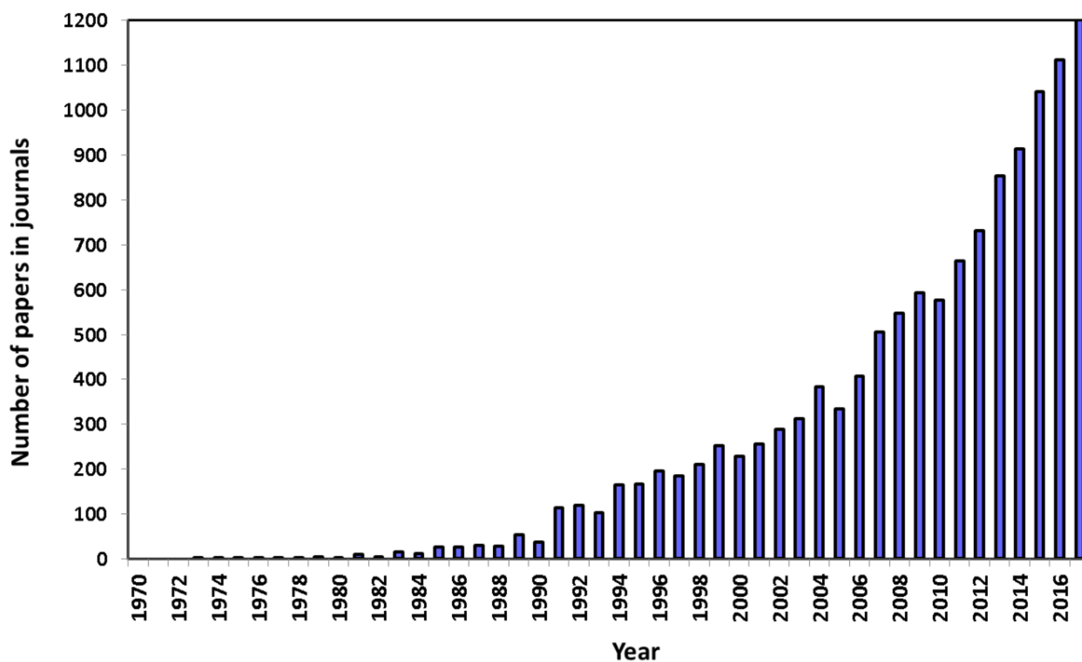


Figure 1.1 Number of papers published per year since 1970 with 'indoor air' as the topic, based on figure by Colbeck & Nasir (2010). Time series updated by I. Colbeck.

Even though the public perceives the risks from outdoor air pollution as being significantly higher than those from indoor contamination, people are far more exposed to pollution indoors than outdoors (Jones, 1999). The increased insulation of buildings combined with poor ventilation has created environments with elevated exposures to indoor air pollutants. Many studies have shown that concentrations of pollutants in the indoor environment are often higher than those typically encountered outdoors, while the estimated risk to human health from indoor sources is about 1000 times higher than the one resulting from exposure to outdoor pollutants (Lazaridis, 2008; Jones, 1999).

Human exposure to a wide range of contaminants (particulate matter, gases such as ozone, nitrogen dioxide, carbon monoxide, sulphur dioxide, formaldehyde, volatile organic compounds, radon etc.) of both indoor and outdoor origin has been linked to adverse health effects. During the 1990s airborne particulate matter (PM) was identified as one of the most important air pollutants (Schwela & Kotzias, 2005). PM is mostly not a single compound like a gas but rather a mixture of compounds with different origins, both inorganic and biological, as well as different chemical and physical properties (Putaud et al., 2004). A large number of studies have investigated the risks that PM poses both to human health and ecosystem functions, but most of these studies have typically focused on the non-biological fraction of suspended particles (e.g. Neuberger et al., 2004; Pope et al., 1999; 1995).

In recent years, the biological fraction of PM (bioaerosols) has become of increasing interest owing to the potential toxigenic, allergenic and infectious nature of airborne micro-organisms (Douwes et al., 2003, Fung & Hughson, 2003; Spengler et al., 1994; Burge, 1990). Even though the impacts of biological aerosol exposure on human health and wellbeing are considerable, our knowledge of the microbiological quality in indoor air lags behind that of total particulate matter and its chemical constituents and relatively little is known about the composition and abundance of airborne micro-organisms (Peccia & Hernandez, 2006). Therefore, it is crucial that reliable and efficient monitoring methods for detecting, identifying and quantifying airborne biological particles be developed.

The aim of this review is to summarise and discuss the state of knowledge in the bioaerosol microbiome research of the indoor environment. Investigation includes commonly occupied microenvironments with typically low encountered bioaerosol levels. Industrial occupational settings associated with high contamination levels and exposure potential (e.g. livestock or waste management workplaces) are not included.

1.2 Bioaerosols and their characteristics

1.2.1The biological fraction of aerosols

Aerosols are liquid or solid particles suspended in a gaseous medium with size ranges from 0.001 to 100 μm. Aerosols represent a complex and dynamic mixture of organic and inorganic substances with potential toxic, carcinogenic, inflammatory, allergenic and other adverse properties (Seinfeld & Pandis, 2006; Hinds, 1999).

The biological fraction of aerosols present in the environment is commonly termed 'bioaerosols'. More specifically, bioaerosols are defined as aerosol particles of biological origin including viable and nonviable micro-organisms (e.g. bacteria, fungi, algae, yeasts, archaea, viruses), as well as their aggregates and fragments, and other types of biomass, including microbial derivatives (bacterial endotoxins, fungal mycotoxins, microbial volatile organic compounds - MVOCs, peptidoglycans, β(1→3)-glucans), pollen and fragments from animals and plants (dander, plant fibres, insect debris) (Peccia & Hernandez, 2006; Douwes et al., 2003; Matthias-Maser & Jaenicke, 1995; Cox & Wathes, 1995; ACGIH, 1989).

Bioaerosols are present in both indoor and outdoor air and they play a vital role in the earth system atmospheric processes (Fröhlich-Nowoisky et al., 2016; Després et al., 2012). Particles of biological origin form a significant portion of atmospheric aerosols, accounting for approximately 25 – 30% of aerosolized matter suspended over urban and rural land surfaces (Jones & Harrison, 2004; Matthais-Maser et al., 2000; Matthias-Maser & Jaenicke, 1994) and up to 80% in pristine rainforest air (Fröhlich-Nowoisky et al., 2016), while sometimes reaching close to 50% numerically of all aerosol particles (Mandal & Brandl, 2011). Estimates of the biological content in atmospheric $PM_{2.5}$ (particulate matter less than 2.5 μm in aerodynamic diameter) range from 4% to 11% of the total biomass (Womiloju et al., 2003). The significance of bioaerosol concentrations in the atmosphere is being reinforced by the fact that the biogenic fraction is much greater as a percentage of the organic carbon fraction of particulate matter (Boreson et al., 2004). Furthermore, according to Srikanth et al. (2008), bioaerosols contribute about 5 to 34% of indoor air pollution.

1.2.2 Microbial airborne particles

The majority of work investigating bioaerosols has focused on the microbial fraction and mainly on the airborne bacterial and fungal micro-organisms. Unlike aerosols being characterized by mass concentration (μg/m^3), a reference of micro-organisms as mass is not common. The number of micro-organisms per air volume is normally given as number

concentration in units such as CFU/m^3 (colony forming units) or cells/m^3, bacterial or fungal genome equivalents/m^3 or copies of specific genes/m^3 (which in the case of multi-copy genes does not correspond to the number of micro-organisms).

Bacteria are single-celled prokaryotic organisms of various shapes that are present in air environments as either vegetative cells or endospores. They tend to grow in colonies in their natural habitats and when aerosolized, they are often aggregated as clusters or chains or attached to other materials. Bacteria may be carried by other aerosol particles, such as water droplet residues, plant materials, or the skin fragments of animals. Some examples of common bacterial taxa detected in the built environment are *Streptococcus, Staphylococcus, Corynebacterium, Propionibacterium, Enterobacteriaceae, Bacillus, Pseudomonas, Acinetobacter* etc. (Adams et al., 2015).

Fungi, also called molds, grow in the form of multicellular filaments, called hyphae, (apart from yeasts, which are single-celled fungi and do not produce true mycelium) on surfaces when sufficient moisture is present. Most species multiply by the characteristic formation of spores. Fungi are disseminated through aerial distribution by the release of spores that are well adapted to various air environments. Fungal spores may be aerosolized as single spores but are more often agglomerated. Moreover, some species have mechanisms for an active release of spores into the air (Grinshpun, 2010). Some common fungal genera found in indoor air are *Aspergillus, Cladosporium, Alternaria, Penicillium, Stachybotrys, Fusarium, Malassezia* (Adan & Samson, 2011).

Archaea comprise a group of prokaryotic single-celled microorganisms and even though they have been associated with extreme environments, their presence has been detected in the indoor environment (Pakpour et al., 2016). Compared to bacteria and fungi, however, very little is known about their propagation mechanisms in the air (Fröhlich-Nowoisky et al., 2014).

Viruses are obligate intracellular parasites. They can be transmitted in air through large (respiratory) droplets that travel only short distances or through droplet nuclei (small-particle residue < 5 μm) of evaporated droplets that can remain suspended in the air for significant periods of time. Reports on the presence of viral aerosols in indoor air are scarce but some viral pathogens which can be easily transmitted in indoor environments are influenza viruses, rhinoviruses, coronaviruses, adenoviruses, noroviruses etc. (Ijaz et al., 2016).

Few more examples of microbial airborne particles typically encountered indoors are given in section 1.2.4 (Table 1.1). Also, some information about microbial derivatives and non-microbial bioaerosols (pollen fragments, plant debris, animal dander, and mite excreta) can be found in section 1.3.

1.2.3 Bioaerosol size

Biological aerosols are ubiquitous and abundant in the atmosphere, covering a wide size range. Most studies have focused on the total concentration of bioaerosols. However, particle size plays a critical role in the transport and depositions mechanisms of aerosols. Microbial airborne particles are being governed by the same mechanisms as non-biological particles with larger bioaerosol particles typically settling out of the air much more quickly compared to the smaller ones, that tend to remain airborne for extended periods of time. Moreover, particle size determines personal exposure and deposition in the human respiratory tract with the subsequent human health impacts (Nazaroff, 2004; Thatcher & Layton, 1995).

Also the size affects the fate and behaviour of biological particles in relation with the presence of abiotic particles. Various interactions could occur between the two types of particles, of which the most likely would be coagulation and attachment of smaller particles to the larger one. For example, non-biological particles can serve as carriers for fungal allergen molecules (Morawska & Salthammer, 2006).

Owing to the irregular shapes that particles have and the difficulty to measure their actual geometric diameters, the size of aerosols is often characterized by the aerodynamic diameter (μm). Aerodynamic diameter is an expression of aerodynamic behaviour of an irregularly shaped particle in terms of the diameter of an idealized particle and is defined as the diameter of a spherical particle with unit density (1 g/cm^3) and the same settling velocity in air of standard pressure and temperature as the irregular particle (Hinds, 1999). Generally, the rate of deposition onto indoor surfaces, as well as somewhere in the respiratory tract, is smaller for particles with diameters in the range 0.1–1 μm as compared with particles with diameters in the range 1–10 μm.

The size scale for whole microbial agents ranges from nanometres up to ~100 μm, with viruses being the smallest potentially living particles, and fungi exhibiting the biggest sizes (Fig.1.2). As examples we mention that the aerodynamic diameter of influenza A virus is ~0.1 μm, the size of a *Staphylococcus Aureus* (bacterial) cell is ~ 1 μm, the aerodynamic diameter of an

Aspergillus fumigatus spore has been measured to be ~ 2.2 µm (Nazaroff, 2016), while the size of an *Alternaria alternate* spore is ~19 µm (McCartney et al., 1993). A considerable amount of fungal material, such as allergens, glucan, and mycotoxins, can also exist in smaller fragments, which are below 1 µm in size (Reponen et al., 2007). Pollen, algae, protozoa and dander are several tens to hundreds of micrometers in diameter (Reponen et al., 2011). Due to the significant differences in size between types of bioaerosols, orders of magnitude differences in settling times and transport distances are expected. Particle sizes for some common bioaerosols are also presented in an exemplary illustration (Fig. 1.3) by Fröhlich-Nowoisky et al. (2016).

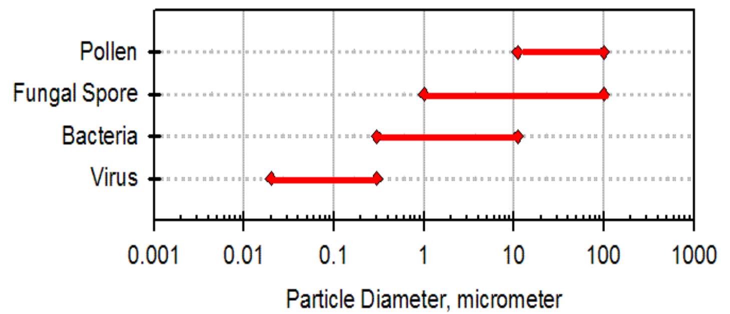


Figure 1.2 Individual particle sizes for common bioaerosols (http://aerosol.ees.ufl.edu/Bioaerosol/Section02.html).

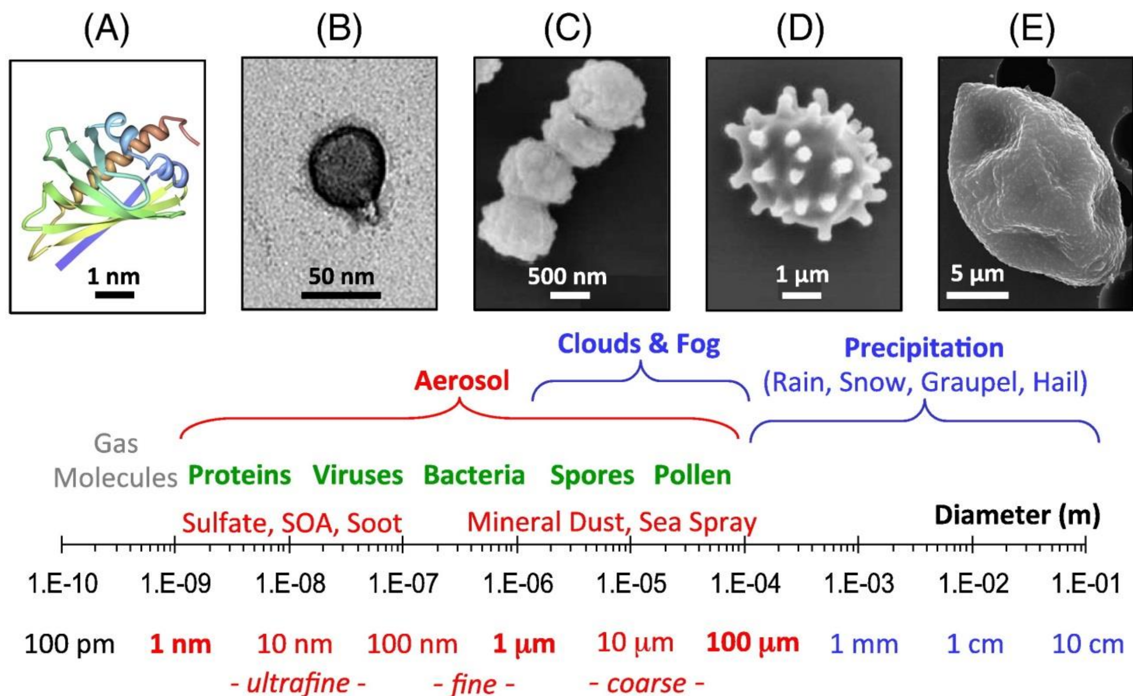


Figure 1.3 Characteristic size ranges of atmospheric particles and bioaerosols: (A) protein (model simulation), (B) virus, (C) bacteria, (D) fungal spore, and (E) pollen grain. Image A is a model simulation of BetV1. Images (B–E) are scanning electron micrographs of representative particles from each of the bioaerosol categories listed. Reprinted from Fröhlich-Nowoisky et al. (2016).

1.2.4 Bioaerosol sources

Built environments are complex ecosystems that contain numerous organisms including trillions of micro-organisms, covering all surfaces, present in accumulated dust and any internal parts of the building structure or its operating systems (inside walls, air-conditioning units, ducts, etc.), as well as covering the occupants and any other living organisms (plants, pets, insects) (Jones, 1999). Moreover, the built environment contains many potential novel habitats for microbial life that have chemical and physical properties not found in the natural world (Hewitt et al., 2012) and indoor environments have been found to harbour microbial taxa not commonly found outdoors (Amend et al., 2010; Tringe et al., 2008). Microbial airborne particles are part of the microbiome of the indoor environment and a result of the interaction with it, as biological particles suspended in indoor air may originate from the built environment microbiome and may, as well, contribute to it by depositing biological material on it.

It is general accepted that aerosol concentrations in the indoor environment are associated with both indoor and outdoor sources. According to Prussin & Marr (2015) the indoor sources of airborne microorganisms in the built environment can be divided in seven major categories: humans, pets, plants, plumbing systems (showers, sinks and toilets), heating, ventilation and air-conditiong (HVAC) systems when improperly maintained, water-damaged or dampness-related moldy materials (e.g. ceiling tiles, walls etc.) and dust resuspension that can occur from any kind of surface (e.g. furnishing, fabrics, mattresses, floor, tiles, carpeting, etc.) (Fig. 1.4). Sources of indoor bioaerosols are often located outdoors (vegetation, soil, water reservoirs etc.) and particles are transferred to the inside via air exchange-induced supply from outdoor air through (a) designed openings, such as windows and doors (natural ventilation), (b) leaks and cracks in the building envelope (infiltration) and (c) the potential presence of a fans induced air distribution system (mechanical ventilation) (Nazaroff, 2016). Bioaerosols can also be transported indoors by the occupants, pets, insects and other animals that can be potential passive transport vectors of microbes from outdoors (Jones, 1999). Examples of reported source-identified emitted microbial agents are shown in Table 1.1. Moreover, sources of microbial particles in indoor air can act both as direct emitting sources and indirect sources from dust perturbations. Once microorganisms, that were previously airborne, deposit on a surface, it cannot be assumed that they have been permanently removed from the air, as there are many opportunities for resuspension and they can re-enter the air.

The significance of both indoor and outdoor sources depends on a number of variables, e.g., air-exchange rate, outdoor air pollution, type of indoor activities, aerodynamic diameter of particles emitted, etc. (Wallace, 1996; Thatcher & Layton, 1995; Owen et al., 1992). Also sources are strongly related to the climate zone and geographic location of the building, the type of environment (urban or rural), as well as to socioeconomic factors (developed or developing world), the lifestyle and hygiene of occupants (Chase et al., 2016; Barberan et al., 2015; Nasir et al., 2013; Amend et al., 2010).

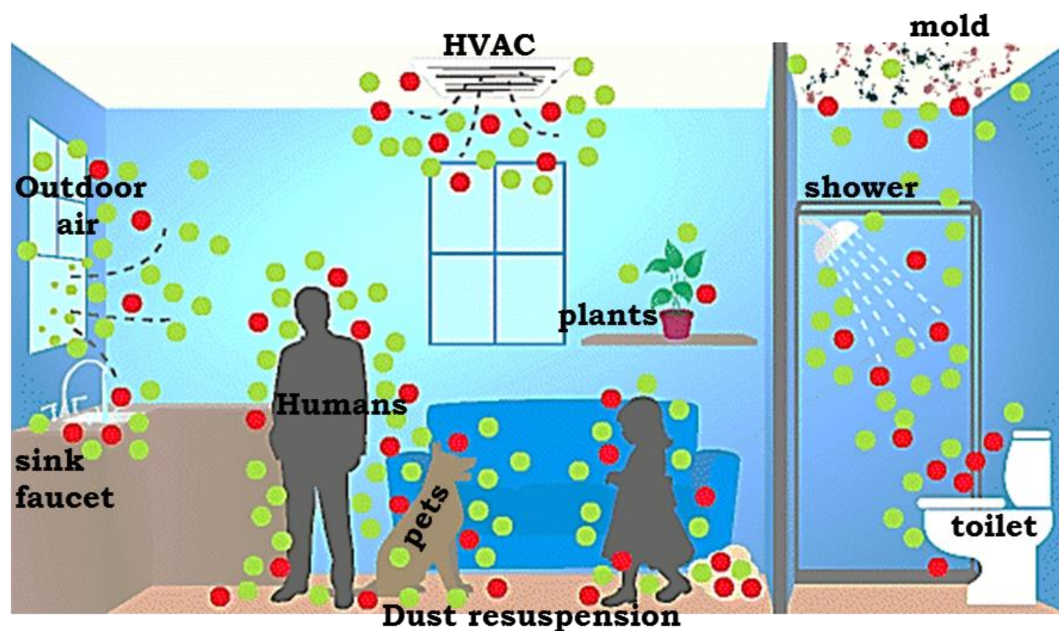


Figure 1.4 Sources of microbial bioaerosols in the built environment. The green and red dots represent microorganisms that may be beneficial or detrimental to human health, respectively. [Adapted from Prussin & Marr (2015)]

One of the most important sources of airborne microbial particles in the indoor environment is the presence of human beings (Adams et al., 2015b; Meadow et al., 2014; Hospodsky et al., 2012; Qian et al., 2012; Mandal & Brandl, 2011). Humans can act as primary sources through direct emission of human microbiota or as secondary sources through resuspension. Humans convey a large fraction of the microbes by emitting oral and respiratory fluid via coughing, sneezing, talking and breathing, and by shedding them from their bodies (skin-associated microbiota). It is estimated that the average human sheds approximately 1.5 million skin cells an hour, carrying with them approximately 15 million bacterial cells (Kelley & Gilbert, 2013). Also, material resuspended from surfaces (indoor dust) as a result of occupants' movements and activities (e.g. walking) are an important source of indoor airborne particles. Even the clothes of the occupants could act as a secondary source of particles that were previously deposited on the fabric (Adams et al., 2015b; McDonagh et al., 2014).

Last, even though occupational-related bioaerosol exposure is not in the scope of this review, it has to be added that numerous sources that can lead to generation of high levels of aerosolised biological material, including pathogenic reagents, exist in certain industrial workplaces associated mainly with food production (e.g. dairy farms, crops storage areas, swine barns, poultry etc.) and waste management (e.g. wastewater treatment plants, compost units etc.) (Lecours et al., 2012; Gilbert & Duchaine, 2009).

Table 1.1 Categories of indoor bioaerosol sources and emission of associated microbial taxa**,** as reported by Prussin & Marr (2015).

Source	Emitted airborne micro-organisms
Humans	
skin shedding	*Micrococcus* spp., *Staphylococcus* spp., *Acinetobacter* spp., Corynebacterium spp., *Bacillus* spp., *Streptomyces* spp., *Rhodotorula*, *Candida*, *Cryptococcus*, *Malassezia* and *Trichosporon*
nasopharynx & oropharynx tracts	*Staphylococcaceae* spp., *Propionibacteriaceae* spp., *Corynebacteriaceae* spp., *Streptococcaceae* spp., *Veillonellaceae* spp., *Prevotellaceae* spp., *Fusobacteriaceae* spp. and *Neisseriaceae* spp.
coughing/ sneezing/ breathing	influenza A virus *Mycobacterium tuberculosis*
Pets	
dogs	*Porphyromonas* spp., *Moraxella* spp., *Bacteroides* spp., *Arthrobacter* spp., *Blautia* spp. and *Neisseria* spp.
cats	*Prevotella* spp., *Porphyromonas* spp., *Jeotgalicoccus* spp., *Sporosarcina* spp., *Moraxella* spp. and *Bifidobacterium* spp.
Plants	*Cladosporium*, *Penicillium*, *Alternaria*, *Epicoccum* and *Pithomyces*
Plumbing systems	
toilet flushing	*Shigella* spp, *Salmonella* spp., *Escherichia coli, Serratia* spp., norovirus, human adenovirus, MS-2 bacteriophage and Torque teno virus
showers & sink faucets	*Legionella, Mycobacterium mucogenicum*, *Pseudomonas aeruginosa, Fusarium* spp. *Aspergillus* spp., *Penicillium* spp., *Paecilomyces variotii*, *Alternaria alternata*, *Cladosporium* spp. and Acremonium spp.
HVAC systems	*Penicillium* spp., *Legionella pneumophila*
Water damaged materials	*Aspergillus*, *Penicillium* and *Stachybotrys atra*
Resuspension of settled dust	*Staphylococcus*, *Corynebacterium*, *Lactococcus*, *Firmicutes*, and *Actinobacteria*. *Cladosporium* spp., *Penicillium* spp., *Aspergillus* spp., *Candida* spp., *Saccharomyces* spp., wood-degrading fungi
Outdoor air (environment)	*Burkholderiales* spp., *Pseudomonadales* spp., *Flavobacteriales* spp., *Streptophyta* spp., *Cladosporium* spp., *Epicoccum* spp., *Penicillium* spp. and *Cryptococcus victoriae*

1.2.5 The role of environmental parameters

Factors that may significantly affect the levels of indoor bioaerosols and contribute to the varying concentrations are ventilation, the moisture content of building materials, relative humidity and temperature.

Even though building air exchange can reduce the concentrations of indoor air pollutants, such as the accumulation of carbon dioxide, provided that the outdoor air is uncontaminated, it can also introduce contaminants in the indoor environments, as discussed previously. Ventilation systems have a significant effect on indoor bioaerosols abundance and composition. Studies to date have shown that increased ventilation rate can contribute to dilution of potential infectious agents (Luongo et al., 2016; Sundell et al., 2011; Li et al., 2007). Also, the filters installed in properly maintained mechanical ventilation systems can remove biological particles from indoor environments, while the lower relative humidity levels in air-conditioned buildings restrain the growth of fungi. Furthermore, it has been shown that the airborne microbial assemblages in naturally ventilated spaces have a higher level of diversity, correlated more with the outdoor air rather than the indoor sources, compared to mechanically ventilated buildings with closed windows (Meadow et al., 2014). This effect is more prevalent for airborne fungal taxa found indoors (except in damp buildings), as they mainly originate from outdoors (Barberán et al., 2015; Adams et al., 2013), whereas airborne bacteria have been less strongly correlated between indoor and outdoor air, and more associated with indoor sources (Lax et al., 2014; Tringe et al., 2008).

Water availability is also one of the primary drivers of the built environment microbial communities. It is well established, as well as one of the first areas of indoor bioaerosol research, that increased moisture levels, condensation and dampness-related conditions can lead to visual presence of mold and increased fungal spore production and emission (Emerson et al., 2015; Gorny et al., 2001; Dales et al., 1997). Also moist surfaces, such as shower curtains and showerheads have been reported to harbour growth of potentially aerosolised opportunistic pathogenic bacteria such as *Legionella* (Bollin et al., 1985) and non-tuberculous mycobacteria - NTM (Feazel et al., 2009).

Temperature and relative humidity are the two most important microclimatic parameters affecting the survival of microorganisms in the airborne state. For this reason these are usually recorded during the collection of bioaerosols. In general, bacteria and fungi are more stressed as the rate of evaporation increases, which occurs as relative humidity deceases and

temperature increases. Thus, increased survival is favored at higher relative humidity and lower temperatures. Fungi and their spores are more resilient than bacteria, being able to withstand greater stresses. The effect of relative humidity is more complex for viruses, varying with virus type, but generally, as temperature rises, virus survival decreases (Tang, 2009). However, recent studies have found weak or negligible correlations between these physical parameters and microbial community measures (Stephens, 2016), possibly due to the use of modern molecular techniques that cannot determine the viability of detected micro-organisms.

1.3 Human exposure to indoor bioaerosols and health effects

Data from epidemiological and toxicological studies conducted to date demonstrate associations between ambient particulate concentrations and increased morbidity and mortality (Englert, 2004; Neuberger et al., 2004; Pope et al., 1995; Schlesinger, 1995). Worldwide mortality data attributable to air pollution (http://www.who.int/gho/phe/en/, WHO 2012) show that 3 million deaths every year are a result of exposure to fine particulate matter ($PM_{2.5}$). Moreover, in 2015, the exposure to $PM_{2.5}$ was ranked as the 5th mortality risk in the Global Burden of Disease study (GBD 2015 Risk Factor Collaborators, 2016).

Considering the major public health impact of aerosols, the microbial fraction of aerosols has become of increasing interest owing to its wide range of health effects (Douwes et al., 2003). According to the World Health Organization (2004; 1999), airborne pathogens are responsible for three of the top six causes (ARIs - acute respiratory infections, including bacterial and viral pneumonia, tuberculosis and measles) of death from infectious disease in the developing world.

The route of human exposure to potential pathogenic microbial agents being transmitted through aerosols is inhalation. The adverse effects of inhalable aerosols are dependent on deposition in the respiratory tract and the ability of the respiratory to remove them, which is directly related to particle size. The $PM_{2.5}$-fine aerosol fraction is of primary air quality monitoring and health-related interest, since it is the most susceptible portion of the bioaerosols to reach the deeper parts of the respiratory system (Mandal & Brandl, 2011). Coarse PM (2.5-10 µm) tends to deposit in the nasal, pharyngeal, and laryngeal regions of the respiratory system, whereas fine (0.1-2.5 µm) and ultrafine (<0.1 µm) tends to deposit in the tracheobronchial region and alveoli (U.S. Environmental Protection Agency, 2004) (Fig. 1.5).

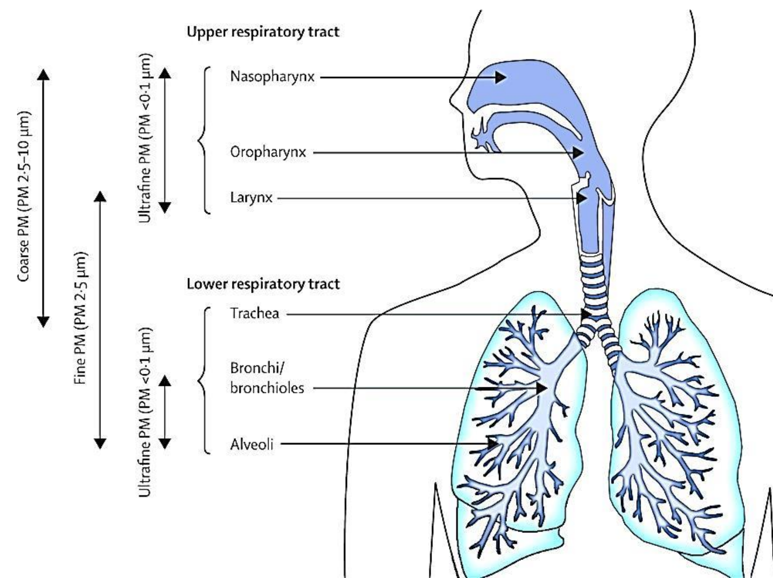


Figure 1.5 Deposition of inhaled particles on the human respiratory tract. Reprinted from Guarnieri & Balmes (2014).

Airborne bacteria may have important impacts on human health, serving directly as human pathogens (e.g. *Legionella pneumophila*, *Mycobacterium tuberculosis, Staphylococcus aureus)* or triggering allergic reactions (e.g. *actinomycetes).* Bacteria in indoor air are the confirmed or presumed causative agents of several infectious diseases (e.g. Legionnaires' disease, Tuberculosis, Staphylococcal infection), some of them fatal, and their components are linked to the development and exacerbation of chronic respiratory illness including asthma (Couzin-Frankel, 2010; Peccia et al., 2008; Fields et al., 2002; Hussman, 1996). Also, high levels of airborne bacteria have been found to be associated with the prevalence of Sick Building Syndrome - SBS (Mentese & Tasdibi, 2016). SBS is caused by poor IAQ and it is linked with the occurrence of various combined symptoms (e.g. headache, fatigue, nausea, dizziness, eye, nose and throat irritation) and reduced occupants/employees performance and well-being.

Airborne fungal material typically found within the indoor environment consistutes a source of potent allergens and inflammogens and is associated with noninfectious airway diseases such as allergies, asthma and hypersensitivity pneumonitis (Tischer et al., 2011; Portnoy et al., 2005; Kurup et al., 2002; Robbins et al., 2000). *Aspergillus* spp., *Alternaria* spp., *Cladosporium* spp. and *Penicillium* spp. are some of the most common allergenic fungi (Simon-Nobbe et al., 2008; Horner et al., 1995). It is estimated that 20–30% of the population in developed countries is affected by allergic diseases related to fungal allergens (Crameri et al., 2006). Yet, some fungal infections occurring e.g. from *Aspergillus, Candida*, and

Cryptococcus spp. pose a significant threat to immunocompetent and immunocompromised patients and are associated with high case-fatality rate (Brown et al., 2012; Pfaller & Diekema, 2004).

Last but not least, **viral aerosols** represent a common cause of infectious diseases acquired indoors. Some examples of infectious viruses being transmitted through the airborne route are the common cold Rhinovirus, Influenza virus A, Measles virus, SARS coronavirus etc. (Tellier, 2009; Yu et al., 2004; Heikkinen & Jarvinen, 2003; Bloch et al., 1985). Table 1.2 provides a list of diseases associated with exposure to indoor microbial bioaerosols.

Table 1.2 Diseases associated with exposure to airborne micro-organisms (Nazaroff et al., 2016).

Disease	**Microbial agent**	**Taxa**
Pneumonia	*Streptococcus pneumonia*	Bacteria
Pulmonary disease	Non-tuberculous mycobacteria (NTM)	Bacteria
Legionnaires' disease	*Legionella pneumophila*	Bacteria
Staphylococcal infection	*Staphylococcus aureus*	Bacteria
Tuberculosis	*Mycobacterium tuberculosis*	Bacteria
Whooping cough	*Bordetella pertussis*	Bacteria
Cold (common)	Rhinovirus	Virus
Gastroenteritis	Norovirus	Virus
Influenza	Influenza virus A	Virus
Chickenpox	Varicella zoster virus	Virus
Measles	Measles virus	Virus
SARS	SARS coronavirus	Virus

In addition to living and non-living micro-organisms in the air, some microbial products may also induce respiratory symptoms. These include endotoxins, β-glucans and mycotoxins (Hurst et al., 2007; Douwes et al., 2003). Endotoxins are high molecular weight lipopolysaccharide components of the cell wall of Gram-negative bacteria with high proinflammatory potency. Gram-negative bacteria are found as normal microflora of soil, water and living organisms, including humans and their pets. Exposure to airborne endotoxins is considered as an important risk factor for human health, as it has been associated with the development of airway inflammation, nonallergic asthma, bronchitis, organic dust toxic syndrome, toxic pneumonitis, mucous membrane irritation and accelerated pulmonary function decline (Madsen, 2006; Michel et al., 1996; Olenchock, 1994). β-glucans are polymers of glucose with variable degrees of branching and molecular weight that originate from most fungi. Several studies have showed that exposure to β-glucans could induce inflammatory reactions and respiratory symptoms (Rylander & Li, 2000; Rylander, 1998). Other common microbial

allergens are mycotoxins, which are low molecular weight secondary metabolites produced by fungi and are toxic to animals and humans (Jarvis & Miller, 2005; Robbins et al., 2000; Hendry & Cole, 1993).

Apart from micro-organisms and their derivatives that can be found in airborne particles, pollen fragments, plant debris, animal dander, and mite excreta contain components that are among the most important allergens. These biocontaminants, because of their size and mass, settle rapidly within the indoor environment and over time they may become non-viable and fragmented by the process of desiccation. Once these smaller and lighter fragments become suspended in air, they have a greater tendency to stay suspended (Menetrez et al., 2001). Desiccated non-viable fragments of organisms are common in the air and can cause allergenic, toxic and inflammatory responses (Green et al., 2006; Gorny et al., 2002), depending upon the specific organism or organism component.

Finally, in today's world, bioaerosol exposure health risk could also occur by deliberately releasing hazardous pathogenic micro-organisms due to political conflicts and regional instability (Relman, 2006). Among the most common biohazardous agents potentially usable for bioterrorism are spores of *Bacillus anthracis* (causative agent of anthrax). Moreover, the densely populated urban networks that characterize big cities form an ideal basis for rapid pandemics by the uncontrolled spread of diseases by the airborne route (Weiss & McMichael, 2004).

Even though the literature has focused on the hazardous aspect of exposure to bioaerosols and the potential adverse health effects yielded, it has to be highlighted that not all airborne microorganisms are contaminants with negative health impacts, and the majority of the air microbiome is actually part of the "healthy" indoor microbiome. Still, even though there is still much work to be done to understand the link between microbial diversity and human exposure, there is emerging evidence that exposure to certain airborne microbial agents can also be beneficial for human health. In particular, recent studies have shown that increased exposure to certain allergens in early life stages has been linked with reduced risk of wheezing illnesses and asthma development (Liu et al., 2015; Lynch et al., 2014). An example supporting this is the lower prevalence of asthma and atopy among children raised in a farming environment (von Mutius, 2016; Ege et al., 2011; von Mutius & Radon, 2008).

Other effects

Finally, some airborne microbial agents are also the causative agents of plant and animal diseases (West et al., 2009; McCartney et al., 2003; Brown & Hovmøller, 2002). Apart from the health issues, bioaerosols are linked to deterioration and degradation of building materials (Ahmed et al., 2017; Kakde, 2015). In addition, offensive odors are also associated with microbial contamination of indoor environments. Fungal and bacterial growth produces products of microbial metabolism, e.g. microbial volatile organic compounds (MVOCs), which give rise to the characteristic odors of 'mold' or 'cellar' (Wolkoff & Nielsen, 2001; Pasanen et al, 1998).

1.4 Bioaerosol collection methods

Bioaerosol sampling involves either active or passive collection techniques and can be time-integrated or time-resolved (offline and inline), stationary (area) or personal (collection of the inhalable aerosol fraction in the breathing zone of the human subject), size-resolved or non-size-resolved (depending on particle size-selective sampling ability), viable or non-viable (depending on the downstream analysis method).

1.4.1 Active sampling

Active sampling is the collection of particles by means of a forced movement of air by a pump. Bioaerosol sampling methods utilize the same principles as those for particle collection from air. The sampling phases include aspiration from the ambient environment into the inlet of a measurement device, the transport through the inlet to the collection area and the collection of the bioaerosol on a specific medium. After collection on a medium (e.g. filter, liquid or agar), the sample can be analyzed for its various characteristics, including gravimetric mass, chemical or biological composition. However, particles sampling for bioaerosol analysis requires use of aseptic techniques, i.e. systematic avoidance of sample contamination throughout the sampling procedures, transportation and laboratory analyses, along with sterilisation of the sampler and collection substrate. In addition, special requirements related to low sampling stresses for the collected micro-organisms are necessary for the purposes of viable sampling.

The four principal collection methods used in active bioaerosol sampling are:

- **impaction**, in which air is drawn through a nozzle and impacted on a collection surface (loaded with nutrient medium or filter) located perpendicular to the air flow due to particle inertia,
- **filtration**, which entails passing air through a membrane filter of a nominal pore size mounted onto a filter cassette and collection of particles due to mechanisms of interception, impaction, diffusion, settling, and electrostatic attraction,
- **impingement**, in which air is drawn through a nozzle into a collection fluid that traps the aerosols passing through it, and
- **cyclone**-based sampling, where particles are captured into a liquid medium using swirling air and centrifugal force.

Another less common bioaerosol sampling method is collection by electrostatic precipitation, in which the airborne particles are electrically charged at the inlet and then subjected to a strong electrical field inside the sampler, which causes their deposition on the collection substrate (Mainelis, 1999).

Bioaerosol samplers can operate at a range of airflow rates, from 2 L/min to as much as 1000 L/min, and, depending on amounts of sampling time, can result in collection of different sampling volumes. Representations of common pump-driven sampling devices that utilise the four collection principles are illustrated in Fig. 6, 7, 8 and 9. Membrane filtration is a widely used assay because of low expense, ease of use and high-capture rate. Some commonly used filter materials are polycarbonate, polytetrafluoroethylene (PTFE), gelatin and mixed cellulose ester (MCE). However, when viability of collected micro-organisms is required, filter-based sampling could lead to desiccation/drying off of microorganisms. Liquid impingement is a more common method used for the collection of viable bacteria and virus particles, whereas many fungal spores are hydrophobic and may impinge with low efficiency. Some samplers have the advantage of performing particle size-distribution of the aerosols collected, such as the six-stage viable or eight-stage non-viable Andersen cascade impactor. Generally particles which are larger than a particular aerodynamic size get impacted onto a collection surface forcing the smaller particles to proceed through the sampler (Hinds, 1982). However, the majority of aerosol samplers designed for bioaerosol collection does not have any specific particle cut-off size and therefore only collect total suspended particles. Another disadvantage related to active samplers is that they can be impractical because of their noise (especially in the indoor environment), weight/size and their requirement of power supply.

Early on, several studies attempted to evaluate the overall efficiency of different samplers in terms of bioaerosol collection and recovery efficiency (e.g. Buttner & Stetzenbach, 1993; Jensen et al., 1992; Nevalainen et al., 1992; Kang & Frank, 1989). Very few recent publications have looked into this issue using the latest analysis techniques. According to a comparative study between the three collection methods; impactor, liquid impinger and filter sampler, by Fahlgren et al. (2011), comparable results were yielded by the different sampling approaches regarding the overall composition of the airborne bacterial community. However, according to Li (2011), liquid impingers and filter samplers are more efficient for molecular analysis, compared to solid impactors, because of high collection efficiency. Due to the wide variation of airborne collection methods in their collection efficiencies, which is reflected in the analysis outcome, Hoisington et al. (2014) suggest that it may be more appropriate to combine a variety of techniques in order to achieve a better representation of the bioaerosol abundance and diversity.

To sum up, considering that there is no standardised method for bioaerosol collection at this time, when adopting a sampling strategy, some of the factors that have to be considered include easy handling of the device (light-weight), sampling capacity which is connected to the airflow and sampling time and options available for sample processing.

1.4.2 Passive sampling

Passive sampling is based on gravitational settling of particles on a collection surface. Surfaces act as sources and sink for air contaminants and therefore reflect what has been in the air averaged over the time period since the surface was cleaned. Unlike active sampling, it does not require the use of any mechanical equipment and thus it is a non-intrusive method. Moreover, it is a practical method for extended periods of sampling. Concentrations of biological particles vary greatly in time and space (Brandl et al., 2008; Hyvärinen et al., 2001) and short-time air sampling may not necessarily reflect this variation properly.

Collection can be on a specified collection substrate, such as settle plates (Adams et al., 2015c) and dustfall collectors (Noss et al., 2008; Würtz et al., 2005) or on an actual indoor surface (e.g. on a furniture), from which the sample can be retrieved using suction-based methods (e.g. vacuums) or, most commonly, wipe methods (e.g. swabs). An alternative sampling approach that has started being increasingly popular is the convenient use of dust recovered from HVAC filters (Noris et al., 2011). These filters can serve as integrated air samplers and dust recovered from filter portions can be utilized for bioaerosol analysis. Nevertheless, because the effective

volume of air sampled cannot be determined in passive techniques, the collected sample cannot be expressed in volumetric units; hence, these data are considered semi-quantitative. In addition, retrieval of dust sample from the collection surface is subject to interpersonal variation, for instance in the strength of swabbing.

Yamamoto et al. (2011) found relatively good correlations between active and passive sampling techniques, while the findings of Hoisington et al. (2014) showed that the bioaerosols recovered from the settled dust and HVAC filter dust were much more diverse, compared to the shorter term samples collected from four different active samplers. Considering that the microbial characterisation of particles is affected by the sampling approach, strongly supports the argument that a combination of techniques might give a better representation of the actual airborne load.

1.4.3 Online techniques

Both types of active and passive sampling techniques described above constitute time-integrated types of sampling. Unlike many characteristics of particles that can be measured in real time, e.g. particle mass or number concentration, by readily available methods, there are very few methods for real-time measurements of bioaerosols. In recent years, significant efforts have been made in development of online monitoring systems based on laser-induced fluorescence (LIF) that make possible real-time biological particle counting and particle size determination (Toprak & Schnaiter, 2013; Hairston et al., 1997). Samples of biological origin are discriminated based on the characteristic emission bands they show under ultraviolet excitation. Two commercially available instruments are the Ultraviolet Aerodynamic Particle Sizer (UVAPS) and the Wideband Integrated Bioaerosol Sensor (WIBS4). However, compositional specificity of LIF detectors is still in its infancy.

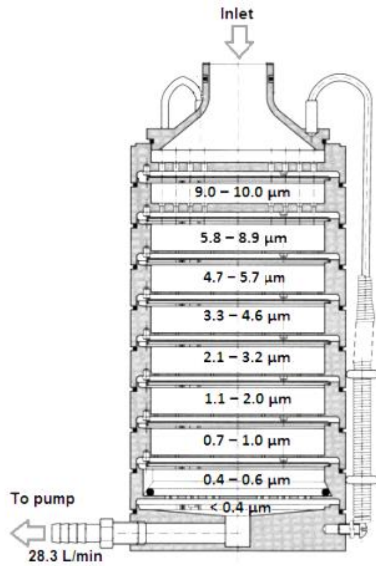


Figure 1.6 The Andersen non-viable cascade impactor consists of 8 stages together with a final filter. The collection stages serve as an impaction surface for the collection of the particles possessing inertia too great to be carried around the collection surface and onward to the next stage. Thus, smaller particles are collected on successive stages. Image adapted from http://www.drugfuture.com/Pharmacopoeia/BP2010/data/895.html

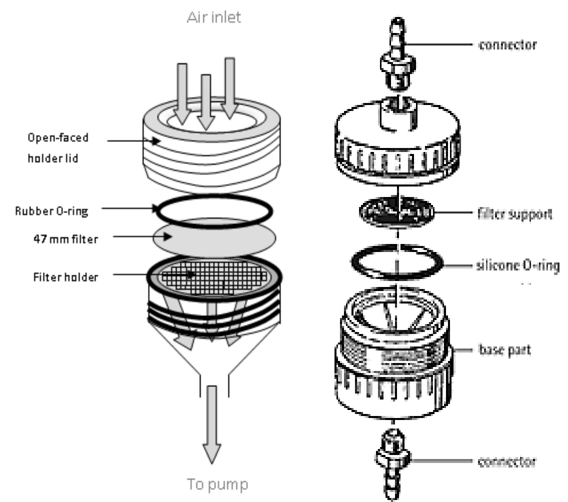


Figure 1.7 Representations of an open-faced and a closed-faced (in-line) filter holder. The filter is held in place by an o-ring and supported on a plastic grid holder. Air passes across the filter, trapping particles. Open-faced filter holders reduce the possibility of losses in the inlet section. The closed-faced type allows connection for some equipment by means of a probe. Schematic representations adapted from https://www.sartorius.com/_ui/images/h70/hb9/8874030235678.pdf

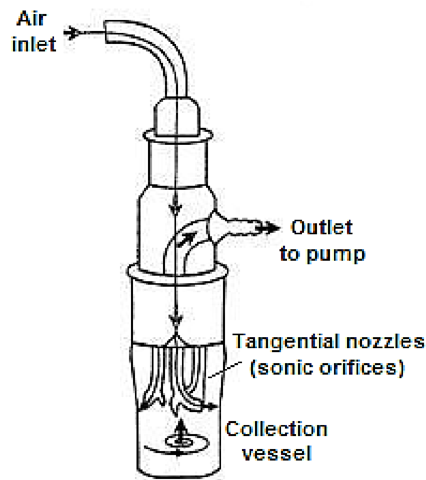


Figure 1.8 The SKC Biosampler impinger. Air is drawn in through a vertical tube and bubbled through a collection liquid. Particles remain in the liquid, whilst air, depleted of particles, passes out to the pump. The BioSampler's three tangential nozzles act as critical (sonic) orifices, each permitting 4.2 L/min of ambient air to pass through for a total flow rate of 12.5 L/min (https://www.skcinc.com/catalog/pdf/instructions/37084.pdf).

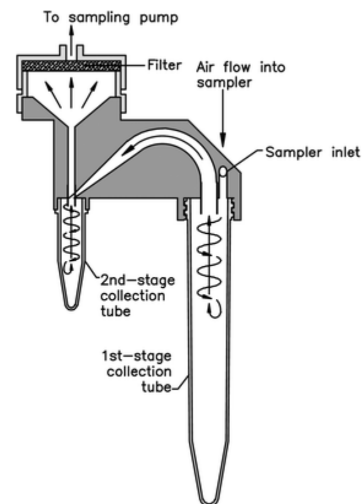


Figure 1.9 A two-stage cyclone developed by NIOSH. As ambient air is drawn into an inlet at 3.5 L/min, the first stage deposits aerosol particles that are >4 µm on the wall of a 15 ml centrifuge tube. In the second stage, 1 to 4 µm (respirable) particles are deposited on the wall of a 1.5 ml microcentrifuge tube and particles that are <1 µm are collected on a 37 mm filter. Reprinted from Cao et al. (2011).

1.5 Bioaerosol analysis methods

1.5.1 Culture-based methods

The interest in air microbiology and study of airborne micro-organisms dates back to Pasteur's work in the mid-19th century (Ariatti & Comtois, 1993). The majority of bioaerosol studies to date have used culture-based microbiological analysis methods (ACGIH, 1999; Cox & Wathes, 1995). This type of analysis requires the prior use of a sampling method with high bioefficiency (survival and recovery of micro-organisms after their collection from the air), mainly achieved by the use of an appropriate type of solid or liquid media. Cultivation of collected micro-organisms is typically followed by enumeration of culturable micro-organisms and/or microscopic identification.

Culture-dependent methods are by far the most widely used procedures for assessing the microbiological content of bioaerosols. A long history of applying culture-based techniques to investigate the biological content in indoor environments, such as schools, offices, residences, health-care settings, etc. (Mandal & Brandl, 2011), has built a fundamental knowledge base for indoor bioaerosols. Culture-based microbiology studies have indicated that some of the bacterial species that tend to dominate the built environment are *Bacillus* spp., *Streptococcus* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Micrococcus* spp., *Staphylococcus* spp., along with a few *Actinomycetes*, such as *Corynebacterium* spp. Also, fungi such as *Aspergillus* spp., *Cladosporium* spp., *Penicillium* spp. and *Alternaria* spp. are common inhabitants of built environments (e.g. Tsai & Macher, 2005; Gorny et al., 1999; Reponen et al., 1994; Miller et al., 1988).

However, it is now well recognized that traditional culture techniques capture only a small fraction of the total microbial diversity resulting in a great underestimation of the microbial load (Mandal & Brandl, 2011). It has been estimated that less than 1% of bacteria in any environment can be readily cultivated (Pace, 1997; Amann et al., 1995) and airborne microbes in particular are difficult to grow in a culture because of the stresses linked to conventional collection methods (e.g. the inactivation of bacteria during impaction or desiccation during filter-based sampling) (Wang et al., 2001; Stewart et al., 1995). Moreover, the use of culturing methods for determination of micro-organisms has been criticized, as only the viable micro-organisms and those able to grow on the particular media used will be detected. Yet, micro-organisms that are not culturable under the specific growth conditions imposed in the laboratory may be capable of inducing adverse health effects (also see 1.3). Bioaerosols consist

not only of viable micro-organisms, but also of non-viable, as well as microbial fragments and derivatives, that although they cannot be cultivated, many of them are toxic or allergenic (Liang et al., 2012).

1.5.2 Molecular methods

The advance of cultivation-independent methods overcomes the problems associated with culture-based methods underestimation of the airborne microbial load and have opened new fields of research providing access to far more microbial diversity than has been ever viewed. DNA-based molecular methods allow all micro-organisms (live, dead, viable but non cultivable) in a sample to be examined regardless of the viability or culturability of the cells. Molecular biology approaches provide more sensitive and specific microbial data and demonstrate a great potential for biological aerosol detection, especially in low concentrations airborne micro-organisms (Peccia et al., 2011; Metzker, 2010; Peccia & Hernandez, 2006; Alvarez et al., 1994).

The fact that collected bioaerosols need not be viable or culturable for molecular analysis maximises the potential of sampling techniques as sampling stresses imposed to collected micro-organisms are not of concern. The first step in processing aerosol samples for molecular analysis is to isolate the DNA from the collected sample and therefore it is critical that a sampling volume that can recover sufficient amount of DNA for downstream analysis is collected. High efficiency of the applied DNA extraction protocol is also required. Application of molecular techniques, such as Polymerase Chain Reaction (PCR), DNA fingerprinting methods (e.g. PCR-Denaturing Gradient Gel Electrophoresis), DNA sequencing and real-time quantitative PCR (qPCR) for quantifying and describing the total diversity and composition of microbial communities to the study of airborne microbiology has started to provide an insight on the investigation of microbial ecology of the atmosphere and indoor air.

The advent of **Next Generation DNA Sequencing** (NGS) or so-called high throughput sequencing (HTS) or second generation sequencing, which has replaced the still applicable Sanger sequencing technology (first generation), provides the possibility of rapidly generating millions of large datasets of sequences from both culturable and unculturable micro-organisms and has enabled the building of fundamental knowledge on microbial ecology genomics (Shokralla et al., 2012). Two widely used HTS techniques are sequencing of PCR-amplified marker gene, also called amplicon sequencing (field of metagenetics), or whole-genome

shotgun sequencing (field of metagenomics). Amplicon sequencing targets a specific conserved genomic region, such as the 16S ribosomal gene in bacteria and archaea, the internal transcribed spacer (ITS) region in fungi and the 18S rRNA gene in eukaryotic communities, to identify the micro-organisms, while whole-genome sequencing uses all the genetic material present in a DNA sample (Ranjan et al., 2016). So far, the amplicon sequencing has been mostly exploited in indoor air research. Nonetheless, for identification of viral aerosols only shotgun metagenomics can be applied, as there is no homologous genomic region across all viruses (Posada-Cespedes et al., 2016; Prussin et al., 2014). The current sequencing technologies are mainly based on Illumina MiSeq and HiSeq platforms, while 454 Roche/ GS FLX Titanium pyrosequencing platform that has been utilised for many studies has been discontinued (see Table 1.3). Data generated with high-throughput sequencing present computational challenges and require the use of bioinformatics tools (Caporaso et al., 2010; Schloss et al., 2009).

Thus far, the literature has investigated the microbial communities using molecular methods in outdoor air (Innocente et al., 2017; Genitsaris et al., 2017; Gandolfi et al., 2015; 2013; Seifried et al., 2015; Dannemiller et al., 2014; Bertolini et al., 2013; DeLeon-Rodriguez et al., 2013; Yamamoto et al., 2012; Bowers et al., 2012; 2011a;b; 2009; Franzetti et al., 2011; Jeon et al., 2011; Li et al., 2010; Boreson et al., 2004) and indoor air of various enclosed spaces (see section 1.7). Studies using clone library Sanger sequencing (e.g. Pitkäranta et al., 2011; Tringe et al., 2008) have provided microbial identification at the species level but lack coverage to fully describe the microbial diversity. High-throughput amplicon sequencing (pyrosequencing, Illumina) have provided in-depth sequence coverage of the microbial communities to the genus level and have yielded an increasing number of bacterial and fungal datasets regarding the built environment. Application of metagenomic HTS (i.e., whole genome shotgun sequencing) has been limited and has only been applied indoors in the hospital environment (Tong et al., 2017; King et al., 2016) and outdoors in urban areas (Be et al., 2015), enabling mapping of prokaryotic, eukaryotic and viral sequences to characterise the general airborne content. In addition, Yooseph and colleagues (2013) presented a metagenomic framework, in their study, suitable for studying airborne microbial communities both in indoor and outdoor samples.

In general, examination of the literature findings published so far has shown that the four dominant phyla in indoor air identified from high-throughput sequencing data are: Actinobacteria, Proteobacteria (Alpha, Beta and Gamma), Firmicutes and Bacteroidetes. These phyla have been common to all air samples regardless the sampling site. For instance, this

common microflora was also encountered in a study comparing three different types of indoor sites; museum, hospital and office (Gaüzère et al., 2014). It should be noted that the airborne microbial abundance revealed in these sites included also several pathogen-related species (e.g. *Acinetobacter* spp., *Micrococcus* spp.). Regarding fungal bioaerosols, the two main phyla observed are Ascomycota and Basidiomycota. Still, it has been reported that detected archaeal sequences in air samples (Euryarchaeota and Crenarchaeota phyla) constitute a minimal portion (< 0.1% or < 1%) of the assigned reads (Leung et al, 2014; Robertson et al. 2013). Relatively little is known about viral communities' structure in general and there have been few studies reporting concentrations of particular virus species in the air, such as influenza (Yan et al., 2011). In the limited number of samples that have been analysed by metagenomic sequencing, archaea accounted for 3 - 4% of all sequences, while detected viruses comprised 3 - 8% of total diversity. The particular air samples were from a hospital environment (Tong et al., 2017).

Quantitative Polymerase Chain Reaction (qPCR) provides highly sensitive quantitative information on the number of copies of a target gene or a target organism (genome) in an environmental sample (cubic meters of air). qPCR has the advantage of counting even agglomerated organisms, rather than determining them as one colony-forming unit. Application of qPCR method requires a priori knowledge of the species of interest and specifically designed primers. The use of qPCR with universal fungal or bacterial primers can provide a general estimate of total bacterial genomes or fungal spore equivalents in a sample. However, it should be noted that when using universal primers, the determination of multi-copy genes, such as the 16S rRNA gene, is not directly related to the cell number due to the presence of multiple ribosomal operons (1 - 15 depending on the species) per (bacterial) genome. Moreover, the use of ITS universal fungal barcode region as a target in qPCR entails biases due to the known variability of the internal transcribed spacer region even within species or over the lifetime of the fungi and therefore targeting of specific fungal spores for absolute quantification of specific genera (e.g. *Penicillium*, *Aspergillus, Saccharomyces* species etc.) might be a better approach (Haugland et al., 2004). In the case of viral aerosols, virus specific (e.g. *Influenza virus* A) primers are required.

Apart from the fact that when using universal primers the number of gene copies may not represent the number of micro-organisms, it has to be considered that genes may also occur attached to cell debris. Cell fragments in the indoor air can outnumber the micro-organism

counts (e.g. fungal fragments compared to intact spores) and since both entities can be detected by PCR, our understanding of what is the true microbial abundance is biased (Reponen et al., 2007). Still, due to lack of standardised protocols and the wide variation of assays used in qPCR, these numbers can only be understood in isolation. Finally, despite the fact that DNA quantification by qPCR cannot distinguish live from dead or active from inactive microbial cells, detection of high abundance of a pathogenic microbial agent is still a good indicator of a potential health risk.

On the other hand, the quantification data output from NGS sequencing is reported as relative abundance, i.e. as a percent of the total abundance of reads produced. However, this makes difficult the comparison between different aerosol samples, as the total abundance is most often different. Dannemiller et al. (2014) and Yamamoto et al. (2014) suggested that, for a meaningful quantification, NGS relative abundance values must be transformed to absolute concentration through multiplication of relative abundance by the total concentration of micro-organisms derived by qPCR. The combination of the two molecular technologies could contribute to improving NGS sequencing-based quantitative aerosol exposure assessment.

1.6 Major limitations/implications in bioaerosol research

Studies of aerosols typically focus on physicochemical rather than biological characterization of particles and even though biological particles may represent a significant fraction of the particulates suspended in the atmosphere, we still have a limited understanding of aerosol microbial ecology. Despite the recognition of the importance of bioaerosol exposure on human health, the number of publications on indoor air microbiology is relatively low compared to the advanced field of aerosol science research.

According to Peccia et al. (2011; 2008; 2006) one of the reasons contributing to the limited development of this research area is the poor integration of biology with aerosol science. Aerosol research infrequently focuses on the biological fraction, whereas aerobiology studies often undervalue the important physical and chemical characteristics of particulate matter. Bioaerosol research covers a wide area that combines several disciplines such as aerosol physics, microbiology, environmental science and engineering, occupational and public health and thus requires complementary knowledge and skills.

From the biological science point of view, research into airborne micro-organisms has been restricted by difficulties in their collection and analysis in contrast to the more easily sampled aquatic and terrestrial environments (Peccia & Hernandez, 2006). The major difference between the study of microbiology in these environments is the dilute nature of biological materials in air. Compared to soil and water, where total micro-organism concentrations are on the order of $10^6/\text{cm}^3$ or greater (for instance 10^{12} to 10^{14} of bacteria per m^3 of water or 10^4 - 10^7 per ml of marine water or 10^8 - 10^9 prokaryotes per g of soil is common), bioaerosol concentrations are commonly $<1/\text{cm}^3$ (e.g. 10^3 to 10^5 or $10^6/\text{m}^3$ of bacterial cells in air is common) and often $<1/\ \text{m}^3$ in the case of infectious aerosols (Behzad et al., 2015; Hubad & Lapanje, 2013; Peccia, 2008; Lighthart et al., 2000).

Owing to the low biomass of the air, it is challenging to obtain a representative microbiological sample in order to recover sufficient DNA, particularly when using molecular tools. The challenge of applying the latest molecular techniques on studying the low density micro-organisms in the air is reflected in the slow progress in air metagenomics compared to the microbial ecology fields of soil and marine environments (Fig. 1.10).

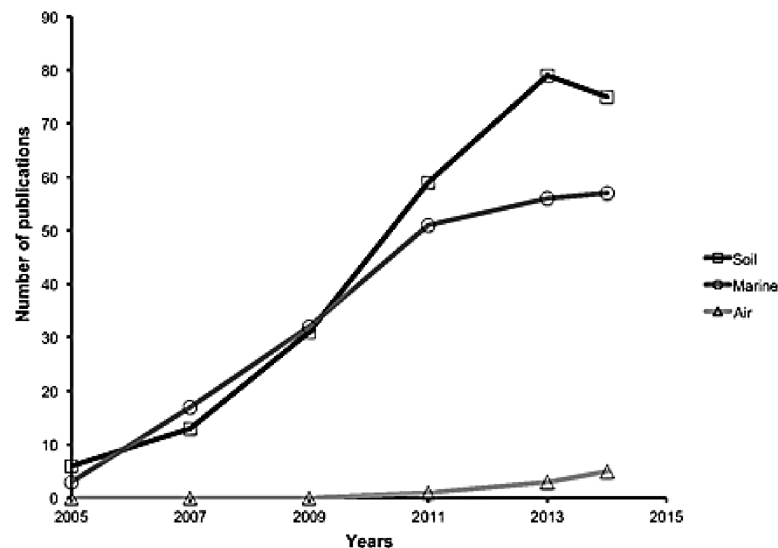


Figure 1.10 Relative number of publications related to metagenomic studies of soil, marine, and air. The graph was generated with the data obtained from Scopus using the search words: “soil metagenomic,” “marine metagenomic,” and “air/airborne metagenomic,” respectively. Reprinted from Behzad et al. (2015).

The efficiencies of both the bioaerosol collection and nucleic acids extraction are the most important factors in air investigations. Collection devices with high flow rates or high

collection efficiencies may be necessary to achieve molecular analysis under optimum conditions (Gauzere et al., 2013; Li, 2011).

Moreover, no standardised methods and protocols are currently available for the collection and analysis of all types of bioaerosols. Data between studies are often difficult to compare due to differences in sampler design, collection time, air flow rate and analysis method. In addition, there are no internationally recognized exposure limit values or Threshold Limit Values (TLV) for bioaerosols (ACGIH, 1999). Causal dose–response relationships between exposure to bioaerosols in indoor air and related health symptoms are still under investigation due to the lack of valid standardised measuring exposure assessment methods. In order to define the environmental hazard biological particles might pose and to establish exposure thresholds, it is crucial that reliable methods for the collection and determination of airborne micro-organisms must be developed (Mandal & Brandl, 2011).

1.7 Bioaerosol studies so far

Research on bioaerosols is a growing field as revealed by the increasing number of scientific articles published during recent years. The emergence of severe acute respiratory syndrome (SARS) in 2002–03, the potential threat of deliberately released agents such as anthrax and concerns about a highly pathogenic influenza pandemic are some of the facts that have resulted in an emerging interest in the role of environmental exposure to airborne biological agents. Furthermore, bioaerosol research has been enhanced by the access to new molecular tools allowing better and faster characterisation.

In general, both culture-dependent and culture-independent studies have identified the presence of a diverse mixture of microbial groups and species in the air (see 1.5.2). While the predominant focus of traditional culture-based studies was on fungi, the vast majority of modern high-throughput sequencing studies have been mostly characterising bacteria in indoor air (Adams et al., 2015; Kelley & Gilbert, 2013). Recently, though, examining both the bacterial and fungal components in the same samples has started being quite common (e.g. Luongo et al., 2017; Hospodsky et al., 2015; Shin et al., 2015; Adams et al., 2014). Microbial data from the recent indoor air microbiome investigations have demonstrated a rich microbial presence of bacterial and fungal taxa, with much less being known about archaeal, non-fungal eukaryotic and viral communities found in indoor environments (Prussin et al., 2014; Yooseph

et al., 2013). Endotoxins from settled dust have also been explored (Noss et al., 2008**;** Bouillard et al., 2005; Thorne et al., 2005).

Interestingly, cultivation techniques are still being used extensively for bioaerosol research (e.g. Shinohara et al., 2018; Heo et al., 2017; Gandhi et al., 2017; Zemouri et al., 2017; Osman et al., 2017; Fan et al., 2017; Madureira et al., 2015; Hwang & Park, 2014), mostly due to their easy cost-effective application, non-requirement of special equipment, knowledge and skills. Yet, the preferential current methods for bioaerosol analysis are the molecular tools, based on DNA isolated directly from collected samples of culturable and non-culturable airborne micro-organisms. In cases of disease-causing agents, though, the cultivation methods must be utilized to establish the cause of the disease. Nonetheless, molecular detection and cultivation assay are not mutually exclusive but rather could be complementary. In order to link the gap between traditional culture and molecular techniques, Hubad & Lapanje (2013) developed a method that utilises both approaches and enables simultaneous monitoring of both total and culturable airborne bacteria. Even though deep sequencing capability of the molecular analysis has widened the understanding about the microbial diversity, according to Venkateswaran et al. (2014), the cultivation assay proved to be essential in their study, since some of the spore-forming microorganisms were detected only by the culture-based method.

The majority of sequencing-based indoor bioaerosol investigations have been conducted in individual sampling locations, i.e. one type of micro-environment. The variety of indoor spaces that have been investigated include university lecture rooms (Meadow et al., 2014; Hospodsky et al., 2012; Qian et al., 2012), schools (Yamamoto et al., 2015) offices (Chase et al., 2016; Gaüzère et al., 2014), residences (Luongo et al., 2017; Emerson et al., 2016; Wilkins et al., 2016; Miletto & Lindow, 2015; Adams et al., 2014; 2013), transportation systems (Triadó-Margarit et al., 2017; Leung et al., 2014; Robertson et al., 2013), health-care facilities (Tong et al., 2017; Park et al., 2014; Kembel et al., 2012), child-care facilities (Nygaar & Charnock, 2017; Prussin et al., 2016; Shin et al., 2015), retail stores (Hoisington et al., 2016), museums (Gaüzère et al., 2014b), a zero-carbon building (Leung et al., 2018), as well as more uncommon environments such as the International Space Station (Venkateswaran et al., 2014). HTS studies investigating bioaerosol dynamics have also been conducted in experimental chambers (Adams et al., 2015b; Meadow et al., 2015). However, this is a scientific field that is still in its early stages and a lot more studies are still required in order to gain a fundamental

understanding of bioaerosols and the factors shaping their diversity and abundance in indoor environments.

Still, very few studies on enclosed spaces have monitored the microbial load and diversity using a culture-independent approach during a long period of time (Emerson et al., 2017; Gaüzère et al., 2014b; Robertson et al., 2013) and there is a lack of information regarding the air microbiome seasonality. The use of alternative sampling methods, such as the use of settle plates or the HVAC filters already existing in the ventilation systems of the sampling sites (see also Section 1.4.2) consist a convenient way of conducting longitudinal studies and are being increasingly utilised (e.g. Luongo et al., 2017, Adams et al., 2015c; 2014; 2013; Hoisington et al., 2014).

Owing to their easy and cost-efficient applicability, settled dust approaches were also deployed by the only two existed large-scale spatial dynamics studies of airborne microbial particles. Barberán and colleagues (2015) conducted a continental-scale survey covering 1,200 households across the USA. Samples were collected by swabbing the upper trim of an interior and an exterior door by volunteer citizens. The particular surfaces served as a passive dust collectors with the settled dust representing the contributions of indoor/outdoor air bacteria and fungi to the indoor air microbiome and results provided an insight into the geographic variability between buildings in urban and rural locations An earlier global survey of fungi from 72 indoor locations (Amend et al., 2010) was also based on collection of settled dust using samplers attached to domestic vacuum cleaners. Interestingly, findings revealed a higher fungal diversity in temperate zones compared to the tropics. Apart from the issue of local-scale variability, another important point that was highlighted by Adams et al. (2015) that conducted a meta-analysis of microbiota data derived from HTS studies in indoor environments, is about the geographic coverage of study locations, which *"tends to be focused around a few heavily sampled locations, with sparse representation elsewhere"*. It has to be added that all the modern studies have been examining microenvironments in developed countries and high-throughput sequencing analysis has not been yet applied in studying bioaerosols in the developing world.

A list of few examples of HTS-based studies dealing with descriptions of indoor airborne microbial composition is presented in Table 1.3. Studies utilising only surface-associated sampling approaches have not been included. Even though surface/dust studies (An & Yamamoto, 2016; Hsu et al., 2016; Afshinnekoo et al., 2015; Barberán et al., 2015; Checinska

et al., 2015; Kettleson et al., 2015; Wood et al., 2015; Dannemiller et al., 2014b; Meadow et al., 2014b; Lax et al., 2014; Dunn et al., 2013; Adams et al., 2013b; Flores et al., 2013; Hewitt et al., 2012; Täubel et al., 2009) have made a big contribution to the field of the built environment microbiology, it has to be specified that surface associated taxa do not necessarily reflect biological particulates suspended in the air (especially the fine fraction) and can only be used as a surrogate measures of real exposure. Moreover, it has to be clarified that the term "microbiome of the indoor environment" or "built environment microbiome" that is used in many published surveys environment (Adams et al., 2016; Stephens, 2016; Konya & Scot, 2014; Kelley & Gilbert; 2013), is broad and does not necessarily mean that that the particular study has included indoor air microbiota sampling and analysis, too. However, some of the studies have combined bioaerosol collection with surface sampling (e.g. Hospodsky et al., 2012). This type of sampling can be complementary to air sampling as it can be utilised for characterising the various sources of airborne micro-organisms and the relative contribution of each (Duchaine & Mériaux, 2001). Source apportionment, as it is known in the aerosol science community, has been increasingly applied in bioaerosol microbiome investigations with the aid of bioinformatics tools, such as the SourceTracker (Knight et al., 2011).

Regarding the main conclusions that have been drawn from the latest studies, human occupancy is considered to be the main source of the airborne bacterial diversity encountered in the indoor environment, including direct human emissions, as revealed by the human oral, nasal and skin-associated microbiota taxa (e.g. *Acinetobacter* sp., *Staphylococcus* sp., *Propionibacterium* spp., *Corynebacteria* spp.), and physical activities causing resuspension of dust (Hospodsky et al., 2012; Qian et al., 2012). Meadow and colleagues (2015) demonstrated that humans release their own personalized microbial cloud with distinct bacterial communities that can be used to identify individual occupants, while Luongo et al. (2017) showed that the bacterial taxa found in airborne dust can predict the sex of the occupants. Fungi, on the other hand, show little direct influence of human occupancy (Adams et al., 2014). The fungal composition is mainly structured by the dispersal from the outdoor environment, except in damp buildings, and airborne fungi and their spores (e.g. plant-associated taxa) have the potential to be blown into a naturally ventilated building more than bacteria (Adams et al., 2013). This is in line with Hospodsky et al. (2012), who suggested that bacterial populations in indoor air show greater similarity to the populations found in floor dust than to populations from outdoor air. In the absence of occupants, though, it has been shown that ventilation does

influence airborne bacterial community composition (Meadow et al., 2014; Kembel et al., 2012; Li et al., 2007).

The above observations make it clear that indoor air quality cannot be presented as being linked only to sources indoors, but should entail a description of both the indoor and outdoor environment. In order to avoid an incorrect and incomplete assessment of the air microbial content in the built environment, the role of the outdoor air on the indoor particles cannot be underestimated and is critical to consider indoor and outdoor environment as parts of one system. Thus, the degree of connectivity between indoor and outdoor airborne microbial communities is an issue that needs to be further investigated. Moreover, the determination of the origins of micro-organisms in indoor air requires integrated knowledge of physical indoor aerosol processes with molecular biology-based tools.

Importantly, there is still little knowledge concerning the size-distribution of the airborne microbial particles. The majority of bioaerosol studies, regardless whether they employ active or passive collection techniques, lack particle-size specificity. Even though PM monitoring devices commonly used in physicochemical studies of aerosols always come with a cut-off size specification, typically at 2.5 or 10 μm aerodynamic diameter, bioaerosol sampling is most commonly performed for total suspended particles. One important knowledge gap in bioaerosol surveys is the lack of exposure assessment and potential estimation of the related dose. Sampling the total suspended particulate matter and not the inhalable fraction does not provide an estimate of the actual exposure and cannot be linked with observed health effects. Moreover, bioaerosol investigations using active collection techniques have been dominated by short time sampling (e.g. 1 – 2 hours or few minutes), also known as “snapshot” sampling and the question whether the temporal variability of bioaerosol concentrations reflects a representative view of the airborne load to which individuals are exposed still remains. It has been also claimed that stationary sampling can only provide a partial insight in personal exposure assessment and there is a need for inclusion of personal samplers in studies (Toivola et al., 2002) since exposure differs greatly with activity, location and time spent on each activity (e.g. while commuting). Nevertheless, it is evident that more knowledge is needed about understanding the link between bioaerosol exposure and human health.

The number distribution and size-resolved diversity of airborne micro-organisms in different particle size fractions by applying molecular methods has been only reported in a limited number of surveys in non-occupational environments (Yamamoto et al., 2015; 2014; 2012;

Sippula et al., 2013; Qian et al., 2012). One basic reason for this is the high demand for increased sampling volume. The biological material distributed per each size bin is equivalent to only one fraction of the total sampled volume (depending on the number of size bins) and therefore the collected total biomass has to be high enough in order to yield sufficient amounts of retrievable DNA for downstream analysis of all size fractions. For instance, in Qian et al. (2012), eight-stage non-viable cascade impactors had to sample air cumulatively for four consecutive days in classrooms. Aerodynamic diameter strongly influences the fate of biological particles and human exposure and therefore, size-resolved data are of fundamental value for developing insights regarding health effects of exposure to bioaerosols.

Furthermore, the technical aspect regarding sampling methodology continues to be different in every study, making difficult the cross-comparison of molecular results between studies. Selecting a sampling strategy that can overcome the low biomass issue is critical. Factors such as sampling approach selection, air volume sampled (determined by air flow rate and sampling duration in active samplers) and collection substrate/media (e.g. filter membrane, collection fluid) must be addressed to standardise a sampling protocol and additional work is needed to adequately characterise indoor samplers. In addition, the choice of DNA extraction protocol, molecular assay design (e.g. choice of primers, sequencing technology), as well as bioinformatic processing have an equally important impact on produced microbial data.

From the outline presented above it can be concluded that there are still critical gaps in bioaerosol research. Interestingly, as pointed out by a recent published review (Stephens, 2016), even though the application of molecular techniques have initiated a new era in indoor air research and unravelled the microbial diversity in far greater depth than was previously possible, the main conclusions derived from the latest studies may "*have not really advanced knowledge beyond what we already knew from over 100 years of applying culture-based methods*". There are still many issues that need to be addressed and much more information is needed to better understand the role of air in disseminating microbes in indoor environments and to improve the monitoring and management of indoor air quality. Several joint research initiatives have emerged in recent years exploring the air microbiome within the spaces in which we live and work such as the Alfred P. Sloan Foundation's program on the "*Microbiology of the Built Environment*" (microBEnet: www.microbe.net), the "*Hospital Microbiome Project*" (hospitalmicrobiome.com), the *"Microbiomes of the Built Environment"* by The National Academies of Sciences, Engineering and Medicine (nas-sites.org/builtmicrobiome/), the "*Wild Life of Our Homes*" (robdunnlab.com/projects/wild-life-

of-our-homes/) and the "*Metagenomics and Metadesign of Subways and Urban Biomes"* (MetaSUB: metasub.org).

The present study

The present project is part of the EU Research Programme "Human EXposure to Aerosol Contaminants in Modern Microenvironments" ("HEXACOMM", FP7-PEOPLE-2012-ITN), which is a collaboration of several research groups investigating many aspects of indoor aerosols. The overall goal of the programme is "*to apply scientifically-based modelling and experimental methods to relate concentrations of aerosols in European domestic environments to its sources and human exposure implications*", based on the hypothesis that "*aerosols indoors constitute an important pathway of human exposure at levels detrimental to health based on recent research findings*". In this study, this hypothesis will be tested by focusing attention to bioaerosols.

The aim of the present study is to investigate the abundance and diversity of bioaerosols and to evaluate the significance of the indoor microbial load in the context of human exposure using molecular techniques. Several research questions are going to be addressed within case studies of HTS-based characterisation of bioaerosols from different types of indoor microenvironments; educational, residential and transportation. The results of this project could improve and expand our comprehension of the specific characteristics of bioaerosols in the indoor environment. Moreover, the data generated by this study could make a significant contribution to improving air quality by facilitating the design and development of effective methods for prevention and mitigation of the impacts of bioaerosols on human exposure.

Table 1.3 Examples of high throughput sequencing-based indoor air studies with sampling details included.

Study details				Sampling method					Molecular analysis	
Reference	**Study location**	**Target micro-organisms**	**Target PM fraction**	**Aerosol sampling instrument**	**Filter/ Collection substrate**	**Sampling time for each sample* /study period**	**Flow rate**	**Environmental parameters monitored**	**Sequencing technology**	**Dominant microbial taxa detected in indoor air samples**
Meadow et al. (2014)	University classrooms (8), indoors and outdoors (USA)	Bacteria	-	Button samplers (SKC)	25 mm Cellulose ester filter (1.4 µm pore size)	8 h ×2 (samples pooled) /over 9 days	4 L/min	Ventilation rate, occupancy	Illumina MiSeq of 16S rRNA	*Sphingomonas* spp. *Acinetobacter*-related OTUs
Hospodsky et al. (2012)	University classroom (1), indoors and outdoors (USA)	Bacteria	PM_{10} and $PM_{2.5}$	PM_{10} and $PM_{2.5}$ Personal Exposure Monitors (SKC)	37 mm Polycarbonate track-etched filter (0.8 µm pore size)	22.2 h ×2 cumulatively /over 7 days	10 L/min	Temperature, relative humidity. CO_2 concentration air exchange rate, PM_{10} & $PM_{2.5}$ mass	454 GS-FLX Titanium Pyrosequencing of 16S rRNA	*Proprionibacterineae, Micrococcineae Staphylococcus, Corynebacterineae*
Kembel et al. (2012)	Hospital patient rooms (researcher-occupied), indoors and outdoors (USA)	Bacteria	-	BioSampler liquid impinger (SKC)	Sterile molecular-grade water	1 h ×2 (samples pooled) /over 2 days	12.5 L/min	Temperature, relative humidity, absolute humidity and air flow rate	454 GS-FLX Titanium Pyrosequencing of 16S rRNA	*Betaproteobacteria*
Qian et al. (2012)	University classroom (1), indoors and outdoors (USA)	Bacteria and fungi	impactor cut points: 9.0, 4.7, 3.3, 2.1, 1.1, and 0.4 µm	8-stage non-viable impactor (only 6 stages used, New Star Environmental)	uncoated Polycarbonate track-etched filter	22.2 h cumulatively /over 8 days	28.3 L/min	Temperature, relative humidity, occupancy, air exchange rate, size-resolved PM mass & real-time counts	454 GS-FLX Titanium Pyrosequencing of 16S rRNA	*Propionibacterineae Xanthomonadceae Micrococcineae Sphingomonadales*
Gauzere et al. (2014)	Museum, office, hospital (France)	Bacteria	-	Air handling unit filtration connected to ventilation	Cellulose high efficiency particulate air (HEPA) 13 filter	4 weeks	$6.3 m^3/h$	-	454 GS-FLX Titanium Pyrosequencing of 16S rRNA	*Actinobacteria, Paracoccus, Acinetobacter, Micrococcus, Pseudomonas, Staphylococcus* spp.
Gauzere et al. (2014b)	Room in the Louvre Museum (France)	Bacteria and fungi	-	Experimental bioaerosol collector (a cyclone-like device)	Molecular-grade Water	40 min /over 6 months	1000 L/min	Temperature, relative humidity, occupancy, size-resolved real-time particle counts	454 GS-FLX Titanium Pyrosequencing of 16S rRNA	*Actinobacteria, Paracoccus, Acinetobacter, Pseudomonas* spp.

Robertson et al. (2013)	New York subway stations (7), indoors and outdoors (USA)	Bacteria and Eukarya	-	Omni 3000 fluid impinger (InnovaPrep LLC)	Phosphate-buffered saline & 0.005% Tween Solution	20 min /over 1.5 year	300 L/min	Temperature, relative humidity	454 GS-FLX Titanium Pyrosequencing of 16S rRNA & Sanger universal sequencing	*Staphylococcaceae*, *Moraxellaceae*, *Micrococcaceae*, *Agaricomycetes*, *Trichocomaceae*, wood rot fungi
Leung et al. (2014)	Hong Kong subway network (7 lines), indoors and outdoors	Bacteria	-	Sioutas Cascade Impactor (SKC) with a D-plate accelerator	25 mm Cellulose nitrate filter (0.2 µm pore size)	2 h ×4 (samples pooled)	9 L/min	Temperature, relative humidity, CO_2 concentration	Illumina MiSeq of 16S rRNA	*Micrococcus, Enhydrobacter, Propionibacterium, Staphylococcus, Corynebacterium, Sphingobium*
Adams et al. (2013)	Residences in a university-housing complex, indoors and outdoors (USA)	Fungi	-	Passive collection with suspended open-faced empty petri dishes	-	4 weeks /for summer & winter	n/a	Temperature, relative humidity	454 GS-FLX Titanium Pyrosequencing of ITS1 region	*Cryptococcus victoriae* *Cladosporium* sp. *Epicoccum* sp. *Penicillium* sp. *Cryptoccocus* sp.
Adams et al. (2014)	Residences in a university-housing complex, indoors and outdoors (USA)	Bacteria	-	Passive collection with suspended open-faced empty petri dishes	-	4 weeks /for summer & winter	n/a	Temperature, relative humidity	454 GS-FLX Titanium Pyrosequencing of 16S rRNA	*Staphylococcus* spp. *Sphingomonas* sp., *Corynebacterium* sp.
Shin et al. (2015)	Daycare centers (5) & Elementary schools (5), indoors and outdoors (Korea)	Bacteria and fungi	-	Filter cassettes	47 mm Cellulose ester filter (0.45 µm pore size)	10 h ×2 /over 2 months	24 L/min	Temperature, relative humidity	454 GS-FLX Titanium Pyrosequencing of 16S rRNA and ITS2 region	*Micrococcus, Paracoccus, Staphylococcus, Enhydrobacter, Hyphodontia, Thanatephorus* spp.
Wilkins et al. (2016)	Households (19) (Hong Kong)	Bacteria	-	Sioutas Cascade Impactor (SKC) with a D-plate accelerator	25 mm Cellulose nitrate filter (0.2 µm pore size)	12 h	9 L/min	-	Illumina MiSeq of 16S rRNA	*Propionibacterium, Acinetobacter, Staphylococcus, Corynebacterium, Methylobacterium*
Yamamoto et al. (2015)	Primary school classrooms (7), indoors and outdoors (USA, China, Germany & Copenhagen)	Fungi	0.4 - 1.1, 1.1 - 2.1, 2.1 - 3.3, 3.3 - 4.7, 4.7 - 9.0, > 9.0 µm	6-stage non-viable impactor (New Star Environmental Inc.)	Polycarbonate nuclepore filters	3 - 4 days (6h) cumulatively	28.3 L/min	Temperature, relative humidity, occupancy, air exchange rate	454 GS-FLX Titanium Pyrosequencing of ITS region	*Cryptococcus, Alternaria, Cladosporium, Wallemia* spp.

* ×2 denotes duplicates, ×3 denotes triplicates

Chapter 2

Investigation of the effect of filter-based sampling parameters on the recovered abundance and diversity of indoor bioaerosols

2.1 Introduction

Despite the public health importance of indoor bioaerosols in the context of human exposure (Douwes et al., 2003), the microbial content of indoor air has yet been poorly explored. The abundance and diversity of airborne micro-organisms remains inadequately described and little is known about the distribution of micro-organisms over airborne particles of different sizes. Molecular technologies based on DNA extraction and subsequent PCR, allowing an in-depth characterisation of airborne microbial communities, are increasingly being applied to bioaerosol science for microbial identification and quantification and demonstrate a great potential for exploring aerosol microbial ecology (Behzad et al., 2015; Kelley & Gilbert, 2013; Peccia et al., 2011). While these methods have been widely applied to relatively high-biomass microbial habitats like terrestrial and aquatic environments, application in indoor air research has been challenging, owing to the low biomass present in aerosols causing difficulties in obtaining sufficient microbial biomass available for DNA assay (Peccia & Hernandez, 2006).

Moreover, no standardized methods and protocols are currently available for the collection and analysis of bioaerosols. Several different bioaerosol collection methods and instruments that employ different mechanisms, such as filtration, impaction and impingement, have been developed and utilised in indoor air studies However, no standard method has been agreed upon to date. Data between studies are often difficult to compare due to differences in sampler design, collection time, air flow rate and analysis method.

Filtration is the most widely used technique for aerosol sampling and has been documented to be the most commonly used in bioaerosol molecular studies (Peccia & Hernandez, 2006). Filter-based sampling is a simple, low-cost and convenient assay that can provide aerosol collection efficiencies near 100% and, apart from being adaptable to a variety of analysis methods, it is also compatible with a variety of aerosol instruments. Several different types of filters in both fibrous and membranous forms exist, manufactured with a variety of pore sizes (0.01 - 10 µm). 47-mm diameter is a commonly deployed size for filter disks, owing to the

lower pressure drop for a given flow rate compared to smaller filters (e.g. 37-mm or 25-mm). It has been reported that the filter material and sample extraction method are factors that generally affect the performance of a filter-based sampler (Burton et al., 2005), and, thus, the selection of filter material should be considered when adopting a sampling strategy. However, there is no sufficient data defining the role of filter type (material or pore size) in molecular downstream analysis in terms of efficient DNA recovery.

In addition, selecting the optimum time for sample collection, representing the volume of air sampled, is a crucial parameter in bioaerosol collection efficiency as the typically low bioaerosol concentrations found in indoor air often lead to inadequate DNA recoverability. Therefore, molecular-based studies have often utilised relatively long (filter-based) sampling duration in combination with various flow rates, such as eight hours at 4 L/min (Meadow et al., 2014), 24 hours at 10 L/min (Emerson et al., 2017) or even several days at 28 L/min (Qian et al., 2012). Thus far, various studies have been conducted investigating the effect of sampling time on bioaerosol collection of filter samplers but the focus of most of them was on bioefficiency, examining the effects of prolonged sampling on viability and culturability of the collected cells (Woodward et al., 2004; Durand et al, 2002; Wang et al., 2001; Lin, 1999; Lin & Li, 1998). However, this is not of concern when analysis is carried out on both living and dead cells using molecular detection methods. To our knowledge there has been only one culture-independent study investigating the impact of sampling duration on the amount of recovered DNA that showed that extended sampling periods might lead to progressive degradation of the collected bacterial DNA (Luhung et al., 2015). Nevertheless, this result was about sampling times in the range from 8 up to 20 hours and thus there is a lack of information regarding shorter times. Furthermore, the impact of filter-based sampling parameters on the yielded microbial diversity has been also ignored to a large extent.

Finally, even though, bioaerosol filter-based sampling is most typically non- size-selective, filters can also be used as collection substrates on multi-stage impactors, allowing us to explore the different size fractions of bioaerosols. Yet, there is a lack of knowledge regarding the particle size distribution of the microbial load collected via filtration, and the DNA yielded by total airborne particulates filter-based sampling has not been evaluated in relation with size-resolved collection methods.

The present study reported here aimed to investigate the effect of filter-based sampling parameters including filter material, pore size and sampling time on bioaerosol molecular

downstream processing for characterisation of the bacterial abundance and diversity in indoor air. Moreover, the overall performance of filter-based bioaerosol collection was further investigated in relation with size-selective sampling. Replicated sampling experiments using some of the most commonly used filters were carried out in an indoor environment under controlled conditions. Samples were assessed in terms of DNA recovery by universal bacterial qPCR quantification and in terms of bacterial diversity by Illumina MiSeq sequencing of 16S rRNA gene.

2.2 Methods

2.2.1 Experimental setup

A mechanically ventilated office located within a university building in the southeastern UK (Colchester, Essex) was selected for all air sampling in this study. The volume of the room is approximately 57.8 m^3 (L = 4.8 m, W = 4.3 m, H = 2.8 m). Apart from the easy access and securing of required experimental conditions, this location was selected because of consistent low occupancy (1-2 people) and activity levels (mainly computer work), offering low temporal variation of the background aerosol concentrations, along with absence of any prevalent biological sources. Since the focus of this work was to compare different sampling parameters under as similar as possible background conditions, all experiments were carried out under no occupancy. Human occupancy is a major source of indoor bioaerosols and the absence of it contributes to less variations. Even during equipment performance monitoring and sample collection, care was extended to avoid and to prevent any movements or activities that would result in high temporal variation due to generation or re-suspension of particles. However, it should be noted that variation occurring due to outdoor air blown via the ventilation system or air leaked in through the door could not be controlled. All sampling runs were conducted during working hours. The temperature of the room, controlled by the HVAC, was set at the thermal comfort zone range (21-23℃). Temperature and relative humidity measurements were routinely recorded along with filter sampling.

All filters used for sampling were sterilised by autoclaving (121℃ for 15 mins) and were loaded into 50 mm single-stage closed-faced membrane filter holders without any size-cut (Sartorius AG, Goettingen, Germany). Prior to sampling, filter holders were washed thoroughly with 10% bleach, rinsed with ultrapure water (Milli-Q, Millipore) and 70% (v/v) ethanol, placed in autoclavable pouches and sent for autoclaving After sampling, the filter

holders were sealed with parafilm and placed back in the same autoclaved bags, sent back to the lab where filters were placed in sterile Petri-dishes, sealed with parafilm and stored at -20°C until further analysis. When carrying out all investigations, manipulations of the filters were performed with autoclaved equipment in a Biosafety Level II cabinet. Unexposed filters (blank controls) were also analysed alongside those used for sampling to provide procedural controls.

Effect of sampling time and filter material

The impact of sampling duration and collection membrane type on DNA recovery from filters was investigated by conducting side-by-side sampling experiments. The following four commercially available 47-mm diameter filters with 0.4-µm pore size were tested: polytetrafluoroethylene hydrophobic (PF), nuclepore track-etched polycarbonate hydrophilic (PC), cellulose acetate (AC) and nitrate cellulose (NC). All filters were provided by Whatman (UK). The four different types of samples were filtered simultaneously at a flow rate of 28 L/min for sampling periods of 8 h, 4 h, 2 h, 1 h, 30 min, 20 min and 10 min corresponding to 13.6, 6.8, 3.4, 1.7, 0.85, 0.57 and 0.28 m^3 of collected air respectively. Each sampling set was repeated three times during the same day, with the exception of the 4-hour and 8-hour samplings that due to their long duration were carried out on three different days.

Effect of filter pore size

Further assessment of the performance of different filter-based sampling parameters was carried out using the polycarbonate filter (PC). In order to examine the effect of membrane pore size, two different pore-size 47-mm diameter filters, 0.2 µm and 0.4 µm, were loaded into closed-faced samplers and three sets of samples operated in parallel, consisting of three replicates each, were collected. Due to the higher back pressure generated by the 0.2-µm pore size filter and consequent reduction in pump capability, pumps could not achieve a consistent flow rate of 28 L/min and therefore samples were collected at 24 L/min for 70 min (equivalent to sampling volume collected at 28 L/min for 1 h).

Effect of size-resolved sampling

The impact of total suspended particles filter-based (non- size-resolved) sampling on DNA recovery of airborne micro-organisms was also evaluated in relation with size-fractionated collection method. Three sets of 48 hours-duration experiments, which employed the use of a multistage impactor operated in parallel with three filter samplers (PC 47-mm diameter, 0.4-µm pore size), were carried out. This prolonged sampling time was chosen to allow for

sufficient collection of bioaerosol material for molecular analysis. For collection of size-distributed aerosol samples, a modified version of the viable Andersen impactor (Thermo Scientific Electron), operated at a flow rate of 28.3 L/min, was utilised. The Andersen sampler has six stages with lower cut sizes (aerodynamic equivalent diameter) of 0.65, 1.1, 2.1, 3.3, 4.7 and 7.0 µm and is designed for culture-based sampling (Andersen, 1958). For our study, the impactor was modified by placing PTFE-made discs (86-mm diameter, 6 mm thick) in the impactor' s glass petri dishes. This solid surface can be loaded with any type of filter, replacing the traditional collection on agar or membrane overlay on agar that has been used alternatively, for the purposes of non-viable sample collection and downstream applications. 90-mm nuclepore track-etched polycarbonate filters (0.4 µm, Whatman) were used as sampling substrates in the impactor.

All sampling experiments are summarised in Figure 2.1.

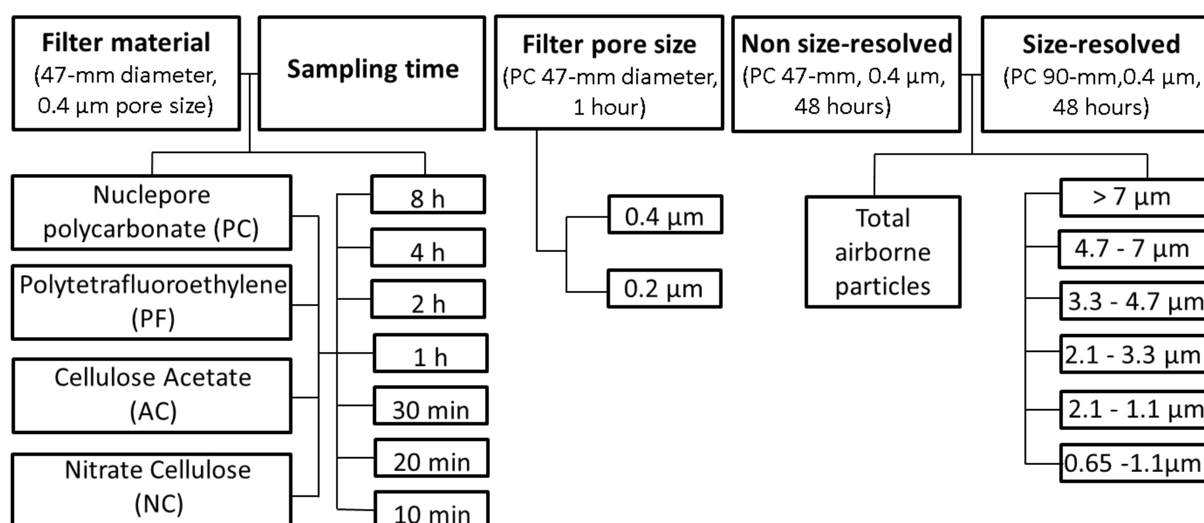


Figure 2.1 Workflow diagram of the filter-based sampling parameters comparisons.

Limitations

Finally, it has to be noted that due to a strong seasonal effect observed, resulting in difficulties recovering sufficient biomass yields in our 1-hour duration sampling efforts during winter and spring, experiments presented here are the ones conducted during summer 2016.

2.2.2 Sample processing

2.2.2.1 Development and optimisation of DNA extraction protocol from filter-samples

DNA was extracted directly from the filters using a combination of mechanical (bead beating), chemical (detergent) and physical treatments (heat treatment) to disrupt effectively the cells. For purposes of sample handling facilitation and in order to avoid sample losses caused by cutting and splitting 47-mm filters, the 2ml-volume bead-beating tubes, used most commonly in DNA extraction protocols, were replaced with 5-ml tubes, an approach that has been adopted by the commercially available Mobio PowerWater DNA extraction kit. Unless stated otherwise, Axygen® 5-mL Self Standing Screw Cap tubes (#SCT-5ML-S, sterile, RNase-/DNase-free) filled with 0.75 g of sterile 0.1-mm zirconium/silica beads (Biospec, USA) were used. Lysing beads had been washed with sodium hypochlorite (0.05%), rinsed with milliQ water, dried overnight and then baked at 300 ^{o}C for 4 hours, prior to use. Filling of tubes was followed by an autoclaving cycle at 121 ^{o}C for 15 mins. Lysis buffer used consisted of 2.5% (v/v) sodium dodecyl sulfate (SDS), 10mM Tris-HCl pH 8, 25mM Na_2EDTA pH 8, 100mM NaCl and molecular biology grade water. Using higher SDS concentration was avoided due to the known PCR inhibitory effects of ionic detergents. The extraction buffer was sterilised by autoclaving and aliquot used for each batch of samples, was also filter-sterilised using Minisart™ Plus Syringe Filters (0.2 µm, Sartorius, UK) prior to extraction. Bead beating of samples was carried out using Vortex-Genie® 2 vortex equipped with a MOBIO Vortex Adapter for 5-ml tubes. Use of a vortex adapter is a cost effective way to achieve cell lysis that can replace bead beating/homogenisation instruments. Vortex-Genie® does not indicate speed range, but, according to manufacturer's specifications, the maximum speed corresponds to 3200 rpm. The subsequent purification method used was based on the classic phenol:chloroform-based procedure described in Sambrook & Russel protocols (2001). An advantage of using this method is the solubility of specific materials such as the polycarbonate and cellulose acetate membranes in phenol/chloroform solution. DNA purification was followed by isopropanol precipitation which is the preferred method for precipitating DNA from large volumes since only one volume is required. In order to minimise co-precipitation of salt that might interfere with downstream applications, overnight incubation (commonly used in nucleic acid precipitation methods involving ethanol or polyethylene glycol) was avoided and isopropanol precipitation was carried out at room temperature. To enhance the recovery of small amounts of nucleic

acid, co-precipitant glycogen (20 mg/ml, Thermo Scientific) was also added prior to incubation (Williams et al., 2001; Hengen, 1996).

DNA extraction

47-mm filters were aseptically inserted into sterile 5-ml lysing tubes using autoclaved forceps by rolling the filter into a cylinder with the top side facing inward. Cells were lysed by bead beating in 1-ml extraction buffer at Vortex-Genie® 2 with a 5-ml tube adapter (MOBIO) at maximum speed for 12 minutes. For complete cell wall disruption, filter samples were incubated in a 75°C water bath for thirty minutes. The cell lysate was washed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) pH 8.0. The supernatant was isolated after centrifuging at 2,000 × g for 5 minutes in a swing bucket centrifuge (Heraeus Megafuge 40, Thermo Fisher Scientific) and transferred into new sterile 2-ml microcentrifuge tubes. The supernatant fractions from the PF and NC filters, that are not soluble in phenol/chloroform, were subjected to further centrifugation (11,337 × g for 5 mins in Minispin, Eppendorf) in order to remove the retained phenolic compounds and the upper aqueous phase was conveyed into new tubes (2 ml). Moreover, an extra purification step was performed by adding 750 µl chloroform to all samples, followed by centrifugation at 11,337 × g for 5 mins in microcentrifuge (Minispin, Eppendorf). The nucleic acids were precipitated from the aqueous phase using equal volume of isopropanol and 2.5 µl of coprecipitant glycogen (20 mg/ml, Thermo Scientific) and incubated for about 100 mins at room temperature. Samples were then centrifuged at 11,337 × g for 30 mins in a microcentrifuge (Minispin, Eppendorf), the supernatant was removed and the pelleted DNA was washed with 70 % (v/v) ice-cold ethanol, air-dried and resuspended in 35-µl sterile water (Fermentas, Thermo Scientific).

90-mm (PC) filters were cut into two pieces and split into two 5-ml bead beating tubes and cells were lysed as described above, except bead beating duration was 15 minutes and half-filter samples were pooled in the end.

All DNA protocols were carried out in a laminar-flow hood, using aerosol resistant pipette tips (ZAP^{TM} Premier Filter Tips, Alpha Laboratories, UK) to reduce the likelihood of contamination. Negative control isolations lacking sample were carried out in parallel throughout all extractions to identify any contamination of the final DNA extracts.

2.2.2.2 16S ribosomal RNA gene quantification

Bacterial DNA recovery from filter samples was assessed by quantitative PCR with universal 16S rRNA primers Bakt_341F/Bakt_805R (Herlemann et al., 2011). Even though the number of copies of the 16S rRNA gene is not directly related to cell number due to the presence of multiple ribosomal operons in the bacterial genomes, it is used as a measure of microbial biomass.

Standard curve preparation

To quantify 16S rRNA gene abundance, a standard curve was generated by serially diluting bacterial genomic DNA extracted from *Escherichia coli* DH10b (also known as TOP10). The numbers of copies for the qPCR standards were calculated using the following equation:

$$Number\ of\ copies = \frac{\text{DNA Concentration}\ [ng/\mu l]\ \times\ 6.022 \text{ x } 10^{23} [\text{copies}\ mol^{-1}]}{DNA\ length\ [bp]\ \times 660\ [g\ mol^{-1} bp^{-1}]\ \times 1 \text{ x } 10^{9} [ng/g]}$$

where DNA concentration (ng/ul) was quantified fluorometrically using Quant-iT™ PicoGreen® Assay kit, 6.022×10^{23} (copies mol^{-1}) is the Avogadro's number, 660 (Da) is the average molecular weight for a base pair of double-stranded DNA and DNA length (bp) is the genome size of *E. coli* (4,686,137 bp; accession number: CP000948; Durfee et al., 2008).

qPCR amplification

PCR reactions were performed in triplicate on a BioRad CFX96 Real-Time System/C1000 Thermal cycler. Reaction mixture (10 µl) included 5 µl (1×) SensiFAST™ SYBR® No-ROX Kit (Bioline), 0.2 µl forward and reverse primers (10 µM), 3.6 µl microbial DNA-free water (Qiagen) and 1 µl DNA template. The amplification was carried out under the following conditions: 95°C for 3 min, followed by 40 cycles of 5 s of denaturation at 95°C and 30 s of annealing and extension at 60°C, finishing with a melting curve analysis ranging from 65°C to 95°C with 0.5°C increment. Data were acquired by CFX Manager Software which automatically determined threshold values.

Results were reported in units of 16S rRNA gene copies by correcting for the number (7) of 16S rRNA genes per *E. coli* genome and expressed per cubic meters of air (the derived copy number from 1µl DNA used per reaction mix was multiplied by the total volume of the DNA extract -35µl-, in order to calculate the copies per each filter sample, and then divided by the total air sampling volume in m^3 from which the DNA was extracted), except when comparing

different sampling times (i.e. volumes) where results were expressed as 16S rRNA genes per filter.

2.2.2.3 Internal Transcribed Spacer region 2 detection

Fungal DNA recovery from different sampling times was assessed by PCR amplification of the internal transcribed spacer region 2 (ITS2) of the rDNA gene using the ITS3 and ITS4 primers (White et al., 1990). PCR reaction mixtures (25 µl) contained 2 µl of DNA template, 1 µl of each primer (10 µM), 12.5 µl (1×) of MyTaq® Red Mix (Bioline) and 8.5 µl ultrapure water. Cycling conditions consisted of: 3 min of denaturation at 95°C, followed by 35 cycles of 15s at 95°C, 15s for primer annealing at 56°C, 30s at 72°C for extension and a final extension step at 72°C for 7 min. qPCR of ITS region was decided not to be performed due to the known variability of the internal transcribed spacer region even within species or over the lifetime of the fungi.

Agarose gel electrophoresis and Ethidium bromide (EtBr) staining

The presence and the size of PCR amplicons was verified via 1.5% (w/v) 1xTAE agarose gel electrophoresis. DNA was electrophoresed in 1×TAE buffer [40 mM Tris acetate, 20Mm glacial acetic acid and 1mM EDTA (pH 8.0)] for 40 minutes at 105 V. A molecular weight DNA marker (GeneRuler DNA Ladder Mix ready-to-use, 0.1µg/µl, 50µg, Thermo Scientific) was added into one well to provide a reference for the determination of the fragment lengths. DNA was stained in Ethidium bromide (1 µg/ml) for 30 min and visualised using Alpha Imager®EP uv transilluminator (Alpha Innotech Ltd.).

2.2.2.4 16S ribosomal RNA gene amplicon sequencing

In order to examine the effect of filter-based sampling parameters on DNA recovered at the compositional level, DNA isolated from selected samples was further processed for characterisation of the microbial diversity using Illumina MiSeq technology. Investigation included PC filter samples collected at different sampling times (1 hour versus 8 hours), with different pore-sizes (0.2-µm versus 0.4-µm) and at different cut sizes (total suspended particles versus size-resolved sampling). The V3 and V4 hypervariable regions of the bacterial 16S rRNA gene was amplified by barcoded PCR with primers Bakt_341F and Bakt_805R (Herlemann et al., 2011), in accordance with the Illumina protocol. Library preparation was carried using Illumina's Nextera XT Index kit and the recommended Library Preparation Workflow ("16S Metagenomic Sequencing Library Preparation", Part

#15044223 Rev. B). Negative controls, both no template (n=3) and blank filters (n=3), were also included through all steps in order to check for contamination

Preparation of 16S rRNA libraries for Illumina MiSeq sequencing

Templates from genomic DNA were amplified using the Bact_341F/Bact_805R primer pair (Herlemann et al., 2011) with added overhang adapter sequences (Appendix S2), as described in the Illumina dual-indexing protocol. Cycling conditions for "Amplicon PCR" (PCR1) were: 3 min at 95°C, 30 times 30 s at 95°C followed by 30 s at 55°C, then 30 s at 72°C and finally 5 min at 72°C. Each reaction was done in a 30µl volume consisting of the following components: 15 µl (1×) MyTaq Hot-Start Red Mix (Bioline), 1.2 µl of each primer (5 µM), 3 µl template and 9.6 µl Microbial DNA-Free Water (Qiagen). 5 µl of the PCR product was run on agarose gel to verify the size. AMPure XP beads (Beckman Coulter, USA) were used to purify the amplicons away from free primers and primer dimer species.

A subsequent 8 cycle amplification step was performed to add dual 8 base indices and Illumina sequencing adapters using the Nextera XT Index Kit. "Index PCR" (PCR2) reaction mixture (50 µl) contained 5 µl of PCR1 product, 25 µl (1×) MyTaq Hot-Start Red Mix (Bioline), 5 µl Nextera XT Index Primer 1 (N7xx), 5 µl Nextera XT Index Primer 2 (S5xx) and 10 µl PCR Grade water (Qiagen). Cycling conditions were as described before. An extra AMPure XP beads clean-up step was used to purify the final products before quantification.

Libraries were quantified using Thermo Picogreen assay on FLUOstar Omega Microplate Reader (BMG LABTECH), normalised and pooled into a single composite sample that was then cleaned using a QIAquick PCR Purification spin column (Qiagen, UK). Final pool was quantified with Nanodrop ND 1000 and the expected size was checked on an agarose gel, while the quality trace of the final library was verified on a Bioanalyzer DNA 1000 chip (Agilent technologies).

The final pool was denatured in 0.2 N NaOH, diluted to a final concentration of 9 pM and combined with 20% PhiX Control library (v3, Illumina) to modulate the overall sample base composition to help facilitate a successful run before being loaded into a v3-chemistry 600-cycle kit reagent cartridge (Illumina, Inc.). 2×300-bp paired-end sequencing was performed on Illumina MiSeq platform at the School of Biological Sciences, University of Essex.

Bioinformatic processing

The quality of the raw 16S rRNA gene sequencing data was evaluated with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Single-end bioinformatic analysis was performed on the forward sequencing reads. Sequences were trimmed to a minimum nucleotide Phred quality score of 20 and minimum sequence length threshold of 180 bp using the fastq_quality_trimmer from the FastX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Quality-trimmed sequences were then processed using the open-source analysis package Quantitative Insights Into Microbial Ecology, QIIME version 1.9.1 (Caporaso et al. 2010). For each library, fastq files were merged and converted into a single file containing sequences from all samples in fasta format using multiple_split_libraries_fastq.py. Chimeric sequences were identified and removed using UCHIME algorithm (Edgar et al., 2011) implemented in VSEARCH (Rognes et al., 2016) with the RDP trainset 16 (02.2016) for 16S rRNA gene (Cole et al., 2013). Clustering of sequences into operational taxonomic units (OTUs) was performed using open-reference OTU picking protocol with UCLUST method (Edgar, 2010) at 97% similarity against the RDP trainset 16 database (02.2016). The same database was employed to assign taxonomy to representative sequences of the OTUs by using QIIME's UCLUST consensus taxonomy assigner. Any resulting singleton OTUs were filtered-out using filter_otu_from_otu_table.py with minimum sequence count of 2. OTUs that appeared in blank controls were subtracted from the data. After removal of contaminants, chloroplast, archaeal sequences and unassigned reads, the resultant OTU table was normalised to the sample with the lowest number of total sequence reads to account for differences in sequencing depth between samples. The OTU table was also subjected to abundance-based filtering in order to minimise the error associated with spurious OTUs and artefactual reads and focus analysis on taxa driving the composition. Low-abundance OTUs, represented by 5 or fewer sequences, were removed using the min_count_fraction flag in QIIME for filter_otus_from_otu_table.py.

2.2.3 Data analysis

2.2.3.1 qPCR quantification data

Shapiro-Wilk test was used for normality testing of qPCR data and non-parametric tests were used when the assumptions for the parametric equivalents were not achieved. In particular, Kruskal-Wallis test was used to compare differences in the DNA recovery across the four tested filter materials and to compare bacterial yields retrieved across different sampling

times. Kruskal-Wallis test was followed by Mann-Whitney U tests used as post hoc tests for multiple comparisons. Independent samples t-test was also used to compare the performance of the two different pore-size filters tested and to determine if there are significant differences between the total concentrations recovered from the impactor and the filter-based samplers. Statistical significance for all tests was set at p-value < 0.05. 16S rRNA gene abundance plots were generated using Microsoft Office Excel. Boxplots and all statistical analyses were implemented using the R platform (R Core Team, 2014).

Particle size distribution data

Particle size distribution data were plotted using normalised concentration ($dN/dlog\ d_p$) that is independent of the size bin width. *dN* is the bacterial concentration (i.e. 16S rRNA gene copy number) in the size range and *dlog dp* is the difference in the log of the size channel width. *dlog dp* is calculated by subtracting the log of the lower bin boundary ($d_{p,l}$) from the log of the upper boundary ($d_{p,u}$) for each channel.

$$dN/d\log d_p = \frac{dN}{\log d_{p,u} - \log d_{p,l}}$$

To quantitatively characterise the representative particle-size distributions, geometric means (d_g) and geometric standard deviation (σ_g) of aerodynamic diameters were calculated by assuming a 50 µm cut-off size for the top May impactor stage and 20 µm for the upper size bound of the Andersen impactor, using equations (1) and (2), (Hinds, 1999):

$$d_g = \exp\left(\frac{\sum n_i \ln d_i}{N}\right) \quad (1)$$

$$\ln \sigma_g = \left(\frac{\sum n_i (\ln d_i - \ln d_g)^2}{N}\right)^{1/2} \quad (2)$$

where n_i is the number of 16S rRNA genes in each (i) particle-size interval, d_i is the geometric midpoint of the interval, N is the total number of genes and the summation is carried out over all i.

The computations were executed using Microsoft Office Excel Visual Basic GM calculator (https://sourceforge.net/projects/gmcalculator/) application (Yamamoto et al., 2014).

2.2.3.2 Sequencing data

Alpha diversity

Rarefaction curves showing the captured diversity as a function of sampling depth were constructed based on the number of observed OTUs in the samples prior normalisation, using the alpha_rarefaction.py QIIME workflow script, in order to assess the adequacy of the sequencing effort. Except for rarefaction curves, all subsequent analysis was conducted on the normalised filtered data by focusing on the most abundant OTUs. Within-sample (i.e. alpha) diversity was evaluated using OTU richness and Shannon's index. Richness accounts for the number of different OTUs present in each sample and calculated by alpha_diversity.py script. The Shannon diversity index (H') takes into account both richness and relative abundance of each OTU ($H' = -\Sigma\, p_i\, ln(p_i)$, where p_i is the proportion of the ith OTU) and was estimated by Vegan library within R environment (R Core Team, 2014). To determine if the measures of alpha diversity were significantly different between types of samples, Mann-Whitney U test was used. Correlations between bacterial richness and qPCR-based abundance were assessed using either Pearson's correlation on normally distributed variables or Spearman's rank coefficient analysis when data were not normally distributed. The number of core OTUs, defined as the OTUs that are present in all samples, was determined using compute_core_microbiome.py within QIIME. The percentage of overlapping OTUs among samples was calculated by Venny 2.1 (Oliveros, 2007).

Beta diversity

In order to assess differences in the composition of the bacterial communities among samples (i.e. beta diversity), the abundance-based (weighted) and incidence-based (unweighted) phylogenetic UniFrac metrics of community dissimilarity were used (Lozupone & Knight, 2005). The metrics were computed using beta_diversity_through_plots.py and the resulting distance matrices were visualized with Principal Coordinates Analyses (PCoA) 3D-plots, generated by EMPeror software (Vázquez-Baeza et al., 2013), within QIIME. Each point in the plots represents the composition in the sample, such that those samples that are closer together share more taxa in common than those further apart. The statistical significance of differences in bacterial composition among groups of samples was determined using permutation-based multivariate analysis of variance (PERMANOVA) (Anderson, 2001) and analysis of similarities (ANOSIM) (Clarke, 1993) with 9999 permutations, performed with compare_categories.py. Statistical significance was set at $p\text{-value} < 0.05$.

Taxonomic composition

The taxonomic distribution of OTUs across samples was determined using QIIME script summarize_taxa.py and visualised with barplots and heatmaps generated in R version 3.1.2 statistical computing environment (R Core Team, 2014).

2.3 Results

2.3.1 The effect of filter material and sampling time on bacterial DNA recovery

Bacterial DNA was successfully recovered from all filters and quantified by qPCR. The reaction efficiency of the standard curve for bacterial load evaluation ranged from 84.2% (slope=-3.768) to 88.4% (slope=-3.635), with the correlation coefficient $R^2 > 0.99$ and the y-intercept value varying between 32.21 and 32.78, among runs. No-template controls included in each run yielded Ct values within the range between 35.88 and 37.81. Figure 2.2 presents the average bacterial concentration recovered for each type of filter, among the three experimental repeats performed for each sampling time, as determined by qPCR. The maximum sampling duration (i.e. 8 hours) yielded the highest bacterial quantities for all filters (average = 5.08×10^4 16S rRNA gene copies per filter), compared to the other sampling times tested. Contamination background in the blank (autoclaved) filters (42 - 128 16S rRNA genes per filter) demonstrated, on average, 80% less bacterial amount compared to the lowest mean recovered bacterial abundance corresponding to 10 minutes sampling duration.

As determined by Kruskal Wallis test, there was no statistically significant difference in the bacterial DNA recovered among the four types of filters (p-value = 0.61, >0.05). However, when examining the effect of sampling duration on recovery of airborne microorganisms collected using filter-based sampling (Fig. 2.3), the data presented clearly that increased sampling times (i.e. volumes) leads to statically significant increased bacterial yield (Kruskal Wallis test, $p < 0.001$). However, subsequent Mann-Whitney U pair-wise tests (Table 2.1) indicated that there were no substantial differences observed between the 4 hours and 2 hours

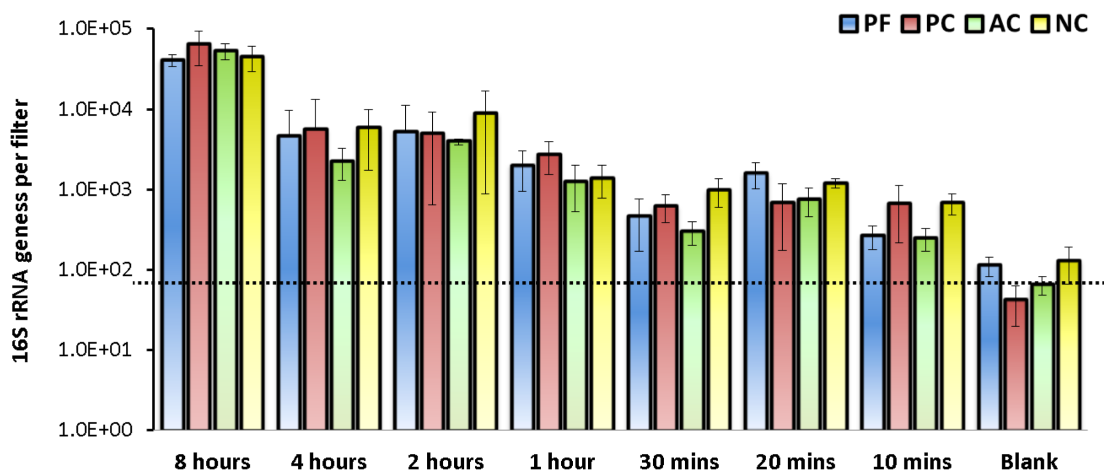


Figure 2.2 Quantitative real-time PCR data of 16S rRNA gene for indoor air samples collected at 28 L/min over different sampling periods using PF (PTFE), PC (polycarbonate), AC (cellulose acetate) and NC (nitrate cellulose) filters. Results are presented as 16S rRNA genes per filter (i.e. DNA extract) and not per cubic meter of air (volume of sampling air is not constant across different sampling times) so that data can be comparable. Error bars represent standard deviation (n=3 repeats). Dotted line indicates the average bacterial load found on blank filters. Axis y is on log scale.

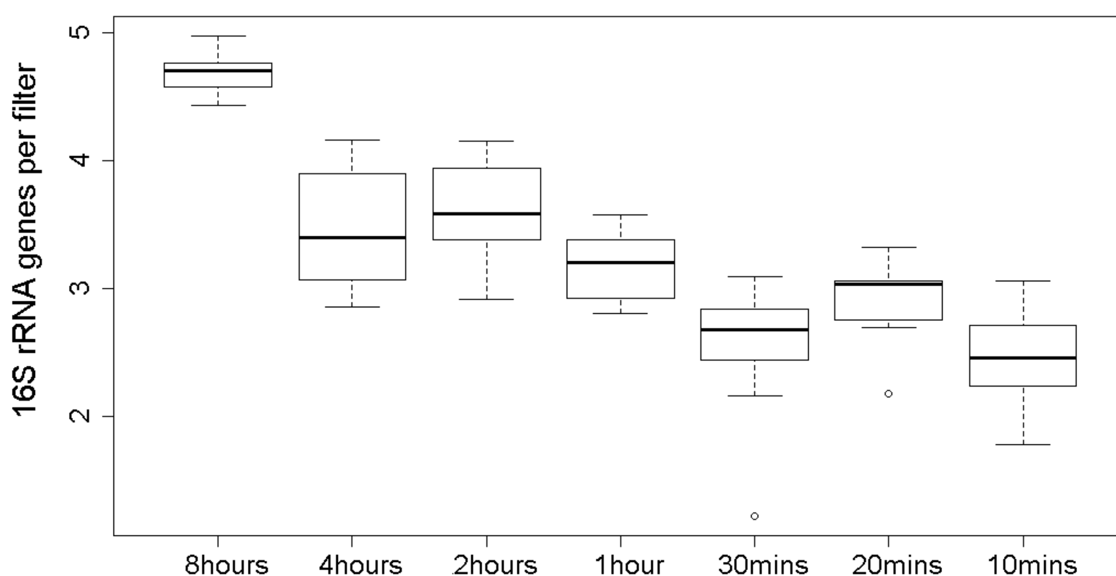


Figure 2.3 Bacterial abundance (log16S rRNA gene copies per filter) for air samples collected using different sampling times, as determined by qPCR of 16S rRNA gene. Each box includes values from replicated (x3) bioaerosol collection using four different filters (n = 12), with blank filter values subtracted. Box boundaries indicate the first (25th percentile) and third (75th percentile) quartiles. The median is given as the horizontal line within the box, while the whiskers (dashed lines) indicate the maximum and minimum values. Outliers are indicated with circles beyond the whiskers. Axis y is on log scale.

($p > 0.05$) or 1-hour ($p > 0.05$) durations. Moreover, 30 minutes exhibited lower concentrations than the 20-mins experimental set ($p < 0.05$), showing that short-time sampling is strongly affected by temporal variability.

Table 2.1 Mann-Whitney U test pair-wise comparisons (p-values) between bacterial yields recovered from different sampling times. Bolded values indicate non-statistically significant differences (i.e. p-value > 0.05).

	8 hours	**4 hours**	**2 hours**	**1 hour**	**30 mins**	**20 mins**	**10 mins**
8 hours	-	<.001	<.001	<.001	<.001	<.001	<.001
4 hours	-	-	**0.343**	**0.219**	<.001	0.008	<.001
2 hours	-	-	-	0.016	<0.01	0.001	<.001
1 hour	-	-	-	-	<0.01	0.045	<.001
30 mins	-	-	-	-	-	0.024	**0.319**
20 mins	-	-	-	-	-	-	0.003
10 mins	-	-	-	-	-	-	-

2.3.2 The effect of filter material and sampling time on fungal DNA detection

Application of DNA extraction protocol was successful in recovery of fungal yield on all types of filters. Detection of fungal DNA isolated from air samples collected over the different sampling times is shown in Fig. 2.4.

Blank samples did not yield any detectable signals and intensity of PCR bands as a measure of recovered biomass was found to increase with the sampling time reflecting the captured sampling volume of air from 10 minutes up to 1 hour. Similar intensity was observed among the bands corresponding to the samples collected over 1 hour, 2 hours and 4 hours, in accordance with results described in 2.3.1. Weaker signals exhibited by the 8-hour samples were attributed to PCR inhibition.

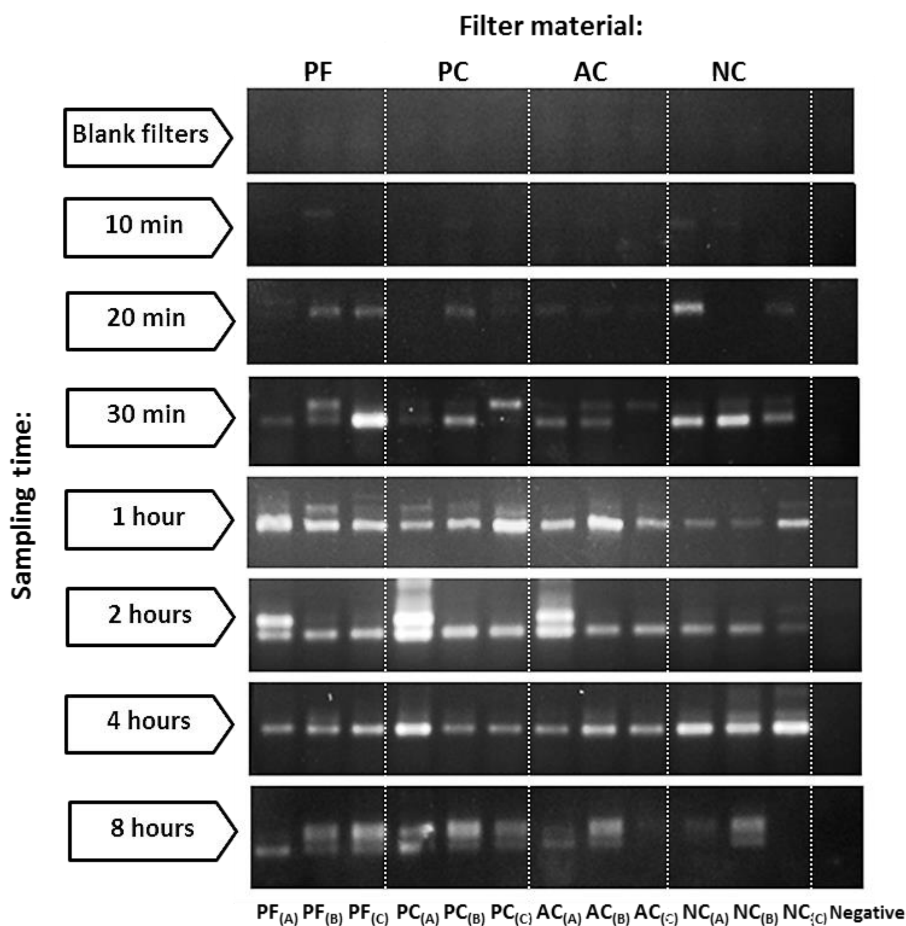


Figure 2.4 Gel electrophoresis images showing the results obtained from PCR amplification of ITS2 region of the DNA recovered from different sampling volumes of air, using four different types of filters (PF, PC, AC, NC). PCR product size is 385 bp.

2.3.3 The effect of filter pore size on bacterial DNA recovery

PC filters were used to further evaluate the performance of different sampling parameters. In the next set of experiments, the possible effect of filter pore size on bacterial DNA recovery of collected microorganisms was investigated. The qPCR amplification efficiency of the standard curve used for the bacterial load evaluation was 90.2% (slope = -3.582), with the correlation coefficient R^2 being 0.998, and the y-intercept value was 33.25. The no template controls resulted in a mean Ct value of 36.73. Results for each side-by-side sampling set are presented in Figure 2.5. Error bars indicate relatively high variation in bacterial concentration recovered among replicates.

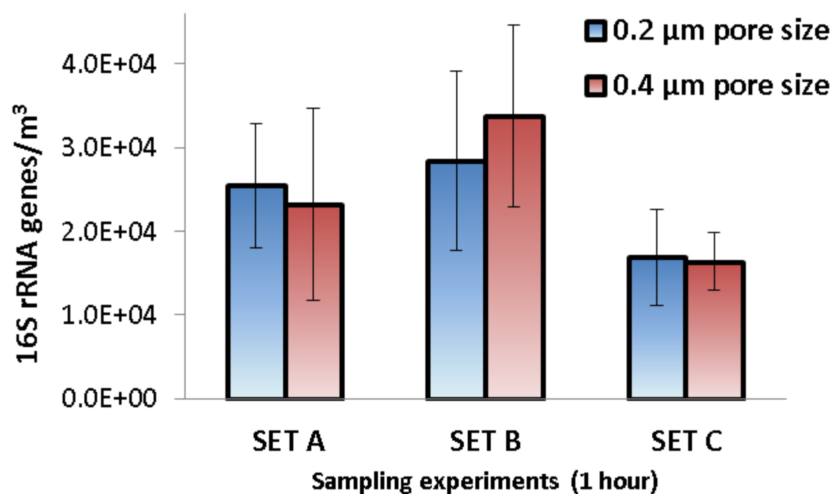


Figure 2.5 Quantitative real-time PCR data of 16S rRNA gene for indoor air samples collected over three 1-hour simultaneous sampling rounds ("sets") using PC filters of different pore size (0.2 µm & 0.4 µm). Results are presented as 16S rRNA gene copies per cubic meter of air. Error bars represent standard error (n = 3 replicates).

Data was normally distributed as assessed by the Shapiro-Wilk test and therefore an independent t-test was run to determine if there were differences in the recovered bacterial abundance between the 2 types of filers. No statistically significant advantage in recovering greater biomass was found for any of the two pore sizes (p-value = 0.905).

2.3.4 The effect of size-resolved sampling on bacterial DNA recovery

The bacterial load recovered by filter-based bioaerosol collection was compared against the bacterial quantities yielded by parallel size-resolved impaction-based sampling. The standard curve used for the qPCR determination of the recovered 16S rRNA gene abundance exhibited 90.5% amplification efficiency (slope=-3.572, R^2=0.998, y-intercept=33.79). Negative (no-template) controls included in the run resulted in a mean Ct value of 37.49. Total bacterial concentrations recovered from the 6-stage impactor varied from 2.79×10^3 (set 1) to 1.70×10^4 (set 3) 16S rRNA gene copies per m^3 of air, among the three experimental repeats. Examination of the size-resolved profiles (Fig. 2.6) reveals that during all sampling sets bacteria were highly enriched in the largest size stage (> 7 µm), while minimum concentrations were recovered in the smallest size range (0.65 – 1 µm). Strong peaks were also observed on the stages corresponding to the 3.3 - 4.7 and 4.7 - 7 µm aerodynamic diameter size ranges for sets 2 and 3 respectively. The d_g values varied substantially, ranging from 4.58 (set 3) to 10.27 (set 1) µm (Table 2.2).

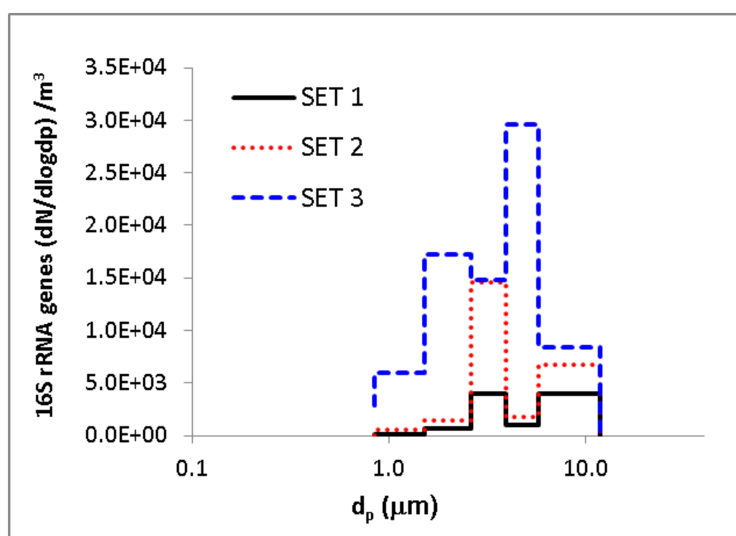


Figure 2.6 The particle-size distribution of airborne bacteria determined by qPCR (y axis, dN/dlogd$_p$16S rRNA genes per m^3 of air) for the three experimental sets. The upper limit of particle size for the stage of > 7 µm was set at 20 µm.

Table 2.2 Geometric mean (d_g), geometric standard deviation (σ_g) of aerodynamic diameters and total bacterial abundance recovered by collection with a modified 6-stage Andersen impactor.

Experiment Repeat	**Geometric mean** d_g (µm)	**Geometric standard deviation** σ_g	**Total concentration** (16S rRNA genes/m^3)
Set 1	10.27	2.73	2.79E+03
Set 2	6.97	2.26	6.04E+03
Set 3	4.58	1.87	1.70E+04

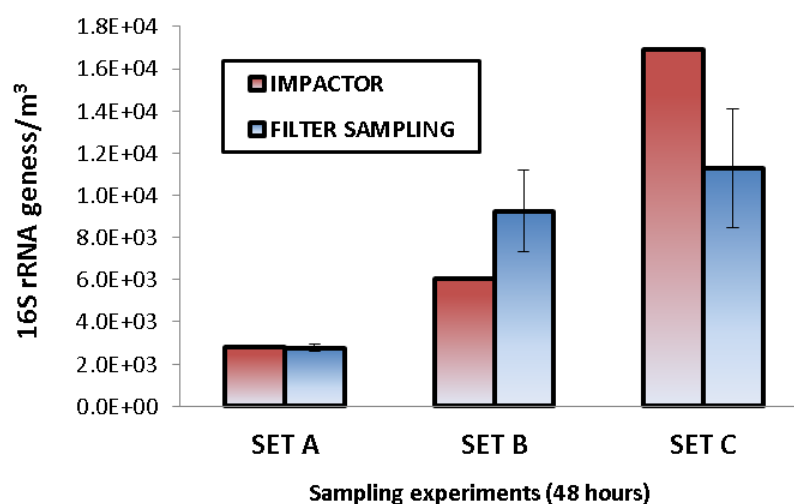


Figure 2.7 Quantitative real-time PCR data of 16S rRNA gene for indoor air samples collected over three 48-hour simultaneous sampling rounds (“sets”) using a modified 6-stage Andersen impactor against three filter samplers. Results are presented as 16S rRNA gene copies per cubic meter of air. Error bars represent standard error (n = 3 replicates).

Results obtained for the DNA recovered from the filter-based sampling are presented in Fig, 2.7. When comparing the total concentrations obtained with the two sampling methods, there was no statistically significant advantage for any of the two samplers as confirmed by independent samples t-test ($p=0.877$).

2.3.5 The effect of sampling time on recovered bacterial diversity

In order to evaluate the impact of sampling duration on the recovered airborne bacterial communities' composition, the samples collected over eight hours (n=3) and over one hour (n=3) were compared in terms of alpha and beta diversity. The MiSeq run generated a total of 244,642 bacterial sequencing reads. After quality trimming, chimera filtering and removal of singletons, a total of 109,429 16S rRNA gene sequences were obtained. These sequences clustered into 3,575 OTUs at the 97% similarity threshold. Removal of chloroplast (4,007 sequences), unassigned reads (3,854 sequences) and subtraction of sequences resulting from the blanks/control samples (9,788 sequences in total, see Appendix S2.1-2 for most abundant taxa) reduced the number to 2,865 OTUs, comprising 53,978 sequences (median/sample = 8,729 ± 1,603 read counts). The numbers of sequences per samples were normalised based on the number of sequences obtained from the smallest library (7,236 sequences). The total of remaining sequences was 43,416. Abundance-based filtering carried out to remove OTUs with 5 or fewer counts further reduced the number to 27,700 sequences represented by 301 OTUs.

The total number of normalized and filtered OTUs detected in individual samples varied from 190 to 209 for the eight-hours samples and from 138 to 154 for the one-hour samples (Table 2.3). The estimated bacterial richness was found to be positively correlated with the 16S rRNA gene abundance, as determined by qPCR (Pearson's $r = 0.95$, p-value = 0.004). The Shannon's Diversity Index also estimated that the greatest level of bacterial diversity was obtained for the eight hours (Table 2.3). However, the difference observed between the two sample types for both OTU richness and Shannon, was not statistically significant, as determined by Mann-Whitney U test (p-value>0.05). It should be noted that the initial difference prior abundance-based filtering, was more substantial, as illustrated in Fig, 2.8A, but most of it was driven by unrepresentative low-abundance OTUs.

Table 2.3 Alpha diversity measures for bacteria based on operational taxonomic units (OTUs) with 97% sequence similarity.

	8 hours (A)	**8 hours (B)**	**8 hours (C)**	**1 hour (A)**	**1 hour (B)**	**1 hour (C)**
OTU richness	209	192	190	154	138	147
Shannon	4.67	4.69	4.74	4.35	4.46	4.52

Computation of the core microbiome revealed that 134 OTUs were common within the 8-hours replicates (53.0% overlap of the total number of OTUs), whereas only 56 OTUs were shared among the 1-hour replicates (21.7% overlap of the total number of OTUs). As the replicated samplings for the two tested collection times were not conducted simultaneously in the experimental room, only a number of 37 OTUs (24.2% of the total number of OTUs) was found to be common between the 8-hours and the 1-hour samples.

The taxonomic composition of the samples at the phylum, class and genus level is illustrated in Fig. 2.8 B, C and D, where variation in the relative abundance of the detected taxa is observed within replicates and between the two types of samples. Results at the genus level (Fig. 2.8D) made evident that the most abundant taxa captured within 1-hour sampling (*Bacillus* 5.55%, *Massilia* 6.66%, *Streptomyces* 4.24%) appeared to be less dominant after 8 hours of sampling (0.37%, 2.64% and 1.80% respectively). On the other hand, the predominant bacteria detected within the 8-hour samples (*Kocuria* 12.45%, *Acinetobacter* 10.82%, *Paracoccus* 7.38%) were recovered at lower proportions after 1 hour sampling (4.48%, 1.79% and 5.82%, respectively).

The phylogenetic dissimilarity of communities (β-diversity) observed between the samples was determined using weighted UniFrac distances. Principal coordinate analysis (Fig. 2.9) showed that the 8-hour samples were grouped together, in contrast to the 1-hour replicates that were scattered and did not exhibit any tight clustering.

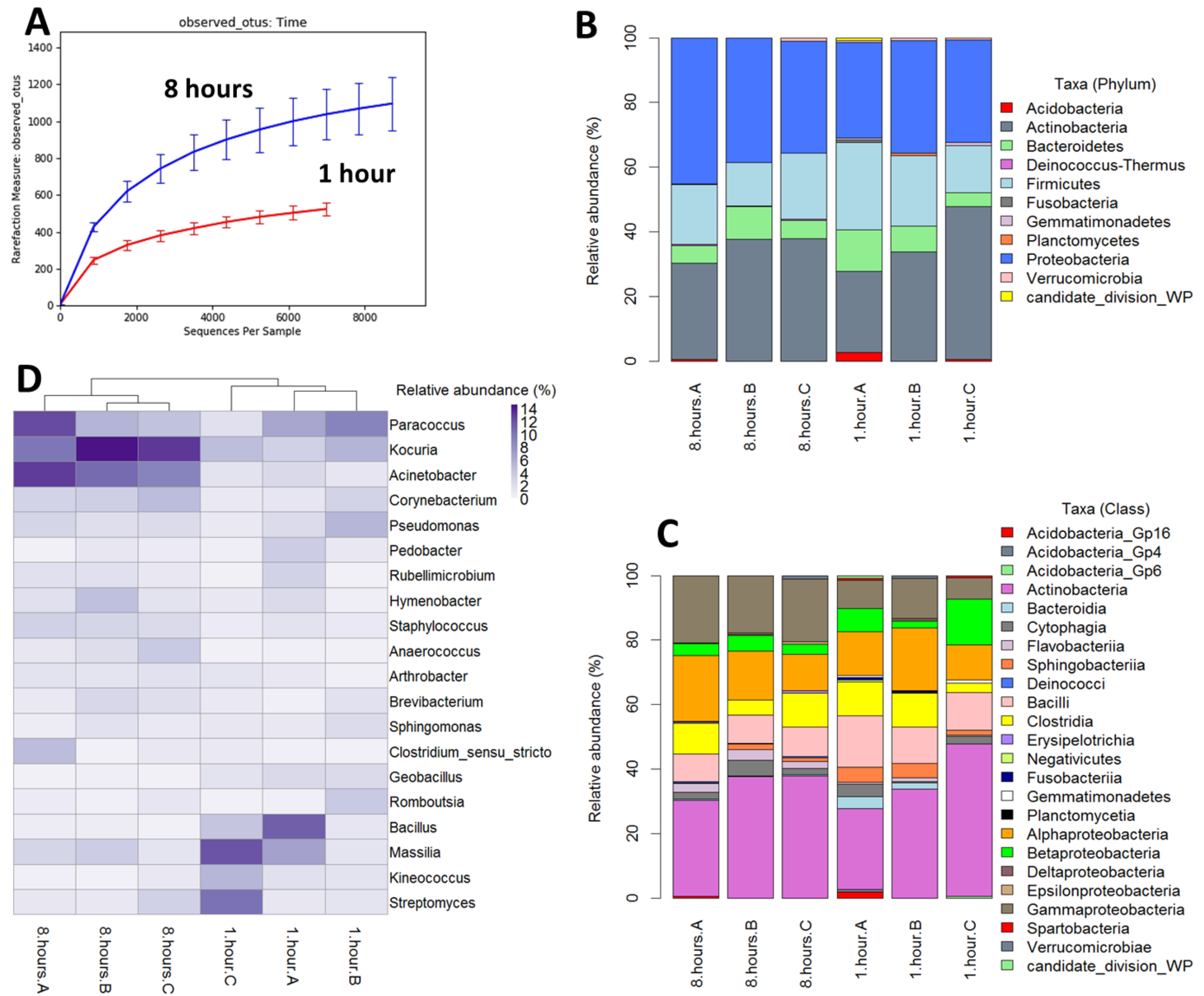


Figure 2.8A. Rarefaction curves of observed bacterial OTU richness based on 97% similarity for triplicate PC filter samples (A, B and C) collected over 1 hour and 8 hours at 28 LPM, prior normalisation and filtering of low-abundance OTUs (n ≤ 5 sequences). Error bars represent standard deviation (n=3). **B.** Relative abundance of bacterial OTUs at the phylum and (**C**) class level per each sample. **D.** Heatmap displaying the relative abundance of the 20 most dominant bacterial genera per each sample.

Both analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) showed that there is no statistical significance in the variation between the two sample types (p-value > 0.05). Similar results were also obtained based on the unweighted UniFrac metric, which is based on the presence or absence of taxa (Appendix S2).

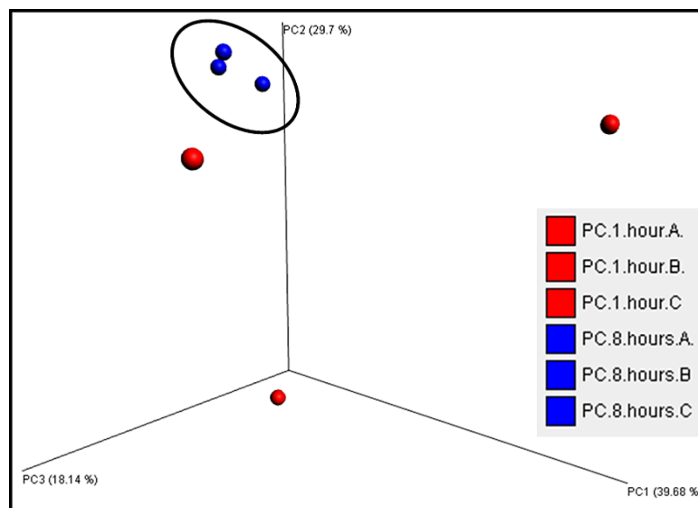


Figure 2.9 Principal coordinate analysis 3D-plot of bacterial beta diversity based on weighted UniFrac distance matrix.

2.3.6 The effect of pore size on recovered bacterial diversity

Sequencing of the two triplicate samples examined, corresponding to 0.2-µm and 0.4-µm pore size PC filter types, produced a total of 373,916 16S rRNA sequences. After quality trimming, chimera removal and singletons filtering, a total 169,489 sequences, comprising 4,111 OTUs, were obtained. A number of 3,456 sequences were classified as chloroplast, whereas 3,598 sequences were unclassified. Following removal of non-bacterial sequences, unassigned reads and subtraction of possible contaminant sequences/OTUs resulting from the control samples, a total of 3,409 OTUs (74,255 sequences, median/sample = 10,787 ± 3,584 read counts) were recovered. The OTU table was normalised to the sample with the lowest number of total sequence reads (10,168) and a total of 61,008 sequences were retained.

Supplementary filtering (exclusion of OTUs with 5 or fewer counts) reduced the number to 42,212 sequences, which were represented by 371 OTUs. Table 2.4 shows the alpha diversity metrics for the recovered bacterial communities. Results indicated that there was no significant difference between the two pore sizes in terms of alpha diversity (Mann-Whitney U test, p-value > 0.05).

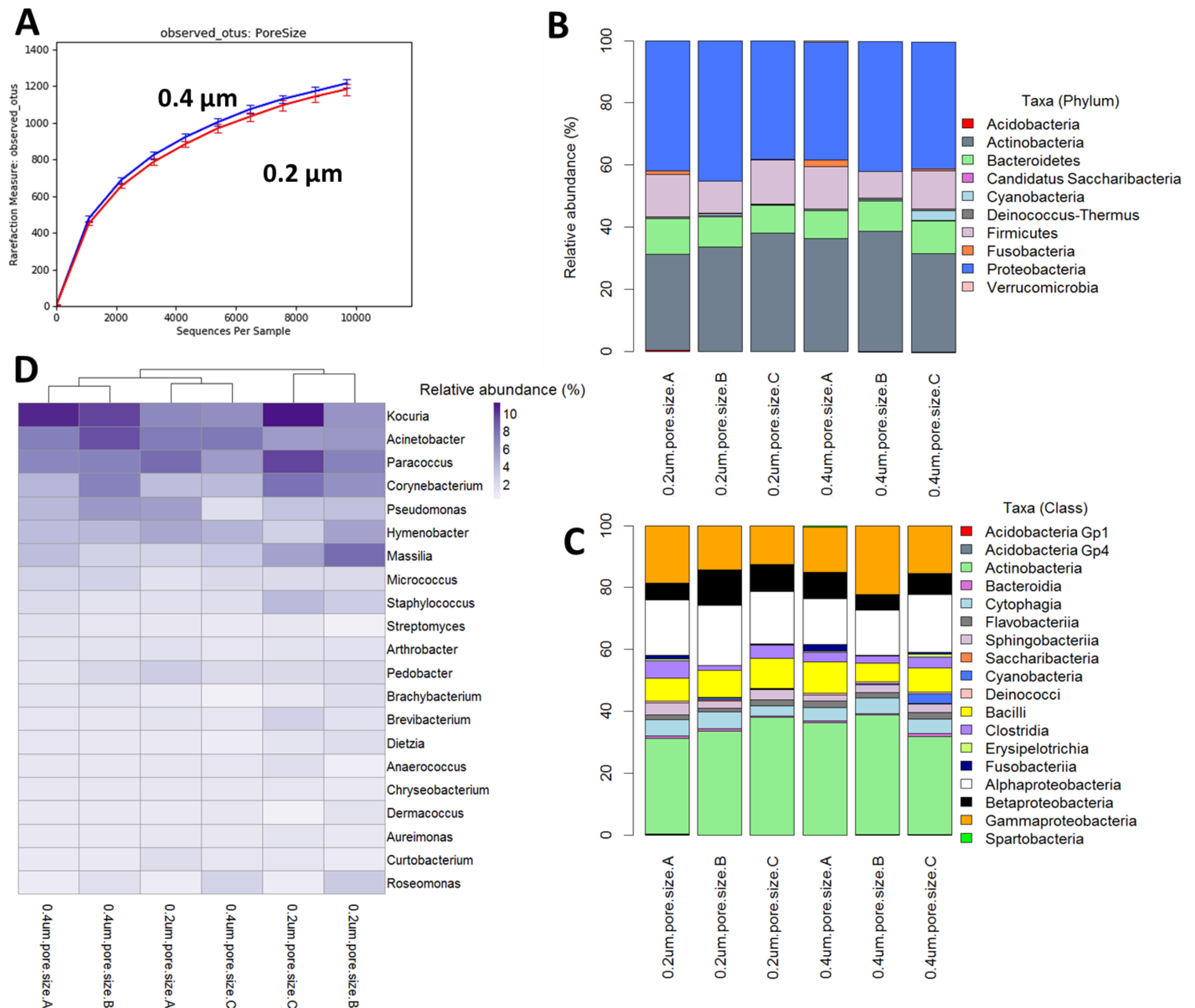


Figure 2.10A. Rarefaction curves of observed bacterial OTU richness based on 97% similarity for triplicate air samples (A, B and C) collected using PC filters with 0.4-µm and 0.2-µm pore sizes, prior normalisation and filtering of low-abundance OTUs (n ≤ 5 sequences). Error bars represent standard deviation (n=3). **B.** Relative abundance of bacterial OTUs at the phylum and (**C**) class level per each sample. **D.** Heatmap displaying the relative abundance of the 20 most dominant bacterial genera per each sample.

The classification of bacteria (phylum, class and genus) identified (Fig. 2.10) revealed only small variations within replicates and between the two types of filters in terms of relative abundance of captured taxa. Core microbiome analysis demonstrated that the number of overlapping OTUs among all samples was 188 (70.4% of the total number of OTUs), while 223 (62.1% overlap) and 232 OTUs (63.6% overlap) were common within the 0.2-µm and 0.4-µm replicates, respectively.

Table 2.4 Alpha diversity measures for bacteria based on operational taxonomic units (OTUs) with 97% sequence similarity.

	0.2 µm (A)	**0.2 µm (B)**	**0.2 µm (C)**	**0.4 µm (A)**	**0.4 µm (B)**	**0.4 µm (C)**
OTU richness	304	292	309	306	311	310
Shannon	5.09	5.02	5.04	5.11	5.12	5.16

Principal coordinate analysis of bacterial beta diversity based on the weighted UniFrac distance matrix (Fig. 2.11) showed that the no-clustering pattern observed for the collected samples (1.7 m^3 sampling volume) was in accordance with the result obtained in 2.3.5 regarding high variation within the one-hour replicates. The same pattern was also observed when the presence/absence of OTUs alone was considered (unweighted UniFrac, Appendix S2). In terms of variation between the two types of samples (0.2 and 0.4 µm pore sizes), both PERMANOVA and ANOSIM analyses confirmed that the pore size used had no significant effect on the recovered community composition (p-value > 0.05).

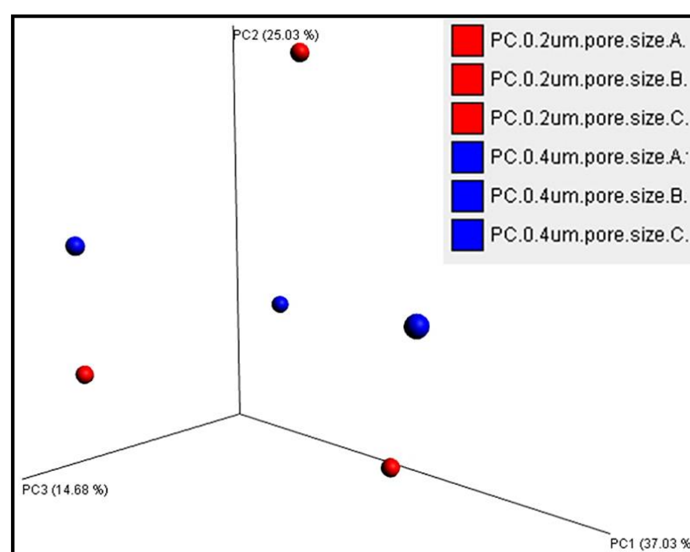


Figure 2.11 Principal coordinate analysis 3D-plot of bacterial beta diversity based on weighted UniFrac distance matrix.

2.3.7 The effect of size-resolved sampling on recovered bacterial diversity

An initial 1,323,234 sequences yielded by Illumina MiSeq for the three experimental repeats (sets) of parallel sampling between the impactor and the filter-based sampler were reduced to 602,141 sequences after quality filtering and removal of singletons. These sequences were distributed across 14,714 OTUs at 97% identity. Removal of archaeal (171), chloroplast (33,579) and unassigned (39,465) sequences, followed by subtraction of sequences in the controls from the samples, resulted in a total of 11,275 OTUs (390,443 sequences, median/sample = 13,606 ± 4,988 read counts). One sample from the 27 collected (Andersen stage 6, set B) yielded a low number of reads (<1000) and was excluded from the analysis. The remaining sequences were normalised (based on the sample with the fewest number of sequences) to 8,606 reads per sample, reducing the total to 223,756 counts. Low-abundance OTUs ($n \leq 5$ sequences) were discarded and a total of 133,955 high-quality sequences comprising 305 OTUs were retained for downstream analysis.

Table 2.5 shows the OTU richness and Shannon's Diversity Index per each sample. The numbers of recovered bacterial OTUs for the three sets largely mirrored results of 16S rRNA gene abundance, as determined by qPCR (Spearman's $\rho = 0.91$, p-value < 0.001). It should be noted that the total number of OTUs recovered from all stages per set is not expected to be approximately equal to the richness from the filter-based collection of total particles, as common taxa exist among the individual size-specific stages. In particular, the number of shared OTUs among all stages was 9 for set A (27 OTUs were common among stages 1 - 5), 53 for set B and 192 for set C.

The number of unique OTUs found for the total of all stages was 259 for set A, 286 for set B and 291 for set C and did not differ significantly compared to the mean numbers of OTUs recovered among the filter-based replicates (n=3) per set (Mann-Whitney U, p-value>0.05). The number of commonly detected OTUs present in all filter-based triplicates was 227 for set A (79.4% OTU overlap among replicates), 240 for set B (84.5% OTU overlap among replicates) and 238 for set C (82.6% OTU overlap among replicates). The OTU overlap between the total unique OTUs recovered by the Andersen impactor and the core OTUs found by the filter-based sampling was high for all three experimental repeats. In particular, 205 OTUs were common for set A (73.0% overlap), 240 OTUs for set B (83.9% overlap) and 238 OTUs for set C (81.8% overlap).

Table 2.5 Alpha diversity measures for bacteria based on operational taxonomic units (OTUs) with 97% sequence similarity.

		Andersen impactor							Filtration		
		Stages*						All stages	Total particles**		
		1	2	3	4	5	6		a	b	c
OTU Richness	Set A	155	96	167	113	90	36	259	256	263	264
	Set B	245	138	245	158	114	-	286	267	264	264
	Set C	267	267	263	263	251	233	291	280	248	276
Shannon	Set A	4.42	3.99	4.37	4.10	3.82	2.16	4.82	4.91	4.96	4.97
	Set B	4.76	4.28	4.81	4.24	3.92	-	4.88	4.93	4.94	4.91
	Set C	4.68	4.88	4.88	4.88	4.92	4.83	5.00	4.92	4.84	4.97

*Stages 1, 2, 3, 4, 5 and 6 correspond to lower cut sizes of 7, 4.7, 3.3, 2.1, 1.1 and 0.65 μm.
** a, b, c denote sampling replicates.

Figure 2.12 illustrates the proportional distributions of bacteria phyla per each particle size fraction and in relation with the total (non-size resolved) particles for the three experimental repeats. All phyla detected at the size-resolved samples were also present in the total particles samples. Looking broadly at the composition of the identified taxa in aerosol particles across the three sets, it can be observed, in particular, that all the predominant phyla (Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes) were present in all size fractions. Among the most abundant phyla, only Proteobacteria were more highly enriched in the larger particle sizes, as observed across the three sets. Fusobacteria that were encountered at lower proportions, were also affiliated with all particle sizes. Apart from Acidobacteria that they were twice consistently detected only at stage 2 (4.7 - 7 μm), Cyanobacteria and Deinococcus-Thermus were also mainly encountered at the larger particle stages (> 2.1 μm). Examining the composition at the class level, there were few consistent trends observed. First, *Alphaproteobacteria* were present at higher relative abundance at stages 1 and 2 (> 4.7 μm). *Deltaproteobacteria*, which were only detected during set A, were only encountered at stage 2 (4.7 - 7 μm). Last, *Clostridia* (Firmicutes) seemed to be more abundant at stages corresponding to particle sizes 3.3 - 1.1 μm.

Heatmaps (Fig. 2.13) show the distribution of the most abundant bacterial genera across particles of different sizes. In general it can be seen that most taxa were present at different relative abundance across various or all stages without exhibiting any size-related pattern. Even though in set A there was a lack of presence of some genera at stage 6 (0.65 - 1 μm), this is most likely associated with the lower biomass recovered (see also Fig. 2.5), compared

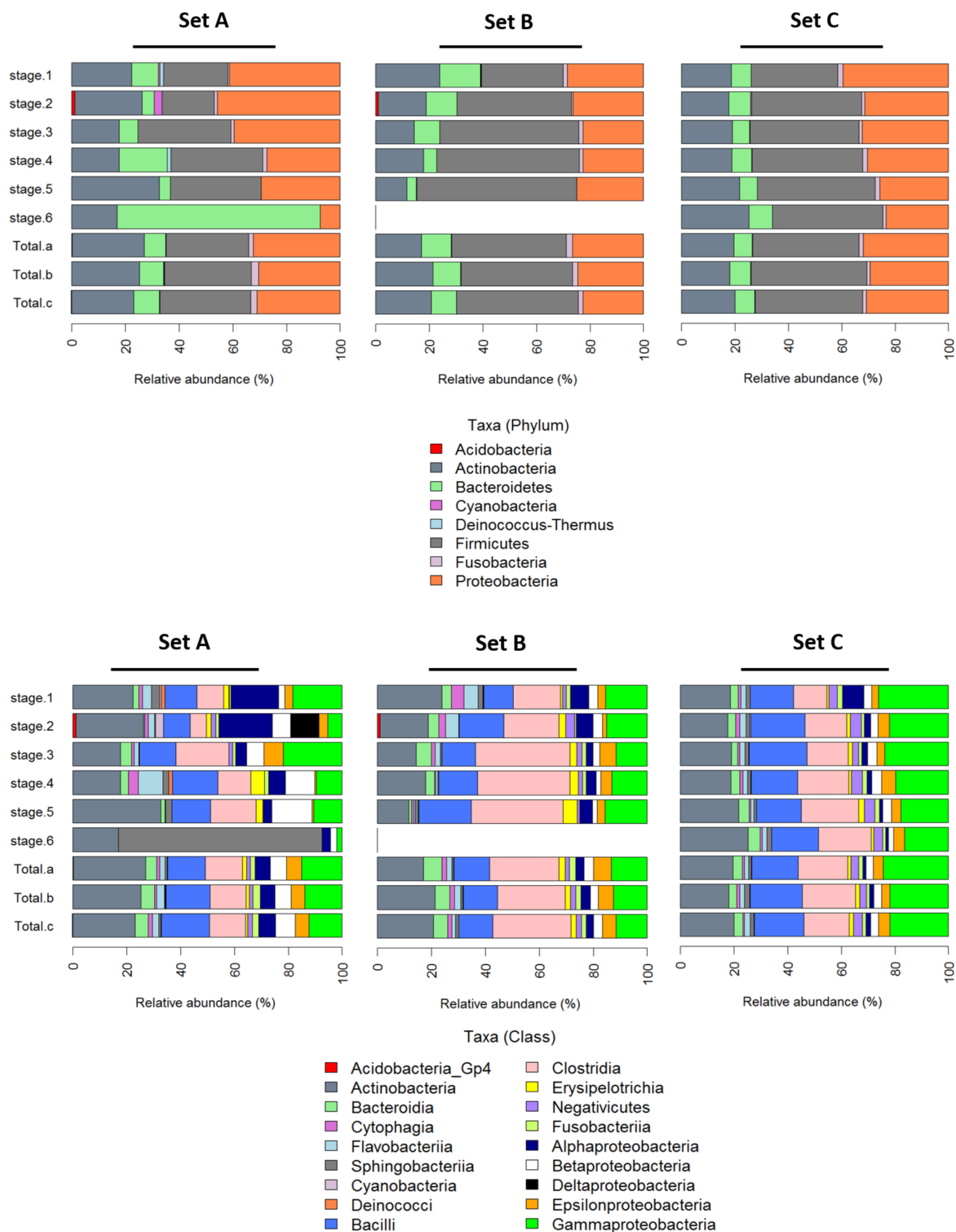


Figure 2.12 Relative abundance of bacterial OTUs at the phylum and class level per each experimental set (A, B, C) across non size-resolved collected total particles (a, b, c) and size-resolved samples (stage 1, 2, 3, 4, 5 and 6 corresponding to lower cut sizes of 7, 4.7, 3.3, 2.1, 1.1 and 0.65 µm). Stage 6 - sample from set B was excluded from the analysis due to very few reads generated.

to set C where, apart from *Paracoccus*, all detected genera were highly abundant at the specific stage. Few genera that were consistently observed at larger proportions at specific size fractions during all three sets were *Streptomyces* (*Actinobacteria*) at stage 6, *Paracoccus* (*Proteobacteria*) at stages 1 and 2 and *Kocuria* (*Actinobacteria*) at stage 5, while *Staphyloccus* (*Bacilli*) was less abundant at stages 5 and 6.

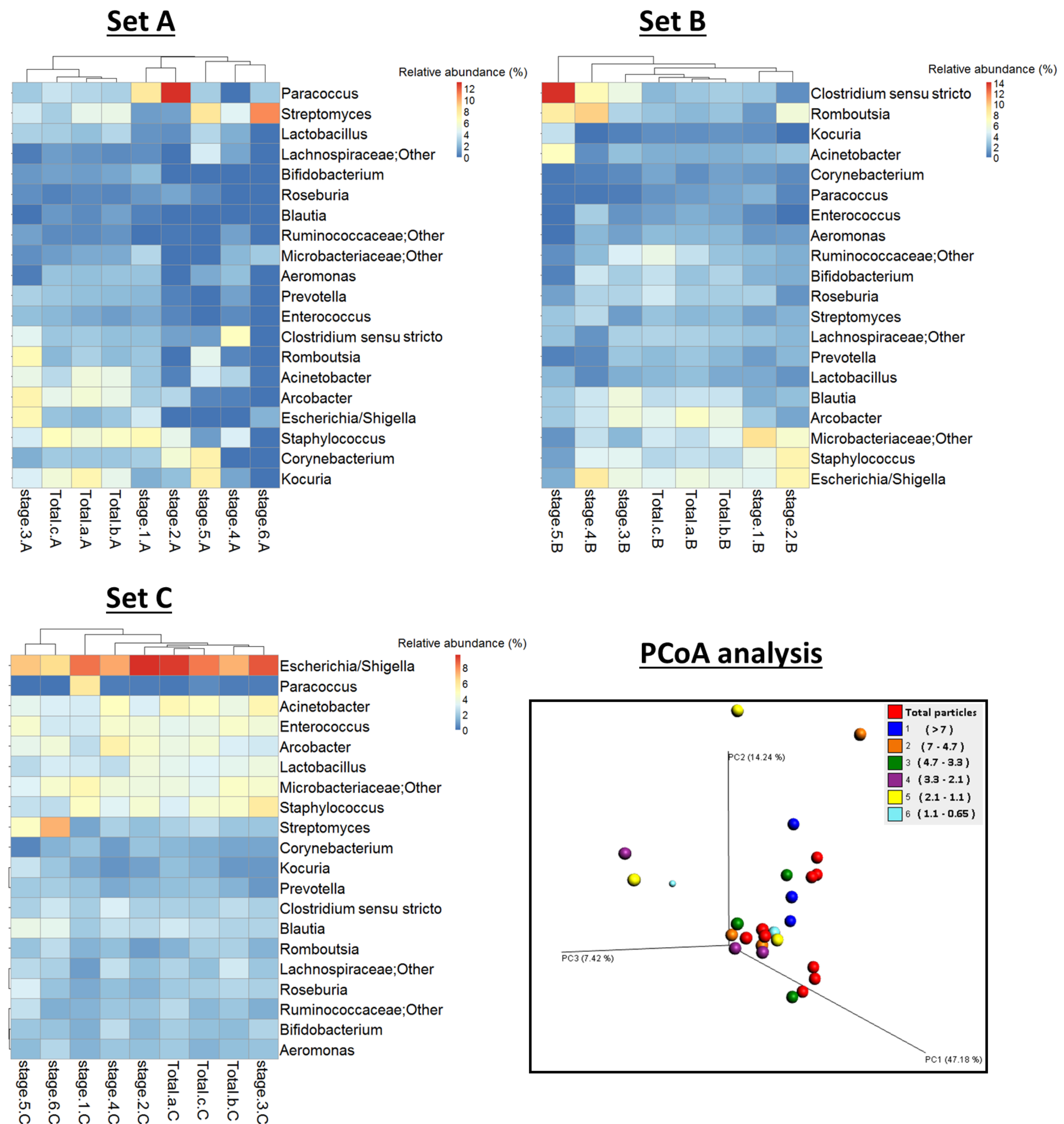


Figure 2.13 Heatmaps displaying the relative abundance of the 20 most dominant bacterial genera per each experimental set (A, B, C) across non size-resolved collected total particles (a, b, c) and size-resolved samples (stage 1, 2, 3, 4, 5 and 6 corresponding to lower cut sizes of 7, 4.7, 3.3, 2.1, 1.1 and 0.65 μm). On the panel on the right, principal coordinate analysis (PCoA) of beta diversity based on weighted UniFrac distance matrix.

Principal component analysis (PCA) was performed in order to assess clustering of samples of different stages, in terms of beta diversity (Fig. 2.13). Replicates of total-particles samples (red spheres) formed distinct groupings per each set. Although most samples were relatively closely clustered, which was even more obvious when only considering the presence/absence of taxa (unweghted UniFrac, Appendix S2), there were few size-specific samples that were far apart from the rest of the Andersen and filter-based samples. However, overall, there were no significant differences found between the two sample types (PEMANOVA and ANOSIM, p-value > 0.05). Moreover, the size-resolved samples collected during the three experimental sets showed no obvious clustering according to the particle size. Permutation-based multivariate analysis of variance (PERMANOVA) revealed, though, that the particle size had a significant, but almost negligible, effect on the recovered community composition (pseudo-$F_{6,11}$ = 3.00, R^2= 0.13 p-value = 0.018). Analysis of similarities (ANOSIM) confirmed that samples recovered from the different stages were not significantly different (R = 0.009, p-value > 0.05).

2.4 Discussion

This study focused on specific technical aspects of bioaerosol filter-based sampling with the aim of optimizing the collection and detection of airborne micro-organisms in indoor environments. The objective of this research was to investigate the effect of sampling-related factors such as sampling time, filter collection material and pore size on the recovery and PCR-based detection of bacteria in indoor air.

The sampling time is one of the master variables that must be addressed to develop an effective bioaerosol collection system and standardize a sampling protocol. One of the main considerations in the culture-based studies was that lengthy air sampling is thought to cause desiccation and loss of viability of collected micro-organisms (Mainelis & Tabayoyong, 2010; Durand et al., 2002; Hensel & Petzoldt, 1995; Juozaitis et al., 1994). However, in the era of high throughput sequencing, the major barrier in molecular studies is non-detect samples due to insufficient collected biomass as a result of low flow rates and limitations on sampling times in some indoor environments. The sampling time should be sufficiently long to obtain a representative sample of the airborne microorganisms present, without exceeding the upper quantitation limit of the sampler. On the other hand, increased sampling time and/or flow rate might not necessarily result in an increase in the quantity of airborne micro-organisms recovered (Luhung et al., 2015). An additional complexity in selecting the optimal collection time is associated with the temporal variability of the bioaerosol concentrations, which may reach several orders of magnitude in the same environment.

In our investigation, the qPCR data from the direct comparisons between the different sampling durations showed that longer sampling times resulted in increased bacterial DNA recovery. However, the similar levels of bacterial quantities yielded from different sampling times, such as 4 and 2 hours or 30 and 10 minutes is most likely associated with the experimental design that could not eliminate temporal variability of the bioaerosol levels. Even though efforts were made to ensure unoccupied conditions and controlled physical parameters (i.e. temperature) during the experiment in order to maintain a low microbially active environment, the sampling runs were not conducted in an experimental chamber (see 2.2) and thus the influence of the outdoor air, which could resulting in large indoor air variability, could not be controlled. Similar trends were also observed when examining the recovery of fungal abundance across different sampling times, despite the fact that captured amounts of fungal DNA were not quantified. Moreover, the fact that the ITS2 amplicons

corresponding to the shorter sampling times could not be visualised on an agarose gel clearly shows that sampling volumes smaller than 1.7 m^3 (i.e. 1 hour sampling at 28 LPM) can lead to inadequate DNA recovery.

In our experiments, sampling times were selected to represent low biomass samples as short-time sampling is a well-known challenge for detecting indoor airborne microbes. A similar study examining molecular analysis-related filter-based parameters investigated whether long durations exceeding the 8 hours (16 LPM) can affect the quality of DNA yield (Luhung et al., 2015). Their results based on qPCR showed that there was no adverse impact on the recovered fungal load, but there were bacterial DNA losses observed, suggesting that the DNA of the more vulnerable species (e.g. Gram-negative bacteria) might be prone to degradation. They also recommended that one way to circumvent this issue would be to pool separately collected samples of shorter duration. Another filter-related issue that has been pointed out by studies is that relatively lengthy sampling can sometimes lead to filter-clogging when sampling in environments with high airborne dust concentrations (Wang et al., 2015). Nevertheless, the choice of sampling time should be a compromise considering that, depending on the study site and the environmental conditions, shorter sampling times might result in insufficient collected biomass (e.g. in our study biomass yields were insufficient when 1-hour sampling experiments were undertaken during other seasons), while prolonged sampling is not preferred for practical reasons of sampling facilitation. It should be noted, though, that sampling volume in bioaerosol collection is also regulated by the flow rate which can be adjusted at higher levels, reducing the sampling duration. In addition, it has to be taken into consideration that concentrations of biological particles vary greatly in time (Brandl et al., 2008; Hyvärinen et al., 2001) and "snapshot" sampling may not fully represent the overall actual microbial load that the occupants of a particular environment are exposed to. Furthermore, it has been documented that short sampling times exhibit poor correlations with observed health effects (Su et al., 2001; Verhoeff & Burge, 1997).

Even though the quantitative data obtained on the optimal sampling time can provide some useful information regarding problems associated with short-term sampling times and use of different types of filters in indoor microenvironments with typically low encountered bioaerosol levels, the data presented here should not be used for their absolute values. Sampling location and environmental variables as well as emitting sources, including occupancy, are expected to differ from study to study and all parameters should be taken into consideration.

Our investigation on the impact of sampling time on the recovered bacterial diversity showed that even though the increased sampling duration yielded a higher number of phylotypes and a greater level of diversity, the difference in alpha diversity metrics between the eight-hours and the one-hour samples did not reach significance. It is worth noting, though, that the number of the low-abundance OTUs, which were preventively removed to preclude the inclusion of artefacts in the analysis, was a lot higher for the 8-hour samples. Inclusion of very low-abundance OTUs, which have been associated with spuriously amplified sequences and characterised by low reproducibility, in microbiome analysis, can lead to overestimation of diversity (Zhan et al., 2014; Kunin et al., 2010). However, it is possible that some of these infrequent OTUs might account for true "rare biosphere" micro-organisms (Skopina et al., 2016; Lynch & Neufeld, 2015).

When examining the compositional differences at the genus level, it became evident that the major taxa driving the community composition might be represented by different relative proportions during short-length and prolonged sampling. This could be related to possible difficulties capturing specific micro-organisms that might require a longer sampling duration, suggesting that particular airborne taxa appearing to be predominant after short-term sampling might not necessarily represent the actual abundance in the sampled environment. In any case, results showed that the representation of a particular OTU/taxon recovered within the composition of a sample is affected by the sampling time used for bioaerosol collection. Moreover, it is worth noting that two of the most dominant taxa (i.e. *Paracoccus* and *Acinetobacter* spp.) found in the 8-hour samples were Gram-negative and were, in fact, detectable at increased proportions compared to the 1-hour samples. This indicates that the bacterial DNA recovery did not seem to be affected by possible degradation attributed to prolonged sampling that has been reported previously (Luhung et al., 2015).

Interestingly, the samples obtained after sampling for eight hours exhibited a higher number of overlapping taxa and lower heterogeneity compared to the 1-hour samples, as revealed by PCoA analysis. Although, in theory, this could be an outcome of the experimental design (PC filter samples derived from successive sampling repeats, alongside the other tested filters), it has to be considered that the three 8-hour samples were collected on three different (consecutive) days (under the same conditions), and, yet, they were tightly clustered. Moreover, the high variability occurring within the 1-hour samples was further confirmed by the community profiles obtained for the two different pore-size (biological) triplicates. The particular findings reflect the difference in starting template DNA concentrations between the

1-hour and 8-hour samples and the issues associated with collection of limited microbial biomass for molecular downstream processing. Although during sample preparation for amplicon sequencing libraries are quantified prior pooling and then mixed in equal (and equimolar) concentrations before being sequenced, it has been demonstrated that the template concentration has a large and significant effect on the resulting composition consistency of Illumina MiSeq-based bacterial community profiles among replicates (Kennedy et al., 2014). Prior findings, which were based on both highly diverse (soil) and less diverse (stool) samples, showed that replicates of higher template-concentrations were more homogeneous and less variable compared to replicates of low template-concentrations. Moreover, they suggested that in cases of low quantities of DNA, pooling of PCR technical replicates prior to sequencing might improve reproducibility of results (Kennedy et al., 2014). Gohl al. (2016) also highlighted the importance of optimised starting template concentrations for the accuracy of amplicon-based microbiome surveys. Furthermore, this issue has been also pointed out in the pre-HTS era by demonstrating the effect of low amounts of DNA on the derived 16S rRNA gene clone libraries (Chandler et al., 1997). Overall, our findings indicate that long-term air sampling has a greater potential of generating more consistent bioaerosol diversity results.

The parallel tests among the four types of filters (PF, PC, AC and NC) did not show any significant advantage in recovering greater biomass for any of the filters. Results showed that the applied extraction protocol is suitable for all types of the four filters tested, for both bacterial and fungal recovery from aerosol filter samples. However, it has to be mentioned that the polycarbonate and the cellulose acetate filter have the benefit of dissolving in phenol: chloroform which makes their purification easier, compared to the PTFE and nitrate cellulose filter that require an extra step of purification (see 2.2). The most recent study, to our knowledge, that compared the performance of different filters was culture-based and concluded that the PC filter collected significantly higher concentrations of bacteria than the PTFE and gelatin filters but did not demonstrate any significant differences in terms of fungal collection (Wang et al., 2015). In the same study, they also attempted to evaluate the efficiency of filter-based personal samplers in relation with short-time sampling intervals, ranging from 15 min up to 8 hours, similar to the times tested in the present investigation. However, they did not observe any consistent effect of sampling time on the recovered bioaerosol concentrations.

Both different pore-size filters tested (0.2 and 0.4 μm) seem to have performed equally in terms of bacterial recovery and the use of a smaller pore size filter did not seem to result in increased bacterial yields. Likewise, results obtained regarding the recovered diversity were in accordance with the qPCR results showing that there was no significance difference between the two filters in terms of microbial composition. Despite the trend observed in bioaerosol studies employing filters with small pore sizes, such as 0.2 μm (e.g. Leung et al., 2018; Wilkins et al., 2016; Lymperopoulou et al., 2016; Adams et al., 2015; Miletto & Lindow, 2015; Meadow et al., 2015; Tanaka et al., 2015; Leung et al., 2014; Park et al., 2014; Bowers et al., 2012; 2011) that is typically used for microbiology applications, filters used for air sampling do not work like a screen or sieve and their capture efficiency is not limited by the size of pores. Collections of aerosols depends on mechanisms of impaction, interception and electrostatic attraction and therefore micro-organisms are deposited within the filters and on the surface of the filters (Hinds, 1982) that have high collection efficiency for particles much smaller than their nominal pore size. The physical collection efficiency for a variety of materials and pore sizes has been investigated in several studies and has been documented to be greater than 90% even for particles at the nano-scale range and, thus, filters allow for the efficient collection of a wide variety of bioaerosols ranging from large fungi and pollens to bacteria and viruses (Soo et al., 2016; Georgakopoulos et al., 2009; Burton et al., 2006; Peccia & Hernandez, 2006). The main drawback related with smaller pore sizes is that they demonstrate higher pressure drops across the filter that a sampling pump might not be able to overcome during air sampling. That is why a slightly lower flow rate in combination with a longer sampling duration (i.e. 24 LPM for 70 min instead of the desired flow rate of 28 LPM for 60 min) had to be used during the pore-size related sampling experiment in our study.

Last, the comparison between impaction-based size-selective sampling and filter-based non-size selective collection of total airborne particles demonstrated that both methods yielded similar levels of total bacterial concentration as well as alpha diversity. Despite the fact that sample extraction and processing of six (90-mm) filters, instead of one (47-mm), should, in theory, result in more potential losses in terms of DNA recovery, our results indicated that both sampling methods performed almost equally. To date most bioaerosol characterisation has been limited to total suspended particles which is not useful for evaluating the potential inhalation human exposure to airborne micro-organisms. Although size-specific sampling is of highly importance for determining the deposition of bioaerosols in the human respiratory tract, it is challenging due to the high demand for increased sampling volume. The biological

material distributed per each size bin is equivalent to only one fraction of the total sampled volume (depending on the number of size bins) and therefore the collected total biomass has to be high enough in order to yield sufficient amounts of retrievable DNA for downstream analysis of all size fractions. For instance, in Qian et al. (2012), eight-stage non-viable cascade impactors had to sample air cumulatively for four consecutive days in classrooms in order to ensure that levels would be above limits of detection. Hospodsky et al. (2014) utilised six-stage non-viable impactors for 25 total hours of sampling under occupied conditions and for about 60 total hours of sampling under no occupancy. In our study, continuous 48 hours of sampling in an unoccupied environment seem to be sufficient for downstream sample processing.

In terms of bacterial composition, even though the majority of samples, resulting from both the Andersen and the filter-based sampling, exhibited high similarity, there were few individual size-specific samples which appeared to be more dissimilar compared to the rest of the samples, indicating that there are some differences in terms of recovered airborne bacterial taxa. It has been previously reported that the HTS-based recovered aerosol microbiome is affected by the sampler type used and that a combination of sampling techniques might achieve a better representation of the bioaerosol diversity (Hoisington et al., 2014). Nonetheless, the differences observed in the present study did not affect significantly the overall community composition and did not result in samples separation based on the collection method.

It is noteworthy that even though the parallel samplings were conducted in the same environment, due to the 48-h period experimental repeats being conducted during different weeks (i.e. 3 consecutive weekends) there was a large variation observed among the three sets in terms of yielded concentrations and relative proportions of recovered taxa. The distribution of collected bacterial taxa over airborne particles of different sizes demonstrated high concentration variability, which was strongly reflected in the numbers of recovered OTUs, and in general peaked towards the coarse size fraction for all sets. Overall, the particle size did not appear to have a large effect on the recovered composition suggesting that the airborne taxa collected were not associated with a particle-size specific pattern. The wide dispersion of taxa observed across multiple size ranges supports the findings of prior molecular studies in indoor environments (Hospodsky et al., 2015; Qian et al., 2012), suggesting that bacteria are mostly not present in airborne particles as single cells but they are

found in aggregates of cells or fragments and they can get attached to abiotic particles and therefore when sampled, they can be detected in any particle-size stage.

In addition, the high percentage of overlapping taxa observed between the two methods and among the filter-based replicates as well as the tight clustering observed between the filter-based replicates further support our aforementioned findings regarding more homogeneous community composition results obtained with lengthy sampling and bigger sampling volumes. Moreover, apart from the high proportions of Proteobacteria and Bacteroidetes recovered from both filter-based and size-specific samples, the detection of the Gram-negative taxa *Escherichia* and the closely related *Shigella*, *Acinetobacter*, *Arcobacter*, *Aeromonas* and *Prevotella* among the most dominant genera identified after 48 hours of sampling, further confirmed that any possible DNA degradation (Luhung et al., 2015) did not seem to affect the detection, recovery and representation of the more vulnerable taxa.

Finally, it has to be taken into consideration that apart from the role of sampling-related parameters in HTS-based bioaerosol surveys, characterising microbial communities is challenging and the molecular analysis itself can have a strong impact on the derived results. Amplicon sequencing methods are affected by various technical factors and inherent biases (Flynn et al., 2015; Nelson et al., 2014; Smith & Peay, 2014; Pinto & Raskin,, 2012; Claesson et al., 2010; Engelbrektson et al., 2010; Kunin et al., 2010) and variation and low reproducibility has been reported even among technical replicates (Wen et al., 2017; Zhan et al., 2014; Zhou et al., 2011).

To sum up, bioaerosol collection is a complex type of environmental sampling which is dependent on various technical parameters. Moreover, sample collection in indoor environments, especially when occupied, is always subjected to limitations due to the intrusive nature of the sampling. In this study we mainly focused on the filter-based sampling parameters but there are certainly many more aspects that need to be explored as well as various different approaches that could be used in order to assess the sampling performance in relation with molecular-based downstream analysis. For instance, instead of comparing different sampling times, another useful approach would be to examine in parallel collection of the equivalent sampling volumes by sampling for the same duration but using different flow rates. As the experiments described in the present study were not performed in a chamber by using artificially aerosolised micro-organisms, results presented here are more representative of the actual conditions, variations and challenges that researchers could face

when sampling bioaerosols in commonly occupied microenvironments with typically low encountered bioaerosol levels. Moreover, sampling methods used in the current work were representative of cost-effective low-volume equipment that can be used for a bioaerosol survey as high-volume sampling devices can be less affordable.

2.5 Conclusions

- Results showed that increased sampling times used for bioaerosol collection yielded increased microbial concentrations and more consistent bacterial community profiling among replicates. Therefore, it would be advisable to use a high sampling volume, achieved either by a longer sampling duration or a high flow-rate sampling device, depending on the study design and objectives, for bioaerosol sampling aimed for HTS-based analysis.

- The filter sampling material did not appear to have any significant effect on the DNA recovered from the filters. However, PC and AC filters seemed to be more user-friendly with regard to the DNA extraction approach used.

- The sampling pore size did not have any impact on the recovered bacterial concentration and composition. However, the use of a bigger pore size is recommended in order to avoid issues associated with flow rate restrictions.

- No significant advantage was found for neither the non size-selective nor the size-selective sampling methods as there were no substantial differences in the yielded total bacterial abundance and diversity between the filter-based and the Andersen-based bioaerosol collection.

The information generated in this study could be helpful for researchers working on the microbial characterisation of aerosols and is expected to assist in developing strategies for monitoring bioaerosol exposure.

Chapter 3

Characterisation of bioaerosols in educational settings

The effect of bacterial DNA contamination of gelatin filters on characterisation of indoor bioaerosols; a university campus case study

3.1 Introduction

The advent of molecular biology techniques and the rapid development of high throughput sequencing technologies, in the past few years, have started to provide insights on the microbiome investigation of the built environment. The airborne micro-organisms as part of the biological composition of suspended particulate matter, defined as bioaerosols, consist a major part of the microbial ecology of the indoor environment. However, the application of molecular methods on aerosol samples entails challenges due to the extremely dilute nature of the air environment that can result into difficulties gaining sufficient biomass, compared to the more easily sampled terrestrial and aquatic environments, and can lead to implications (Mbareche et al., 2017; Behzad et al., 2015; Peccia & Hernandez, 2006, Lighthart, 2000).

Recently, studies have highlighted the problems associated with DNA contamination during sample processing and the significant impact they may have on interpretation of sequencing generated data, especially for samples of low microbial biomass (Kim et al., 2017; Glassing et al., 2016; Lazarevic et al., 2016; Gruber, 2015; Jervis-Bardy et al., 2015; Laurence et al., 2014; Lusk, 2014; Salter et al., 2014; Weiss et al., 2014, Biesbroek et al., 2012). Contamination can occur throughout any step from sample collection to sample processing and contaminants have been identified to be present in laboratory consumables and working reagents (DNA extraction kits, PCR and library preparation reagents) or can be introduced from the wider laboratory environment (air, surfaces especially in labs that work with high-yield DNA, human-related microbiota -skin, oral, respiratory- and clothing). Even though this issue has been well-known by the microbiota research community (e.g. Barton et al., 2006; Evans et al., 2003; Corless et al., 2000; Tanner et al., 1998; Schmidt et al., 1995; Hughes et al., 1994; Kwok & Higuchi, 1989), it has now started being investigated in the context of high throughput sequencing and the inclusion of blank negative controls has become a critical consideration. Moreover, in the case of indoor air microbiome studies, identifying contaminant taxa can be challenging, since they can often be similar to the micro-organisms detected genuinely present in the air samples, as microbial diversity present in indoor air is

linked to human- and environmental associated bacteria (Leung & Lee, 2016; Prussin & Marr, 2015; Meadow et al., 2014; Hospodsky et al., 2012).

An additional factor that has surprisingly received little attention by the environmental microbiology studies is the contamination background from sample collection media and substrates that could compete with the low concentration of collected sample. Air samples are commonly collected through filtration. Filters are either provided sterile by the manufacturer or, most commonly, decontaminated by common lab sterilisation techniques that do not necessarily destroy DNA (e.g. autoclaving). Using blank filter samples, i.e. filters that are subjected to the same procedures as samples except no air is drawn through them, has been an integral part of standard operating procedures for aerosol gravimetric and chemical analysis. Despite their critical role in the quality assurance aspect, most bioaerosol studies do not include or mention the use of blank controls and the importance of the contribution of filter-background contaminants to the HTS-generated data has been underestimated.

In this study we report the detection of contaminants within gelatin filters and we monitor the effect of the DNA background contamination on the microbial profiling of bioaerosol samples throughout molecular sample processing. The gelatin filter has been widely used in culture-based studies (e.g. Hara et al., 2015; Lewandowski et al., 2013; Gołofit-Szymczak & Górny, 2010; Van Houdt et al., 2009; Turnbull et al., 1998; Danneberg et al., 1997; Macher & First, 1984; Koller & Rotter, 1974; Noller & Spendlove, 1956) but since the introduction of DNA analysis methods on bioaerosol research, it has been also utilised in various PCR-based indoor air studies (e.g. Hoisington et al., 2014; Li et al., 2011; Wu et al., 2011; 2010; Nehme et al., 2008). However, this is the first time that contaminants present in the gelatin filter were distinguished from the biological material collected from the air, through the use of blank controls, and detected by molecular techniques.

The aim of the present work was to investigate the impact of the gelatin filter nucleic acid contamination on the molecular-based characterisation of bioaerosols. The suitability of the gelatin filter for characterising the airborne bacterial and fungal load was assessed during a sampling study conducted at various locations in a university educational setting. University premises offer a variety of different types of indoor spaces (educational, residential, occupational and recreational) with different characteristics and levels of occupancy (Table 3.1). In order to characterise the air microbial content and address the question of undesired contamination, PCR-DGGE DNA fingerprinting and quantitative PCR were utilised.

3.2 Methods

3.2.1 Sampling site

Air samples were collected at University of Essex campus (Fig. 3.1), located in south-eastern UK, from the end of February to the end of April, 2015. Sampling was carried out indoors and outdoors for each site. The outdoor environment is highly vegetated with maintained lawns and flower gardens (see picture Supplementary Material). A detailed list of the places with their main characteristics (floor, levels of occupancy, ventilation) is shown in Table 3.1. Sampling sites 3 and 16 were located in a new university building at the final stage of construction. Unique characteristics of this site are the sustainable design of the building, including a rainwater pond recycling water to cool the building and the winter garden dome located at the entrance hall. Due to the high levels of noise generated by the sampling devices, sampling in lecture theatres and seminar rooms was performed under no occupancy. All necessary permissions were obtained for the described field studies.

3.2.2 Sample collection

3.2.2.1 *Air sampling*

Indoor air samplers were located near the middle of the room at a height of 1.5 m, while outdoor air sampling was conducted at the same height with equipment placed on a trolley at a close proximity to the building where the sampling site was located. Triplicate samples were collected at a flow rate of 28 L/min for sampling periods of 1 h, corresponding to 1.68 m^3 of collected air, using Sartorius 47 mm-diameter gelatin filters (3 μm pore size) loaded into single-stage closed-faced membrane filter holders (Sartorius AG, Goettingen, Germany). Gelatin filters were supplied sterile by gamma irradiation and individually packed by the manufacturer. Air for each sampling filter was drawn using a B105 SE pump manufactured by Charles Austen Pumps Ltd. Filter samples were not running simultaneously indoors and outdoors.

Prior to sampling, filter holders were washed thoroughly overnight in 1M NaOH solution. Filter holders were then rinsed with ultrapure water (Milli-Q, Millipore) and 70% (v/v) ethanol, placed in autoclavable pouches (Scientific Laboratory Supplies Ltd, UK) and sent for autoclaving. After sampling, the filter holders were sealed with parafilm and placed back in the same autoclaved bags, sent back to the lab within 2 hours, where filters were placed in sterile Petri-dishes (Sarstedt Ltd, UK) sealed with parafilm and stored at -20°C until further analysis. When carrying out all investigations, manipulations of the filters were performed

with autoclaved equipment in a Biosafety Level II cabinet. Unexposed filters (blank controls) were also analyzed alongside those used for sampling to provide procedural controls. Control filters were treated similarly to sampling filters, except air was not sampled.

Figure 3.1 University of Essex campus overview (https://findyourway.essex.ac.uk/). The letters marked on the map correspond to the sampling locations listed in Table 3.1.

3.2.2.2 ***Surface/dust sampling***

The settled dust approach was used for collection of time-integrated samples of airborne material in order to investigate the microbial diversity in parallel with the air samples. Triplicate samples of settled dust and microbes were collected from a variety of surfaces, depending on the type of the indoor setting. A list of the surfaces sampled in each site is shown on Table 1. Surfaces were sampled using nylon flocked swabs (#552C, COPAN Diagnostics Inc) moistened with sterile PBS buffer. Swabs were supplied sterile, RNAse and DNAse free in dry transport tubes. A 96 cm^2 (12 cm x 8 cm) area determined by a template was swabbed from each surface. Not all surfaces were visibly devoid of standing dust. After sampling, swabs were put back in the transport tubes and stored at -20°C for downstream processing. Negative controls of sterile cotton swabs were also processed.

Table 3.1 List of indoor sampling sites and their main characteristics.

	Indoor environment	Floor	Map location	Sampling date	Occupancy	Ventilation	Temperature (in-out)	Relative humi-dity (in-out)	Types of surfaces sampled
Lecture theatres									
1	**Lecture theatre #1**	2	B	18/02	-	mechanical	22.1±0.1°C 9.4±0.1°C	30.9±0.1% 62.9±0.3%	desks, chairs, floor
2	**Lecture theatre #2**	Ground	B	29/04	-	mechanical	21.5±0.02°C 12.7±0.1°C	38.0±0.1% 67.1±0.2%	desks, floor, window surfaces
3	**Room intended for lecture theatre**	Ground	F	22/04	-	mechanical	23.3±0.01°C 16.2±0.1°C	37.6±0.02% 46.0±0.2%	Chairs
Seminar rooms									
4	**Seminar room #1**	5	A	23/03	-	mechanical	21.8±0.02°C 15.6±0.3°C	25.3±0.1% 34.3±0.7%	desks, floor
5	**Seminar room #2**	5	D	26/03	-	mechanical	22.4±0.03°C 12.7±0.1°C	28.6±0.1% 50.8±0.6%	Desks
Offices									
6	**PC Laboratory**	Ground	C	24/03	7 people	mechanical	24.8±0.1°C 11.0±0.04°C	41.6.±0.1% 43.4±0.3%	desks, keyboards
7	**Research students' room**	2	E	01/04	4 people	mechanical	22.4±0.02°C 9.9±0.1°C	25.8±0.04% 52.4±0.5%	Shelves
Laboratories									
9	**Molecular microbiology lab**	4	A	24/02	8-10 people	mechanical	21.4±0.03°C 11.4±0.02°C	28.1±0.1% 46.4±0.6%	desks, floor shelves
9	**Ecology lab & coral reef unit**	Ground	A	25/03	1-4 people	mechanical	20.6±0.03°C 10.7±0.1°C	29.7±0.1% 44.7±0.4%	desk, working bench
Students' accommodation									
10	**Students' dorm kitchen #1** (flat shared by 14 people)	9	H	18/02	5-10 people	natural (windows opened)	19.3±0.1°C 6.9±0.02°C	35.0±0.2% 70.5±0.2%	bench, kitchen surfaces
11	**Students' dorm kitchen #2** (flat shared by 4 people)	2	K	27/03	3-4 people	natural (windows opened)	23.8±0.1°C 11.7±0.04°C	26.4±0.2% 37.7±0.1%	bench, floor
12	**Students' dorm kitchen #3** (flat shared by 14 people)	12	I	02/04	1-3 people	natural (windows opened)	22.9±0.1°C 13.8±0.2°C	22.0±0.1% 31.4±0.4%	bench, kitchen surfaces
13	**Students' dorm kitchen #4** (flat shared by 6 people)	2	J	06/04	1-4 people	natural	25.0±0.6°C 12.8±0.7°C	48.5±1.2% 55.2±2.4%	bench
Leisure/ other environments									
14	**Gym** (Sports centre)	Ground	G	20/02	variable	mechanical	19.7±0.01°C 8.0±0.1°C	44.9±0.1% 70.3±0.3%	gym surfaces
15	**Coffee/meeting room**	3	A	20/02	2-4 people	natural	22.1±0.04°C 8.8±0.1°C	36.1±0.1% 63.8±0.3%	bench, chairs, floor
16	**Entrance hall** with indoor garden	Ground	F	22/04	variable	natural (door opened)	19.5±0.04°C 16.2±0.1°C	47.6±0.1% 46.0±0.2%	Tables

3.2.2.3 ***Environmental parameters***

Physical parameters were continuously monitored indoors and outdoors using real-time instrument. Parameters of the microclimate in the room (temperature and relative humidity) were recorded every minute using Rotronic CP11 indoor air quality meter

3.2.3 Efficiency of gelatin filters

An additional experiment was conducted to evaluate the efficiency of gelatin filters in a side-by-side comparison with a different type of filter membrane, under vacant conditions. The study room and sampling conditions are described in Chapter #2. The filter tested in parallel with the gelatin was a 47 mm-diameter nuclepore track-etched polycarbonate membrane with 0.45-µm pore size (Whatman, UK). The polycarbonate filter was sterilised by autoclaving at 121°C, according to product information guide. Air samples using the two types of filters were collected, in triplicate, simultaneously at the same flow rate (28 L/min) and for the same sampling duration (1 hour) used for the field sampling. Each sampling set was repeated three times.

3.2.4 Sample processing

3.2.4.1 ***DNA extraction from gelatin filters***

Gelatin filters were aseptically cut into pieces using sterile forceps and placed into autoclaved 5-ml screw-cap tubes, containing 0.75 g of sterile 0.1-mm zirconium/silica beads (BioSpec Products Inc., USA). Gelatin filter is water-soluble at 37°C and tends to solidify at room temperature, thus treatment of filters has to be at high temperature. 2 ml of extraction buffer [10mM Tris-HCl pH 8, 25mM Na2EDTA pH 8, 1% (v/v) sodium dodecyl sulfate (SDS), 100mM NaCl and molecular biology grade water] was added to each tube and samples were incubated at 65°C for 15 min in a water bath. Cells lysis was achieved by bead beating at Vortex Genie with a MOBIO Vortex Adapter at maximum speed for 10 minutes, followed by 30 minutes incubation in a 75°C water bath. The cell lysate was purified with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0). The upper aqueous phase was isolated after centrifuging for 5 minutes at 2,000×g in a swing bucket centrifuge (Heraeus Megafuge 40, Thermo Fisher Scientific), at 40°C, in order to maintain solubility of the gelatin, and the supernatant fractions were aliquoted into two sterile 2-ml microcentrifuge tubes. The nucleic acids were precipitated using equal volume of isopropanol and 2.5 µl of co-precipitant glycogen (20 mg/ml, Thermo Scientific) and incubated for 1 hour at room

temperature, followed by 30 minutes centrifugation (11,337×g for 5 mins in Minispin, Eppendorf). DNA pellets were washed with 70% (v/v) ice-cold ethanol, air-dried and each pair belonging to the same sample was resuspended and pooled in 35 μl sterile water.

3.2.4.2 ***DNA extraction from swabs***

The tips of the swabs were cut directly into autoclaved 2-ml bead beating tubes (pre-filled with 0.5 g beads) using sterile scissors. Cells were lysed as described before except a volume of 500 μl extraction buffer was used for each sample and heating steps prior and after bead beating were omitted. All required centrifugation steps were carried out in a microcentrifuge (Minispin, Eppendorf) at 11,337×g at room temperature and DNA was eluted in 35 μl sterile water.

3.2.4.3 ***PCR-Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rRNA gene and ITS2 region***

DGGE fingerprinting method was implemented in order to validate the presence of distinct microbial communities, prior to processing for high through-put sequencing. Similarity within the bacterial and fungal bioaerosol populations was examined across various sampling sites. Three blank controls were also used included in the analysis.

PCR amplification of the 16S rRNA gene for DGGE analysis was performed using the primers F341-GC and R534, described by Muyzer et al. (1993). ITS3 with a 39-bp GC clamp added to its 5′ end and ITS4 primers, covering the internal transcribed spacer region 2 (ITS2) of the rDNA gene, were also used. ITS3 and ITS4 are considered to be a "Universal Primer" pair for fungi (White et al., 1990). All primer sequences are given on Table 3.2. PCR reaction mixtures (25 μl) contained 2 μl of DNA template, 1 μl of each primer (10 μM), 12.5 μl of (1×) MyTaq® Red Mix (Bioline) and 8.5 μl ultrapure water. Cycling conditions for PCR of 16S rRNA were: 2:15 min of denaturation at 95°C, followed by 35 cycles of 15s at 95°C, 15s for primer annealing at 55°C, 10s at 72°C for extension and a final extension step at 72°C for 5 min. For amplification of the ITS2 region cycling conditions consisted of: 3 min of denaturation at 95°C, followed by 35 cycles of 15s at 95°C, 15s for primer annealing at 56°C, 30s at 72°C for extension and a final extension step at 72°C for 7 min.

DGGE analysis of 16S rRNA gene fragments was performed as described by Muyzer et al. (1993), except gels were silver stained as described by Nicol et al. (2005). PCR products from the fungal ITS primers were analysed in the same way, except using 30 to 70% denaturant gradient.

DGGE data analysis

DGGE profiles were manually assessed and a matrix was constructed using the presence (1) or absence (0) of a band in each sample. Similarity between DGGE profiles was calculated based on Jaccard coefficient and a group average cluster analysis dendrogram was constructed. All analyses of the DGGE data were conducted using the PRIMER 6 software package.

3.2.4.4 ***Sanger sequencing of excised DGGE band***

PCR-DGGE bands of interest, obtained from the DNA profiles of blank filters, were excised and incubated in 20 µl of sterile ultrapure water for six days at 4°C. 5 µl of the eluted DNA was used as a template for PCR amplification using the same pair of primers (534R, 341F-GC, Muyzer et al., 1993) and cycling conditions, except number of cycles was reduced to 30. The amplified products were purified with a GenElute™ PCR Clean-Up kit (Sigma-Aldrich), mixed with the reverse primer (5 pmol/µl) and sent to GATC Biotech AG for sequencing.

The nucleotide sequences obtained were edited and trimmed from low quality bases using the online tool DNA Baser Sequence Assembler v4 (Heracle Biosoft, www.DnaBaser.com). Quality filtered sequences were compared to sequences available in databases using Basic local alignment search tool (Altschul et al., 1990) provided by the National Centre of Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences' affiliations to known genera or species were assessed regarding their similarity.

3.2.4.5 ***qPCR bacterial quantification***

The bacterial abundance of the air samples was estimated by 16S rRNA gene qPCR assay. A 464-bp fragment of the bacterial 16S rDNA (V3 and V4 hypervariable regions) was PCR-amplified with universal primer set Bakt_341F/Bakt_805R (Herlemann et al., 2011). The PCR was performed in a total volume of 10 µl using 5 µl (1×) SsoAdvanced universal SYBR® Green supermix (BioRad), 0.2 µl forward and reverse primers (10 µM) and 3.6 µl microbial DNA-free water (Qiagen). One microlitre out of the 35 µl extracted from each filter was used as template. The amplification was carried out under the following conditions: 98°C for 3 min, followed by 40 cycles of 15 s of denaturation at 98°C and 60 s of annealing and extension at 60°C, finishing with a melting curve analysis ranging from 65°C to 95 °C with 0.5 °C increment. All qPCR analyses were performed in duplicate on a Biorad CFX384 Real-Time PCR System equipped with CFX Manager Software. Genomic bacterial DNA used for standard concentration curve was extracted from pure culture of *B. subtilis* subsp. *subtilis*

DSM10 (ATCC 6051; genome size 4.2 Mbp; accession number: 4215610; Kabisch et al., 2013). The amplification efficiency of the standard curve was 92.4% (slope = -3.518), with the correlation coefficient R^2 being 0.996, and the y-intercept value was 33.752. The no template controls resulted in a mean Ct value of 35.153. Results were converted in units of 16S rRNA gene copies by correcting for the number (10) of 16S rRNA genes per *B. subtilis* genome. In order to compare the air samples (exposed gelatin filters) with the unexposed "blank" filters (control), concentrations were expressed as 16S rRNA genes per filter and not per cubic metre of air. qPCR of ITS region was decided not to be performed due to the known variability of the internal transcribed spacer region even within species or over the lifetime of the fungi.

qPCR data analysis

Based on the qPCR data, the indoor-to-outdoor concentration ratio (I/O) was calculated as a quantitative indicator of the contribution of outdoor sources on indoor air abundance. The normality of data distribution was checked by the Shapiro-Wilk test. The Pearson correlation coefficient (r) was used to measure the strength of the relationship between the sets of bacterial abundance data produced in this study. Independent two samples t-test was used to determine if the controlled experiment's data are significantly different from each other.

Table 3.2 Primers used in the current study.

Primer name	Primer sequence (5' – 3')	Reference
341F with GC clamp	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG	Muyzer et al. (1993)
534R	ATTACCGCGGCTGCTGG	Muyzer et al. (1993)
ITS3 with GC clamp	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCATCGATGAAGAACGCAGC	White et al. (1990)
ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
Bakt_341F	CCTACGGGNGGCWGCAG	Herlemann et al. (2011)
Bakt_805R	GACTACHVGGGTATCTAATCC	Herlemann et al. (2011)

3.3 Results

3.3.1 PCR - Denaturing Gradient Gel Electrophoresis (DGGE)

Examination of gelatin blanks

Bacterial DNA was successfully extracted and PCR amplified from all the indoor and outdoor air samples. However, amplifiable bacterial DNA was also detected in the blank filters that were analysed in order to provide procedural controls (Fig.3.2).

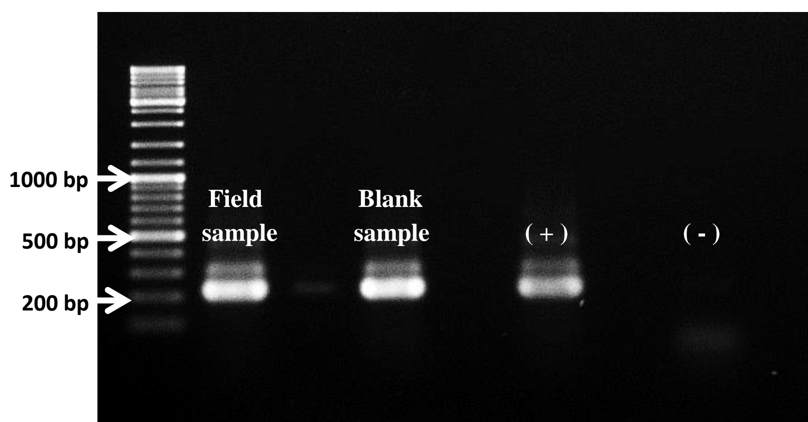


Figure 3.2 Gel electrophoresis image showing the results obtained from 35 cycles PCR amplification of 16S rRNA gene, using 341F and 534R primers, of the DNA recovered from a random indoor site air sample and a blank (unexposed) filter. Positive (+) and negative (-) PCR controls were also run.

In order to investigate the possibility of contamination of the filters in the field throughout the sampling procedures and transportation, gelatin filters taken straight from two new random packs were examined. Moreover, two different types of blank filters, polycarbonate (PC) and polytetrafluoroethylene (PTFE), which are not supplied sterile by the manufacturer but were sterilised by autoclaving, were included in the analysis. A no filter - control sample was also processed in order to check all of the equipment and reagents used for the laboratory procedures. PCR results showed that detection of bacterial DNA was obtained only for the gelatin filters (Fig.3.3).

Bacterial 16S rRNA gene amplicons obtained from the DNA recovered from the three blank gelatin filters were further analysed using PCR-DGGE analysis. Random indoor and outdoor air samples collected during the sampling campaign in various sites were also included in the analysis. The obtained DNA profiles (Fig. 3.4) revealed a high presence of similarly migrating bands in the bacterial community fingerprints.

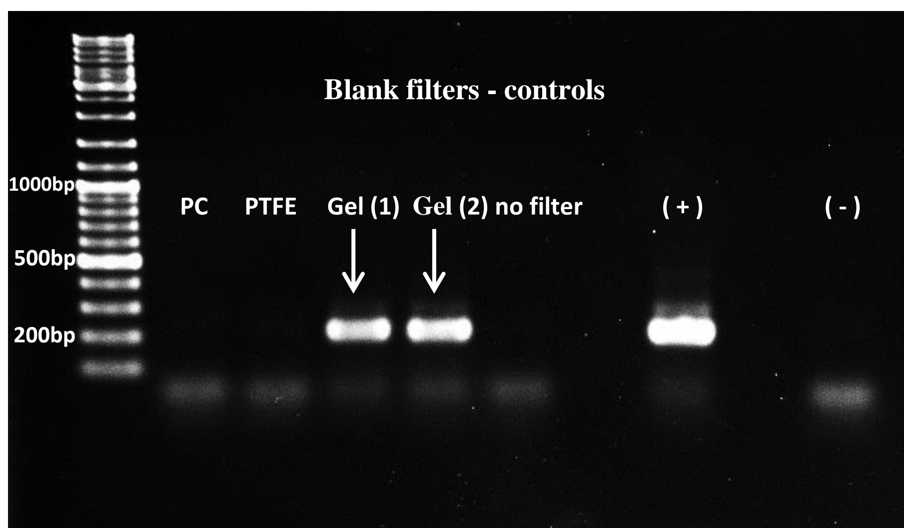
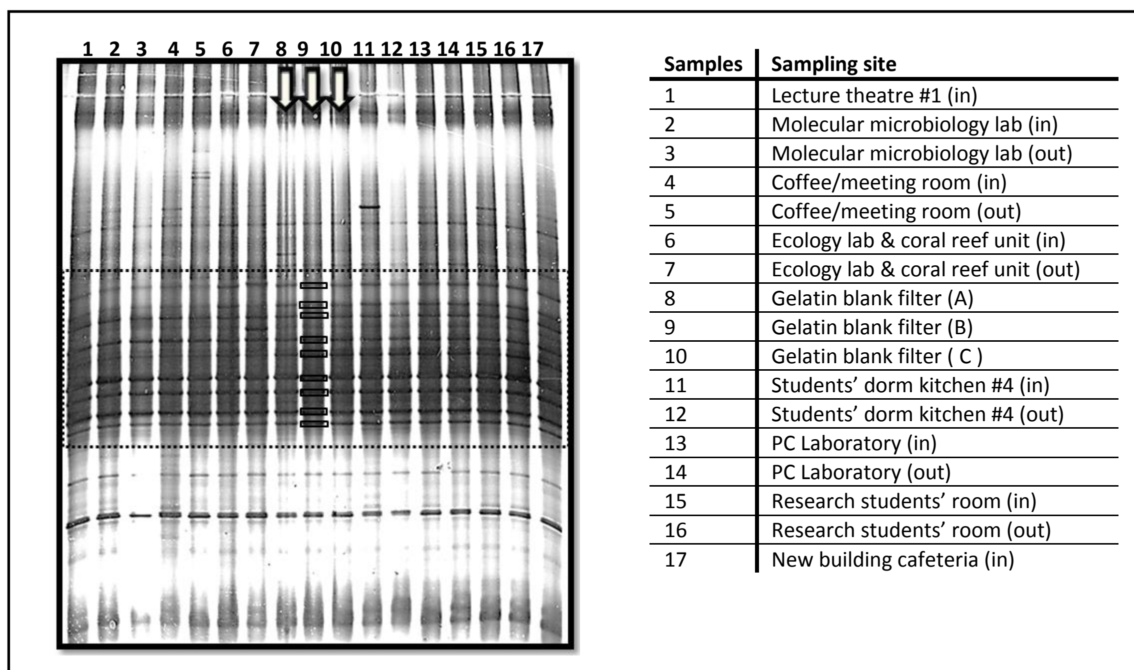


Figure 3.3 Gel electrophoresis image showing the results obtained from 30 cycles PCR amplification of 16S rRNA gene, using primers 341F and 534R, of the DNA recovered from three different types of unexposed 47 mm filters and a procedural control (no filter). Polycarbonate (PC) and polytetrafluoroethylene (PTFE) filters were autoclaved, while gelatin filters (Gel-1, Gel-2) were taken straight from the pack. Positive (+) and negative (-) PCR controls were also run.



Samples	Sampling site
1	Lecture theatre #1 (in)
2	Molecular microbiology lab (in)
3	Molecular microbiology lab (out)
4	Coffee/meeting room (in)
5	Coffee/meeting room (out)
6	Ecology lab & coral reef unit (in)
7	Ecology lab & coral reef unit (out)
8	Gelatin blank filter (A)
9	Gelatin blank filter (B)
10	Gelatin blank filter (C)
11	Students' dorm kitchen #4 (in)
12	Students' dorm kitchen #4 (out)
13	PC Laboratory (in)
14	PC Laboratory (out)
15	Research students' room (in)
16	Research students' room (out)
17	New building cafeteria (in)

Figure 3.4 Bacterial community profiling (DGGE fingerprints) of 16S rRNA gene from the DNA recovered from various indoor and outdoor air samples collected across campus. Marked (white arrows) are the three gelatin blank filters included in the analysis. The highlighted DGGE bands from Gelatin blank filter replicate B (9) were excised and sent for 16S rRNA gene sequencing.

Multivariate analysis based on DGGE banding pattern data (Fig.3.5) showed clear clustering of the air samples with the unexposed filters suggesting that there is a common bacterial background in all gelatin filters.

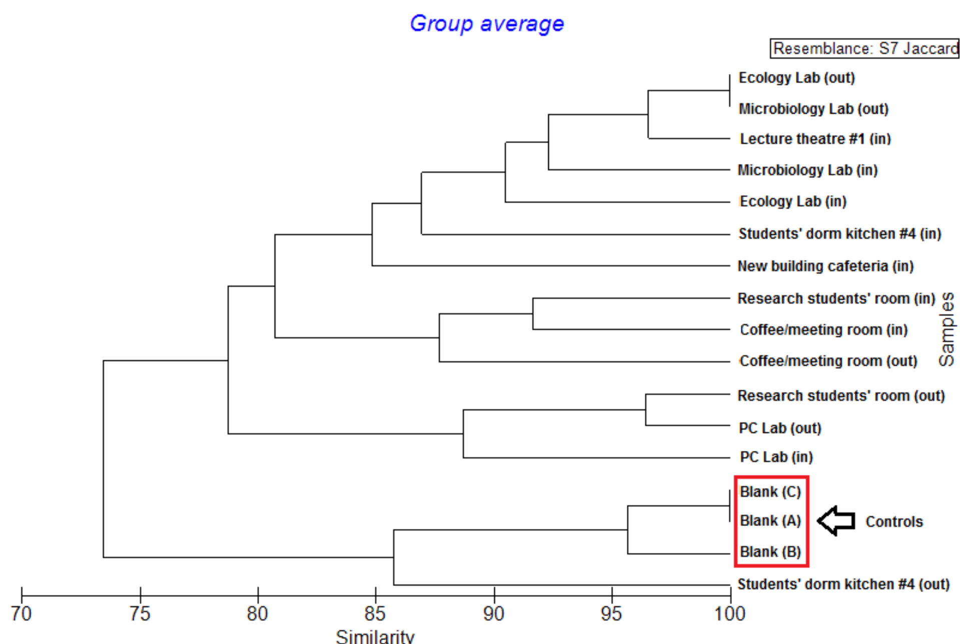


Figure 3.5 Cluster dendrogram of Jaccard coefficient similarity values (average linkage clustering) between DGGE fingerprints of 16S rRNA genes amplified from exposed and blank gelatin filter samples (Fig. 3.4). Percentage similarity is based on the presence of common bands within the profiles.

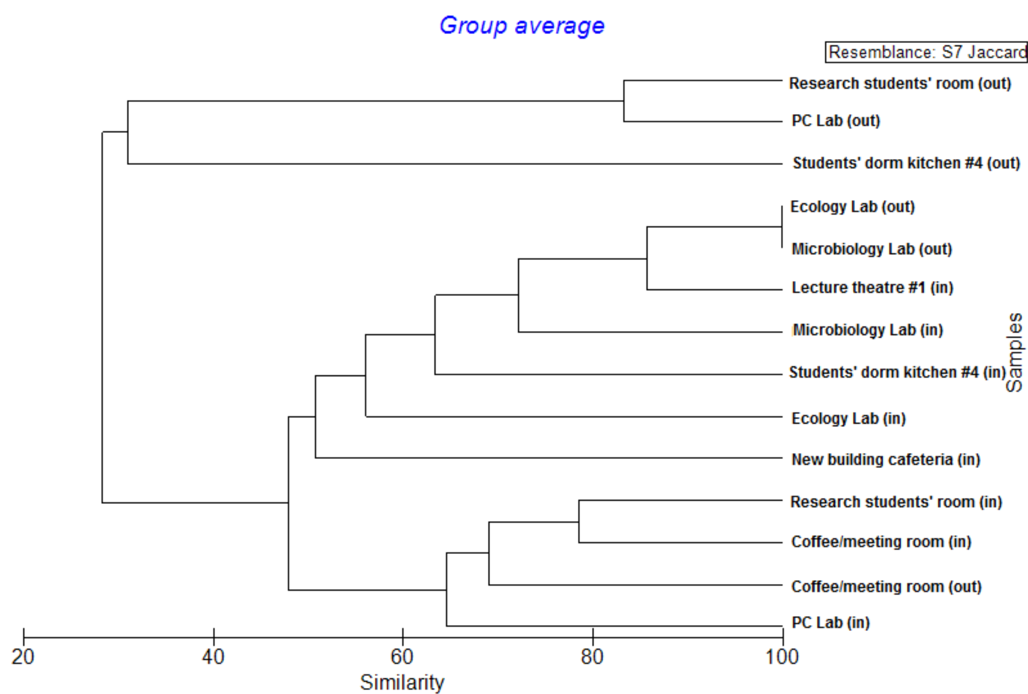


Figure 3.6 Cluster dendrogram of Jaccard coefficient similarity values (average linkage clustering) between DGGE fingerprints of 16S rRNA genes amplified from exposed gelatin filter samples. Percentage similarity is based on the presence of common bands within the profiles.

Subtraction of the blank filters from the analysis increased the dissimilarity (variation ranging from 100% to ~25%) between the air samples (Fig. 3.6).

Sequencing of excised DGGE bands

The DGGE fingerprints of the blanks were further processed by extracting few of the dominant bands from one of the three replicates (marked on Fig. 3.4). Obtained partial 16S rRNA gene sequences retrieved from the excised bands (7 out of the 9 total yielded successful sequencing results) ranged from 124 to 145 bp after quality filtering, except one that was 79 bp long. Comparative sequence analyses revealed similarity values, ranging from 98% to 100% with *Bacillus* spp. sequences (apart from the 72-bp sequence that exhibited 92% similarity) from the NCBI database (Appendix S3). It should be noted that these bands appeared in the common pattern of all sample bacterial profiles.

Moreover, the DGGE method was implemented in order to validate the presence of distinct microbial populations in the actual air samples, in relation to time-integrated swab samples, and examine the composition similarity among the samples. DGGE analysis of bacterial 16S rRNA gene and fungal ITS2 region was applied on triplicates of air and surface samples corresponding to four different sampling locations; one students' accommodation site (Students' dorm kitchen #4 [13] - flat shared by 6), the university gym (Sports centre [14]), one coffee/meeting room [15] and a lecture theatre (Lecture theatre #1 [1]).

DGGE of bacterial 16S rRNA gene

DGGE profiles of 16S rRNA (see Appendix S3) were manually assessed and a matrix was constructed using the presence or absence of a band in each sample. Similarity between DGGE profiles based on the Jaccard coefficient is shown on Figures 3.7 and 3.8. DGGE community profiling validated the presence of diverse airborne microbial populations, at the various sampling sites. The obtained bacterial DNA profiles revealed a high consistence of similarity (~100%) in the bacterial composition among all aerosol sample replicates, indoor and outdoor, which is partially attributed to the bacterial contamination of the gelatin filter (as already discussed). Examination of the DNA fingerprints showed, also, that the common pattern of intense bands observed among the filter samples, does not appear in swab samples, whereas the similarity between air samples and surface samples ranges from 20% (Fig. 3.7) to 35% (Fig. 3.8).

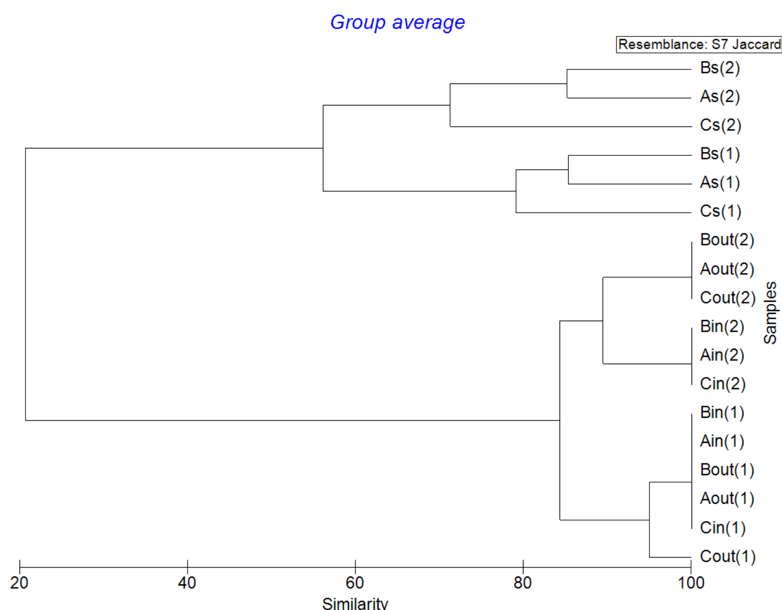


Figure 3.7 Cluster dendrogram of Jaccard coefficient similarity values (average linkage clustering) between DGGE fingerprints of 16S rRNA genes amplified from indoor (in) and outdoor (out) air samples collected with the gelatin filter, as well as surface swab samples (s) (see DGGE image in Appendix S3). Number "1" indicates samples collected at the student's accommodation site, while index "2" corresponds to the university gym. Percentage similarity is based on the presence of common bands within the profiles.

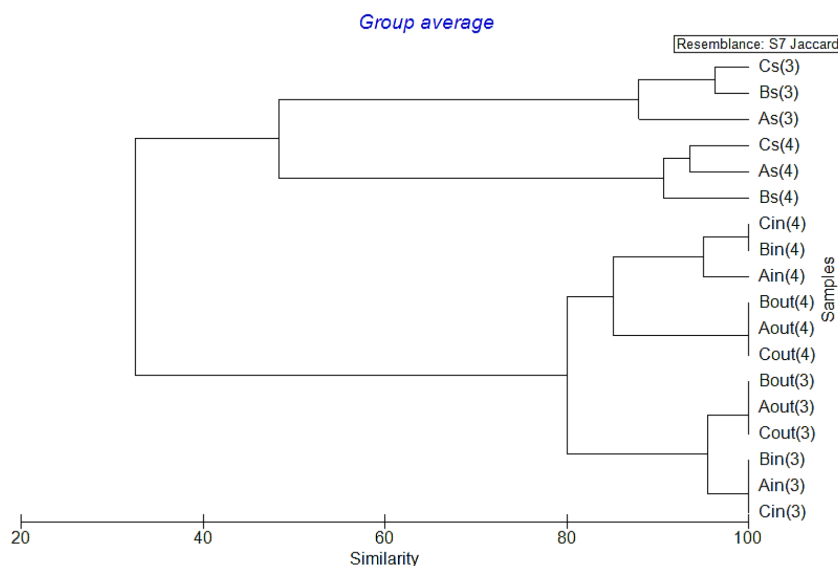


Figure 3.8 Cluster dendrogram of Jaccard coefficient similarity values (average linkage clustering) between DGGE fingerprints of 16S rRNA genes amplified from indoor (in) and outdoor (out) air samples collected with the gelatin filter, as well as surface swab samples (s) (see picture in Appendix S3). Number "3" indicates samples collected at the Lecture theatre, while index "4" corresponds to the university common room site. Percentage similarity is based on the presence of common bands within the profiles.

DGGE of Internal Transcribed Spacer 2 region

Fungal amplicons from the same samples were also analysed using PCR-DGGE analysis (Fig. 3.9 and 3.10).

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19*
Ain(1)	Bin(1)	Cin(1)	Aout(1)	Bout(1)	Cout(1)	As(1)	Bs(1)	Cs(1)	Ain(2)	Bin(2)	Cin(2)	Aout(2)	Bout(2)	Cout(2)	As(2)	Bs(2)	Cs(2)	Marker

20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	
Ain(3)	Bin(3)	Cin(3)	Aout(3)	Bout(3)	Cout(3)	As(3)	Bs(3)	Cs(3)	Ain(4)	Bin(4)	Cin(4)*	Aout(4)	Bout(4)	Cout(4)	As(4)	Bs(4)	Cs(4)	

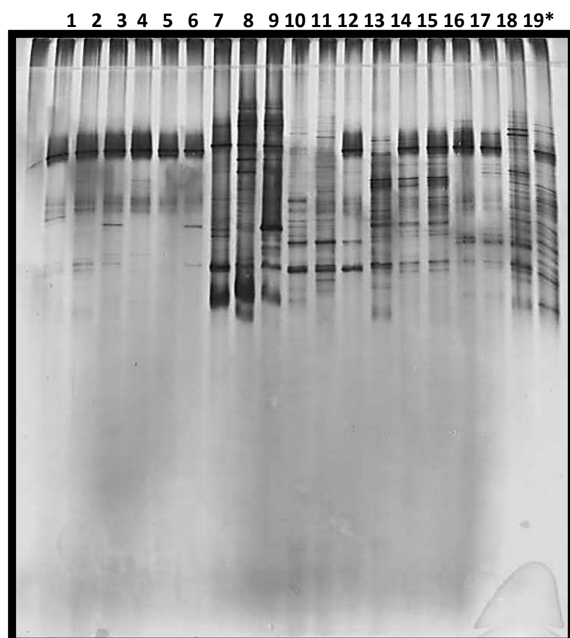


Figure 3.9 DGGE fingerprints of ITS2 region amplified from various indoor and outdoor air samples obtained with gelatin filters, as well surface samples. Number “1” indicates samples collected at the students’ accommodation site, while index “2” corresponds to the university gym.

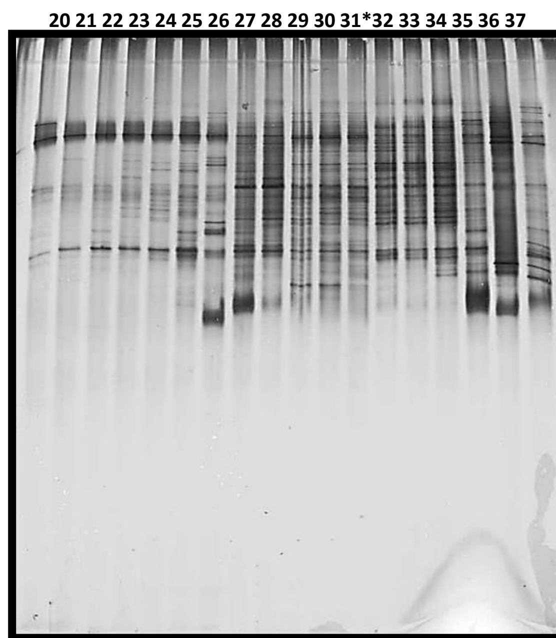


Figure 3.10 DGGE fingerprints of ITS2 region amplified from various indoor and outdoor air samples obtained with gelatin filters, as well surface samples. Number “3” indicates samples collected at the Lecture theatre, while index “4” corresponds to the university common room site. (*Sample #31 - Cin(4) was used as a marker - 19*- in previous gel).

The obtained DNA profiles revealed a high presence of similarly migrating bands in the fungal community fingerprints. However, weak amplicons obtained from air samples #1 (students accommodation site) and #3 (Lecture theatre) did not allow the formation of complete DGGE banding patterns. It should be noted that amplifiable fungal DNA was not detected in the blank filters (and therefore not used for DGGE analysis), which makes the gelatin filter suitable for characterisation of the airborne fungal composition.

3.3.2 qPCR quantification

Bacterial concentration

Average bacterial abundance, as determined by qPCR (Fig.3.11), varied by sampling site and was overall higher in indoor air, relative to outdoor air, apart from two sites (Lecture theatre #2 and Students' dorm #1). The results showed that Seminar room #1 and the Ecology and Coral reef unit Lab were the indoor sites with the highest mean bacterial concentration (1.08 $\times 10^6$ and 9.43 $\times 10^5$ 16S rRNA genes per filter or 6.40 $\times 10^5$ and 5.61 $\times 10^5$ 16S rRNA genes per m^3 of air, respectively).

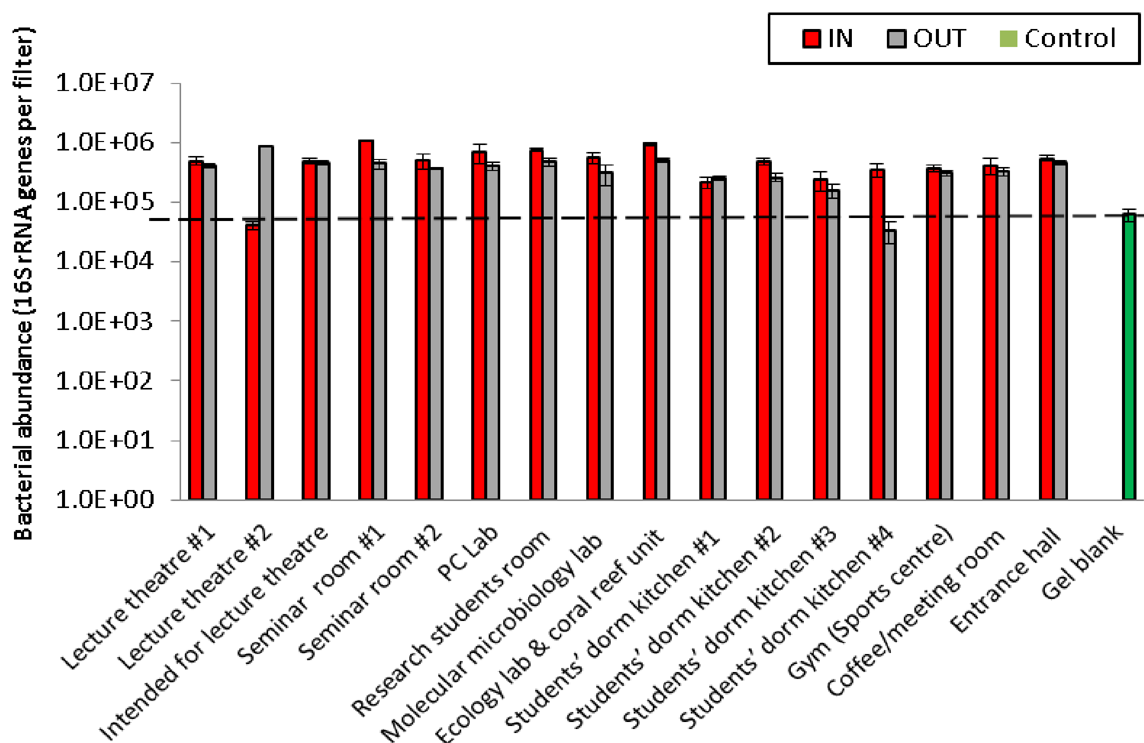


Figure 3.11 Indoor and outdoor airborne bacterial abundance in 16S rRNA genes as determined by 1-hour sampling at 28 L/min using gelatin filters in various sites at a university campus. Results are presented as average over three replicates. Error bars represent standard error. Dotted line indicates the average bacterial load found on unexposed gelatin filters (green bar). Note that y-axis is on logarithmic scale.

Indoor/outdoor relationship

Figure 3.12 presents the indoor-to-outdoor concentration ratio (I/O) for the bacterial abundance per each sampling site, which was estimated based on the mean concentrations. For all indoor environments, the median I/O was higher than 1, ranging between 1.1 (Room intended for lecture theatre) to 2.4 (Seminar room #1), apart from the two sites where the

outdoor air abundance was higher than the indoor. The degree of correlation between the samples was also examined using Pearson correlation coefficient (r) but there was no significant association found between indoor and outdoor air samples (r = 0.08, p-value>0.05).

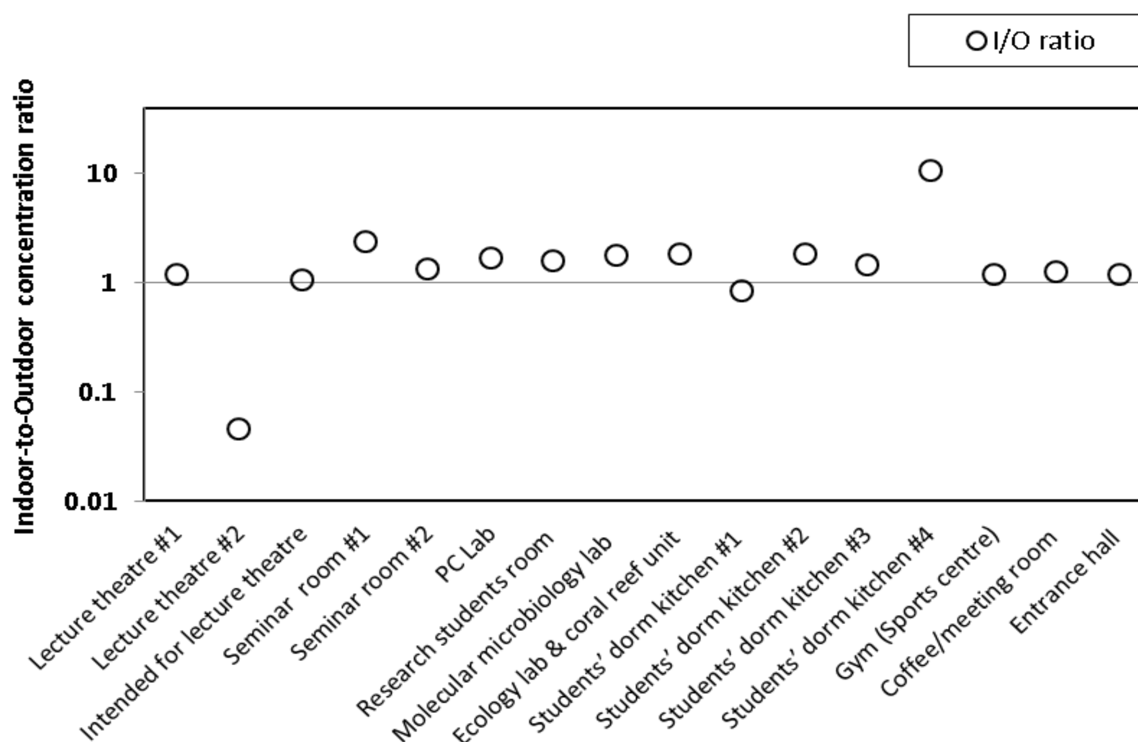


Figure 3.12 Indoor-to-Outdoor bacterial concentration ratio (I/O) per sampling site. A ratio over 1 is a quantitative indicator that indoor bacterial bioaerosols mainly originate from indoor sources, while a ratio below 1 shows that bacteria in indoor air are more likely dominated by outdoor air.

Contamination content

However, based on results mentioned above, part of bacterial concentration reported comes from the filter background, which, based on qPCR results from the DNA recovered from 9 blank gelatin filters, ranged from 3.16×10^4 to 1.06×10^5 16S rRNA gene copies per filter, with a mean value of 6.21×10^4 16S rRNA gene copies per filter. Therefore, the actual bioaerosol concentrations should be estimated by taking into account the bacterial load of the negative controls which, however, could vary per each filter. An overall quantitative comparison between the total bioaerosol samples collected indoors and outdoors across this study and the gelatin blank filters is given in Fig.3.13.

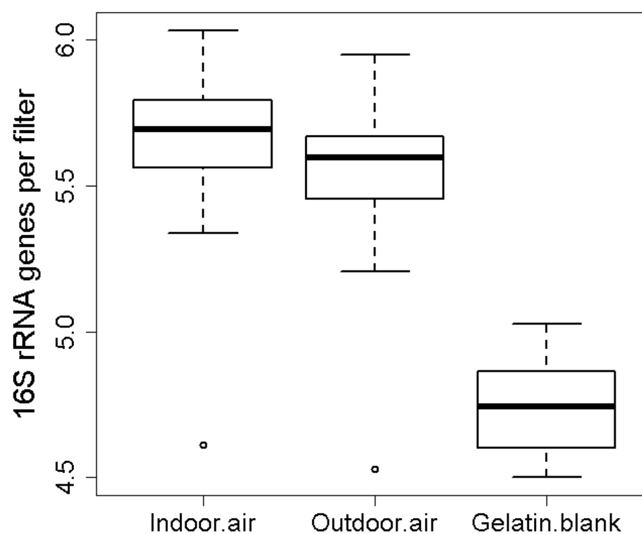


Figure 3.13 Bacterial abundance (log_{10} 16S rRNA genes per gelatin filter) for air samples collected indoors and outdoors in comparison with the unexposed filter controls. Box boundaries indicate the first (25th percentile) and third (75th percentile) quartiles. The median is given as the horizontal line within the box, while the whiskers (dashed lines) indicate the maximum and minimum values. Outliers are indicated with dots beyond the whiskers.

3.3.3 The efficiency of gelatin filters

The bacterial load on the gelatine filter was also assessed by comparative qPCR analysis of air samples collected side by side using gelatin and polycarbonate filters (Fig. 3.14). Bacterial quantities yielded showed a clear advantage for the gelatin filters (average 16S rRNA gene abundance ranged from 6.47×10^4 to 8.44×10^4 copies per filter) compared to the polycarbonate (average abundance ranged from 1.41×10^4 to 3.93×10^4 16S rRNA genes per filter) ones (t-test, p-value = 0.09 < 0.05). Interestingly, even the gelatin blank filters demonstrated higher abundance (mean = 6.21×10^4 16S rRNA gene copies) than the polycarbonate ones. It should be noted that the bacterial load of the polycarbonate blanks was attempted to be quantified but the qPCR-resulted Ct values were close to the Ct value of the no template controls (i.e. the difference was less than 3.3 cycles) and therefore it could not be determined.

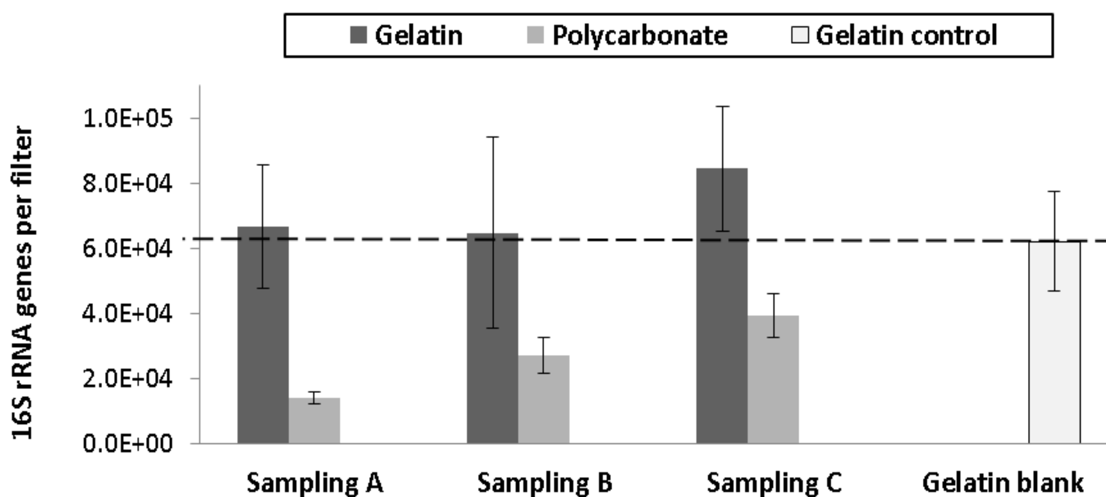


Figure 3.14 QPCR of 16S rRNA gene from three 1-hour simultaneous air sampling rounds (28 L/min) using gelatin filters versus polycarbonate filters. Error bars represent standard error (n = 3). Dotted line indicates the average bacterial load found on unexposed gelatin filters (white bar).

Moreover, we examined the possibility of subtracting the gelatin bacterial background from the air samples based only on quantitative data. Subtraction of the mean value of the 16S rRNA gene abundance estimated for the gelatin blank filter (derived from DNA recovered from 9 unexposed filters) from the average concentrations determined for the gelatin samples, resulted in lower values that the ones yielded for the polycarbonate samples (2.63×10^3 - 2.23×10^4 copies per filter). However, some of the gelatin sample replicates exhibited lower abundance than the one estimated for the blanks and therefore subtraction was not possible, indicating that the bacterial contamination varies per filter and subtraction of the background could lead to false results.

3.4 Discussion

The gelatin filter having a physical collection efficiency almost 100%, despite the fact that the filter pore size is as large as 3 μm (Burton et al., 2006; Parks et al., 1996), can demonstrate high performance in terms of recovery of captured airborne micro-organisms due to its solubility advantage that can overcome the problems associated with removing microbes from the filter prior to extraction. The gelatin material has a low melting point, at approximately 37°C, and thus, it can be easily dissolved in aqueous solution, which can facilitate the DNA isolation from the filter and at the same time minimise potential losses. In our study a direct DNA extraction approach was used by dissolving the filter in the extraction buffer, omitting extra steps of centrifugation and pelleting the dissolved filter prior to extraction that have been used in other studies (e.g. Van Droogenbroeck et al., 2009; Nehme et al., 2008). It has to be noted, though, that maintenance of high temperature is extremely important for preserving the filter in a liquid form during sample processing, as gelatin turns into a colloidal gel at room temperature, and therefore, inexperienced users might encounter solidification problems.

Several other drawbacks with using gelatin filters have been reported. Firstly, gelatin filters are brittle, which can make their handling as well as their use for prolonged sampling quite problematic (Yamamoto et al., 2010; Burton et al., 2005). Also, due to the proteinaceous nature of the filter, inhibition of PCR can be common especially with samples with low numbers of cells (Hubad & Lapanje, 2013). However, this issue was not encountered during our experiments. Fahlgren et al. (2010) reported that gelatin filters contain bacterial DNA and are not considered suitable for molecular analysis, but results were not shown. In 2005, Burton et al. attempted to investigate the performance of the gelatin filter, alongside other types of filters, for collection of viable aerosolised *B. subtilis* in a controlled experimental system. Nonetheless, the microscopic counts revealed the presence of non-viable bacteria, other than *B. subtilis*, in both the exposed samples and the blanks, and therefore the filters were considered contaminated and excluded from the analysis.

In general, gelatin filters have been mostly used in culture-based studies due to their high moisture content, which allows for maintaining viability of the organism collected on the filter, and their easy dissolution when placed on agar. On the other hand, this type of filter has been also widely used for molecular-based studies. Table 3.3 shows a number of

Table 3.3 PCR-based indoor air studies that have utilised gelatin filters for detection of bacteria and fungi.

Reference	Indoor site	Target micro-organisms	Aerosol sampling instrument	Filter details (as mentioned)	Sampling time (for each sample)	Flow rate	Analysis
Dutil et al. (2007)	Dental treatment room	Total bacteria, *Legionella and Mycobacteria*	IOM personal inhalable air samplers (SKC)	25 mm gelatin filter (SKC)	4-h (13 times)	4 L/min	qPCR
Nehme et al. (2008)	Swine confinement building	Bacteria	IOM cassettes (SKC)	25 mm gelatin membrane (SKC)	4 h (triplicates)	2 L/m	qPCR DGGE Sanger sequencing
Van Droogenbroeck et al. (2009)	PC3 negative pressure (0.0015 bar) isolator	*Chlamydophila psittaci*	IOM personal inhalable dust sampler (SKC), PAS6 sampler	25 mm gelatin filter (SKC)	60 min	1-2 L/min	Nested PCR Culture assay
Wu et al. (2010)	Bioaerosol chamber	Influenza A virus (H5N1) nucleotides, *Bacillus subtilis*, House dust mite dermatophagoides allergens	Button Inhalable Aerosol Sampler (SKC)	25 mm gelatin filter (SKC)	1, 2 and 5 min for H5N1 nucleotides, 30 min for bacteria 5, 10 and 15 min for allergens (3 repeats)	4 L/min	qPCR ELISA
Verreault et al. (2010)	Swine confinement buildings	Airborne porcine circovirus Total bacteria	IOM cassettes (SKC)	25 mm gelatin filter (SKC)	4 h (triplicates)	2 L/min	qPCR
Yamamoto et al. (2011)	window frame of a residential building	*Alternaria alternata, Cladosporium cladosporioides, Epicoccum nigrum, and Penicillium chrysogenum*	Personal Aeroallergen Sampler (PAAS)	25 mm gelatin filter (SKC)	3h – 35 days (duplicates)	Gravitational collection	qPCR
Li (2011)	Laboratory	Bacteria	Filter sampler (SKC)	gelatin filter (SKC)	30 min	5 L/min	qPCR DGGE
Chang & Chou (2011)	Bioaerosol generation system	*Legionella pneumophila*	IOM personal inhalable air sampler (SKC)	25 mm sterile gelatin filter (Sartorius)	30, 60, and 270 min	4 L/min 2 L/min	qPCR Culture assay
Wu et al. (2011)	Nursing Care Institution	*Acinetobacter baumannii* *Citrobacter freundii* *Escherichia coli* *Klebsiella pneumonia* *Staphylococcus aureus*	Airport MD8 sampler (Sartorius)	80 mm gelatin membranes (Sartorius)	5 min	50 L/min	qPCR
Yamamoto et al. (2010)	Plastic box (experimental apparatus) and field	Fungi	Plastic filter cassette (clear, styrene, SKC)	25 mm gelatin filter (SKC)	Few secs - mins (laboratory- based), 1 h (field-based)	5 L/min	qPCR
Masclaux et al. (2013)	Pig buildings	Total bacteria Airborne *Staphylococcus aureus* (MRSA and MSSA) Endotoxins	Button aerosol sampler (SKC)	25 mm gelatin filter (SKC)	4 h	4 L/min	qPCR
Chang & Hung (2012)	Biological aeration basins and shower rooms	*L. pneumophila* and *Legionella* spp.	IOM sampler (SKC)	25 mm gelatin filter (SKC)	30, 60, 270 min (3 repeats)	2 L/min	qPCR Culture assay
Hoisington et al. (2014)	Retail store	Bacteria and fungi	Button sampler (SKC), Personal environmental monitor (SKC)	gelatin (SKC)	15 min (triplicates)	4 L/min 10 L/min	454 GS-FLX Titanium Pyrosequencing

PCR-based studies that have utilized the gelatin filter for air sampling. Gelatin has been used for sampling of viral bioaerosols (surveys studying exclusively viruses are not included in the table), e.g. Influenza virus (e.g. Fabian et al., 2009), fungi (Yamamoto et al., 2011; 2010) and pathogenic bacteria (Chang & Hung, 2012; Wu et al, 2011; Droogenbroeck et al.; 2009), mainly in chambers studies. Detection of non-bacteria or specific pathogens could not be possibly affected by a bacterial composition in the filter. In our results, also, we did not encounter any issues when screening fungal bioaerosols since amplifiable fungal DNA was not detected in the blank filters. However, gelatin has also been used for detection of total bacteria (Masclaux et al., 2013; Li, 2011; Verreault et al., 2010; Wu et al., 2011; Nehme et al., 2008; Dutil et al., 2007) with no issue of DNA contamination ever been mentioned. In the study published by Hoisington et al. (2014), gelatin, used for next generation sequencing-based identification of airborne bacteria (and fungi), was claimed to be “DNA-free”. This could just mean that the filters were sterile due to the gamma irradiation applied from the manufacturer, since no further details were mentioned about the way of decontamination.

However, the results we obtained in our study show clearly the fact that gelatin filters contain a bacterial DNA background that cannot be removed with the method of gamma irradiation. The PCR detection of bacterial DNA in the blank gelatin filters, as well as the high similarity observed in the DGGE profiles between the blank and the exposed filters, suggest that all filters share some common bacterial communities. Having excluded any possibility of contamination of the unexposed filters during field and laboratory procedures, our findings suggest that there is background bacterial composition in this type of filter.

Gamma irradiation is a well-established sterilisation technique (da Silva Aquino, 2012; Tauxe, 2001) and since it does not involve any heat or moisture-generating processes, it is compatible with the soluble (at or above 37°C) nature of the gelatin material. However, despite the cell-killing capability of the method, gamma radiation treatment does not result in elimination of PCR-amplifiable DNA. Even though irradiation does cause damage to genomic DNA (e.g. lesions and fragmentation), several studies have demonstrated that microbial DNA extracted from irradiated material is still detectable by PCR (Shehata et al., 2011; Hoile et al., 2010; Dauphin et al., 2008; Trampuz et al., 2006; Dang et al., 2001), which is in accordance with the present findings. Furthermore, a recent survey showed that DNA recovered from irradiated samples could be also utilised for whole genome sequencing (Broomall et al., 2016). It should be noted that, due to the nature of the gelatin filter, other

treatments commonly applied in research labs for decontamination of filters, such as autoclaving or dry heat, cannot be utilised from the users for further sterilisation.

The origin of the bacterial contamination found in the filters is most likely linked to the gelatin production. Gelatin is manufactured from collagen derived from animal connective tissues, skin or bones through a complex multi-stage production process (Gelatin Manufacturers Institute of America, 2012). As gelatin constitutes a nutrient medium for the growth of micro-organisms, it has been demonstrated that bacterial contamination can occur during various different stages of the production process (Sharma et al., 2006a; b; De Clerck et al., 2004a; b; c; De Clerck & De Vos, 2002). The detection of contaminants is of major concern for the production, as many of those can exhibit gelatinase activity (i.e. ability to liquefy gelatin) which can have a deteriorating effect on the gelatin quality.

In spite of the extreme temperature, PH, chemicals and desiccation during the manufacturing process, it has been reported that some types of resistant endospore-forming aerobic bacteria, which are known to survive harsh conditions (Nicholson et al., 2000; Setlow, 1994), can be even found in the final product (De Clerck et al., 2004b; c). In particular, the most common persisting contaminants have been identified as members of *Bacillus* (e.g. *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. fumarioli*, *B. coagulans*) and related endospore-forming genera (*Brevibacillus*, *Paenibacillus*, *Alicyclobacillus*, *Anoxybacillus*, *Geobacillus*) (De Clerck et al., 2004a; b; c; d; De Clerck & De Vos, 2002). Interestingly, many of those have been detected in the stage following the final ultra-high temperature (UHT) purification treatment (De Clerck et al., 2004c). Limitations associated with the technical properties of the gelatin do not allow the application of further decontamination treatments (De Clerck et al., 2004a; c) that could prevent the survival of the particular bacterial spores (Silva et al., 2013; Kreske et al., 2006; Waites et al., 1988). These findings are in agreement with our results, as sequencing of the high-intensity DGGE bands for one of the gelatin blank filters revealed sequences closely related to *Bacillus* spp. It is noteworthy that the issue of contamination with aerobic endospore-formers, many of which can survive UHT sterilisation (Klijn et al., 1997; Brown, 1994; Davies et al., 1975), is a particularly common problem in the industrial sector (Soni et al., 2016; Gopal et al., 2015; Pirttijärvi et al., 2000).

Despite detectable bacterial DNA in the gelatin filter, the differences found in bacterial composition between the blank filters and the exposed samples, as revealed by the DGGE

profiles, indicated that there were different taxa present in the samples, other than just the background contaminants. Moreover, it has to be taken into account that high similarity observed between some of the air samples, such as between the outdoor samples for the Ecology lab and the Microbiology lab or between the outdoor samples for the Students' office and the PC lab, may not only be attributed to the filter background composition. The particular sampling sites are located on the same area (main university campus) and therefore a similar airborne bacterial composition is expected. On the other hand, the more dissimilar profile obtained from the outdoor environment of Students' residence kitchen compared to the other outdoor air samples, could be an outcome of the distance of the specific university accommodation (sampling site 13 - location J) from the main campus.

Multivariate cluster analysis for some of the other sampling sites also revealed few differences among the profiles obtained, which could be due to the different conditions in each place. For instance, the bacterial composition at sampling site #4 varied more between indoors and outdoors (similarity~ 85%) compared to sampling site #3 (similarity ~95% between indoors and outdoors). This is probably attributed to the fact that sampling in the coffee/meeting room (site #4) was carried out under occupied conditions (Hospodsky et al., 2012), whereas the lack of students during sampling in the lecture theatre (site #3) resulted in higher similarity in the bacterial composition of the indoor aerosols with the outdoor air content (Meadow et al., 2014). Cluster analysis with the inclusion of swab samples revealed that there is only 20 to 35% similarity between the air samples and the surface samples, indicating that some of the detected bacteria on the gelatin filter samples could be resuspended from the surfaces. Yet, the intense band pattern observed for the blank controls did not seem to appear in the swab samples suggesting that the microbial content of the filters is not similar to the microbial particles that settle on surfaces.

Although DGGE fingerprinting analysis is a valuable inexpensive and rapid technique that can be used for the initial assessment of the compositional variation among samples and has been utilised by several bioaerosol studies for characterisation of airborne bacteria (e.g. Chatterjee & Singler, 2015; Tanaka et al., 2015; Li, 2011; Xu & Yao, 2013; Lee et al., 2009; Nehme et al., 2008; Negrin et al., 2007), it can only provide limited information. Despite the aforementioned general conclusions that could be drawn from the examination of the banding patterns, one of the main limitations of DGGE is that 16S rRNA gene fragments from different taxa can migrate to the same position within a gel (Gafan & Spratt, 2005; Jackson et

al., 2000). Therefore, the fact that the air samples exhibited bands from at the same positions as the blanks does not guarantee the presence of identical bacterial populations (i.e. *Bacillus*-related taxa). Moreover, a single band could represent multiple co-migrating sequences. Moreover, as *Bacillus* and *Bacilli* in general are bacterial taxa commonly found in the air, these results raise further questions whether some of the common intense bands found in the DGGE profiles of the air samples represent actual captured bacteria and not just the gelatin background-related contaminants. Application of high throughput sequencing could provide a deeper characterisation of the microbial content in gelatin filters.

Analysing blank filters i.e. filters inserted into sampling apparatus but not exposed to air-flow, alongside sample filters to test for contamination is a common practice for field sampling. Detection of DNA on field banks, attributed most likely to filter handling, has been reported previously (Després et al., 2007). Moreover, getting a weak qPCR signal from blanks is not unusual. DeLeon-Rodriguez et al. (2013) observed that field blanks demonstrated at least one order of magnitude lower 16S rRNA gene abundance compared to the field samples. They stated that this was due to the sterilisation approach used for filters and filter holders, which involved autoclaving at 120°C for 30 minutes. Thus, it is likely that DNA from dead (autoclaved) cells remained on the filters and provided a signal during qPCR. In case DNA extracted from field blanks and amplified, is positive, it should be subtracted from the values obtained for the aerosol samples in order to avoid erroneous results. For instance, the gelatin filters appeared to perform better compared to the polycarbonate ones, for the parallel sampling performed, when the bacterial load of the blanks was not taken into account.

Yet, in our study the qPCR determination of the bacterial load was found not to be sufficient for subtraction of the contamination background from the filters and estimation of the actual bacterial concentration in the environmental samples collected. The variable quantification results given for the blank filters indicated that the degree of contamination varies per filter and therefore, a quantitative subtraction could significantly affect the results and lead to inaccurate estimates of the true abundance, especially when working with low biomass samples. This was particularly illustrated in the efficiency experiment that some of the individual replicates exhibited lower concentrations than the average 16S rRNA gene abundance estimated for the blanks and therefore a possible subtraction would lead to negative values. Moreover, even though the samples collected in the field study demonstrated on average quite higher abundance compared to the controlled experiment, there were still

two sites (Lecture theatre #2 indoors, Students' dorm #4 outdoors) that exhibited lower airborne concentrations than the estimated mean abundance for the blanks. In addition, the different levels of contamination per filter would, consequently, affect the determination of the ratio I/O and the conclusions that could be drawn about the contribution of outdoor sources on the indoor air bacterial abundance.

Since examination of every single gelatin filter before and after sampling is not feasible, if the estimation of an approximate concentration is desired, one possible solution could be to increase considerably the sampling volume, either by using a high-flow rate sampling device or by increasing the sampling duration, in order to ensure a significant difference between the blanks and all the samples. Results reported here represent common indoor (and outdoor) settings with typically low encountered bioaerosol levels. The effect of the bacterial DNA contamination of the filter on the quantification of the airborne bacterial load in highly contaminated environments (e.g. industrial workplaces) is expected to be less significant.

3.5 Conclusions

In conclusion, the present study demonstrated for the first time the presence of PCR-amplifiable bacterial DNA in gelatin blank filters and the significant impact that could have on the interpretation of data generated by molecular analysis of bioaerosol samples. Our findings suggest that the gelatin filter is not a suitable collection substrate for sampling of airborne bacteria if culture-independent DNA-based analysis is to be performed. A follow-up investigation is under way as samples have been further processed for high throughput amplicon sequencing of 16S rRNA gene in order to characterise the gelatin filter microbiome and examine its effect on the interpretation of sequencing generated data derived from aerosol samples.

Chapter 4

Characterisation of bioaerosols in the residential environment

Seasonal variation of size-resolved bacterial aerosols in an urban and a semi-urban residential flat

4.1 Introduction

Despite the public perception of bioaerosol exposure being mainly associated with processes related to certain occupational environments in the industrial sector or potential spread of pathogenic agents for bioterrorism purposes, we are all constantly exposed to airborne micro-organisms indoors and outdoors. However, the estimate of the time spent in the built environment by the majority of people in the developed world being about 85 - 90% (Klepeis et al., 2001) underlies the fact that we are far more exposed to airborne microbial particles indoors than outdoors. The recognition of the importance of the indoor environment, due to its association with humans, has led to a rapid increase in the number of studies on the microbiological indoor air quality in various enclosed spaces.

Perhaps the most important environment are the houses where we live and while there is a growing number of culture-independent studies which have explored the airborne microbial content within the residential environment (Coombs et al., 2018; Emerson et al,, 2017; Luongo et al., 2017; Wilkins et al., 2016; Miletto & Lindow, 2015; Adams et al., 2014; 2013) there are still a lot of knowledge gaps that need to be addressed. Although bacterial abundance and diversity of total suspended particulates have been well studied, there is limited information on the size distribution of bioaerosols. Aerodynamic diameter strongly influences the fate of biological particles and human exposure and therefore, size-resolved data are of fundamental value for developing insights regarding health effects of exposure to bioaerosols. To date, only a few prior studies have evaluated the size-resolved diversity of bioaerosols using high throughput sequencing approaches (Yamamoto et al., 2015; 2014; 2012; Qian et al., 2012) and to the best of our knowledge there hasn't been any on domestic environments. In addition, although the size-distribution of airborne particles has been a well-investigated subject in culture-based studies, there is little knowledge about particles > 12 µm (Clauß, 2015). Moreover, even though monitoring the seasonal patterns of airborne microbial communities has been common in outdoor air studies (e.g. Du et al., 2018; Bowers et al., 2013; Franzetti et al., 2011) there is a limited number of longitudinal indoor air studies and

therefore little is known on the seasonality of the indoor aerosol microbial assemblages (Coombs et al., 2018; Adams et al., 2013). The microbial abundance and composition of air not only exhibits seasonal variation, but varies greatly on a daily basis an even within day and it has been shown that sampling in multiple time points is required to characterise microbial communities in indoor air (Emerson et al., 2017). Furthermore, although it is well established that not only the indoor sources, but also the outdoor environment strongly determines the indoor air microbiome, studies tend to be restricted to only one site and variability between multiple buildings in different locations and types of environments has been poorly explored (Amend et al., 2010).

In order to gain an insight into the factors driving the indoor air microbiome in the domestic environment, a long-term sampling study was performed within two residential settings located in two different types of sites; a small town surrounded by rural areas (Colchester, UK) and a far more urbanised big city (London, UK). The two sampling houses were selected to be as similar as possible in terms of building age, interior design (i.e. one bedroom flats), near-outdoor environment (i.e. riverside locations) and occupancy patterns. We aimed at describing the structure of the airborne microbial assemblages and their variability among different environmental conditions, based on time-resolved and particle size-resolved data, using a high throughput sequencing approach.

The overall goal of this study was to investigate the bacterial diversity and size distribution of indoor bioaerosols, in relation with the outdoor bioaerosols, in residential environments situated in two different types of locations ("urban" and "semi-urban") across different seasons using active and passive collection methods. The objectives of the current investigation were to explore whether

1. the type of outdoor environment is going to affect the indoor air bacterial composition in the two study houses and whether people living in more urbanised areas are more likely exposed to different airborne bacteria than those living in less urbanised areas,
2. the seasonality is going to have a strong effect on the overall aerosol bacterial composition and abundance as well as the diurnal patterns of bacterial abundance,
3. the bacterial composition and abundance is going to differ across the different particle size fractions and
4. the type of sampling collection method (active or passive) is going to have an impact on the recovered bacterial community structure.

4.2 Methods

4.2.1 Sampling

Two residential flats with similar characteristics, located in two different types of environments; an urban and a semi-urban area, were monitored over three different seasons using active and passive collection methods.

4.2.1.1 Study environments selection

The sampling campaign took place during 2016 in two residential flats, located in an urban settlement within the district of Stratford, part of the densely populated London Borough of Newham (population: 342,430, ONS, 2017) in East London (UK), and a residential suburb two miles (3 km) from the centre of Colchester, which is the main town of the predominantly rural Colchester Borough (population: 186,635, ONS, 2017) situated in the north east of Essex (UK). The distance between the two sites is approximately 80 km. Volunteer home tenants were recruited for this study. Sampling houses were selected as similar as possible to one another, based upon the willingness of home occupants to participate in the study. Both residences were approximately the same size, consisting of an open plan kitchen and living room space and one bedroom and were situated in riverside residential blocks of similar age. Floor plans and aerial view of the surrounding environment are provided in Figures 4.1 and 4.2. Both apartments were naturally ventilated and were occupied by two adults. Information on the characteristics of each household is provided in Table 4.1. Throughout sampling periods the occupants maintained their normal routines and time-activity information, levels of occupancy and ventilation for each home were recorded. During sampling periods the possible in-house PM sources included typical household tasks and activities, e.g. cooking and cleaning, and no exceptional activities were reported.

4.2.1.2 Sample collection

Sampling was performed within 1-month periods at three different seasons (winter, spring and summer), indoors and outdoors. For indoor sampling, equipment was placed on a sampling trolley at a central position in the living room, at a height of 1.5 m and all sampling apparatuses were connected with tubing to vacuum pumps that were placed in boxes reinforced with soundproofing foam in order to reduce the high levels of pump-generated noise and cause less intrusion to the occupants. Outdoor sampling equipment was set up in the balcony of the semi-urban study house, while for the urban flat, sampling was carried out through the bedroom's window (door to the room was kept closed during sampling). In case

of rainy conditions, a plastic cover was used to protect the outdoor equipment. Sampling periods per each season did not take place simultaneously in both apartments as the whole experimental setup, equipment performance monitoring and sample collection was conducted by one person.

Table 4.1 Information on the characteristics of each household.

House Characteristics	Urban flat (London)	Semi-urban flat (Colchester)
Building construction year	2009	2011
Building distance from closest river	~20 m	~10 m
Flat floor	6	2
No of bedrooms	1	1
Ventilation	Natural	Natural
HVAC	-	-
Dehumidifier	-	1
Floor type	Wood/tile	Wood/carpet
Carpeting	-	1 (bedroom)
Stove type	Electric	Electric
Water damage/ visible mold issues	-	-
Occupants	2	2
Female:male	1	1
Smokers	1	1
Pets	-	-
Houseplants	2	-

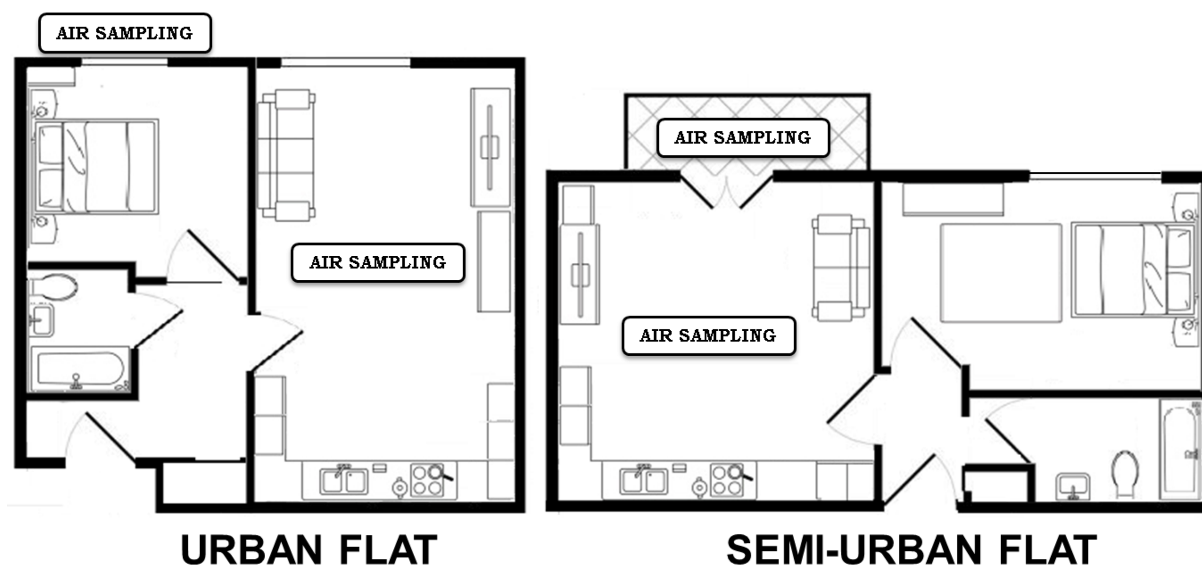


Figure 4.1 Floor plan of the study houses with marked the indoor and outdoor air sampling locations.

Semi-urban site

Urban site

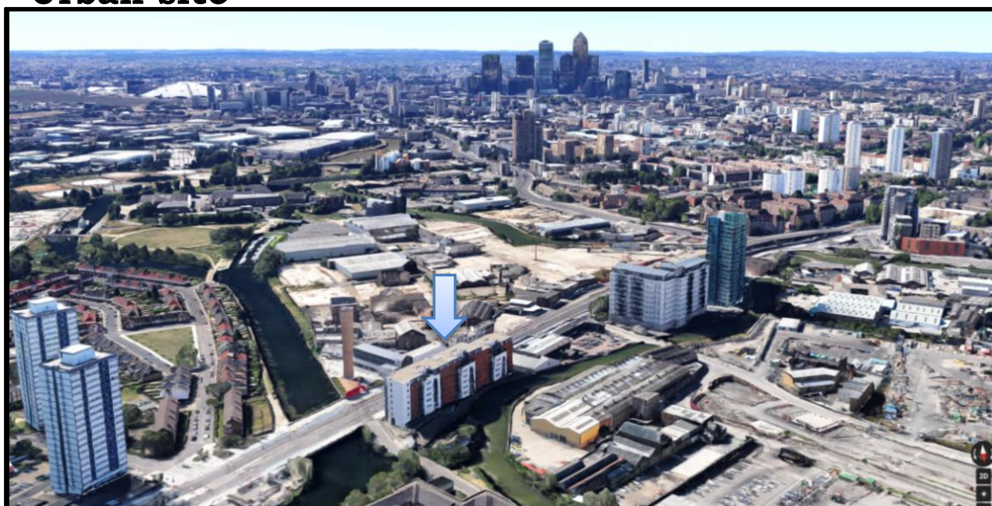


Figure 4.2 Aerial views (3D Google images) illustrating the locations of the study houses (residential blocks are pointed) and the surrounding area in Colchester (Essex) - semi-urban and Stratford (London) - urban sites. See also Appendix-S4.1 for wider scale images of the surrounding regions.

Size-resolved particles sampling

Whole day (12h) collection of size-distributed aerosol samples was carried out between 09:00 - 21:00 for three days per each site and season. For indoor sampling, a modified version of the viable Andersen impactor (Thermo Scientific Electron), operated at a flow rate of 28.3 L/min, was utilised. The Andersen sampler has six stages with lower cut sizes (aerodynamic equivalent diameter) of 0.65, 1.1, 2.1, 3.3, 4.7 and 7.0 μm and is designed for culture-based sampling (Andersen, 1958). For our study, impactor was modified by placing PTFE-made discs (86-mm diameter, 6-mm thick) in glass petri dishes. This solid surface can be loaded with any type of filter, replacing the traditional collection on agar or membrane overlay on agar, for the purposes of non-viable sample collection and downstream applications. 85-mm

Mixed Cellulose Ester filters (0.4-µm, Pall Corporation) were used as sampling substrates in the impactor. For simultaneous indoor and outdoor size-distributed samples collection, two seven-stage May impactors (aerodynamic diameter: 0.5-1, 1-2, 2-4, 4-8, 8-16, 16-32 and >32 µm), that collect particles onto standard microscope glass slides, were deployed. The May impactor, also known as the "ultimate cascade impactor" (Cox & Wathes, 1995), is no longer commercially available and this is the first time that it is used for bioaerosol collection aimed for molecular analysis. Aerosol samples were collected at an air flow rate of 20 L/min. For outdoor sampling at the urban study house, the impactor inlet was connected to a sterile sampling tube (27-mm inner diameter and 50-cm length) which was passed through the window.

Total suspended particles sampling

For each site, the diurnal pattern bioaerosol concentrations of total particles were also monitored by collecting time integrated samples at 28 L/min using 47-mm Mixed Cellulosed Ester membranes (0.45-µm pore size, Pall Corporation), loaded on filter holders, for one hour, three times during each sampling day; morning (~9:00), afternoon (~14:30) and evening (~20:00). Samples were collected simultaneously indoors and outdoors. For the urban study house, extended clamps were used to hold the filter holders out of the window, ensuring that the samplers were directed away from the wall and the inlets facing outwards. A liquid impinger (Biosampler, SKC) was also utilised for daily indoor sampling (1 h) at 12.5 L/min side-by side with the evening filter sample collection (due to insufficient microbial biomass samples collected with the impinge were not included in the analysis).

Passive sampling

Besides active sampling, a passive collection method was also conducted using settle plates. Triplicates of sterile, empty petri dishes were installed in the living room, following method described by Adams et al. (2013), and left for duration of a whole month per each season (February, May and August). The open plates were placed on petri dish lids and hung from the ceiling at a height of approximately 230 cm above the floor, using nylon monofilament fishing line.

A summary with all sampling dates and details is given in Tables 4.2 and 4.3.

Table 4.2 Active sampling dates per each house and season.

House \ Season		Winter	Spring	Summer
Urban (London)	**DAY 1**	29.02.2016	11.05.2016	08.08.2016
	DAY 2	02.03.2016	13.05.2016	11.08.2016
	DAY 3	04.03.2016	16.05.2016	16.08.2016
Semi-urban (Colchester)	**DAY 1**	22.02.2016	24.05.2016	22.08.2016
	DAY 2	24.02.2016	27.05.2016	25.08.2016
	DAY 3	26.02.2016	31.05.2016	30.08.2016

Table 4.3 Samples collected per each house and season.

Sampling method	Collection substrate	Flow rate	Sampling environment	Duration	Replication
6-stage modified Andersen impactor	85-mm Mixed Cellulose Ester filters (0.4 μm pore size)	28.3 LPM	indoors	whole day (12 hours)	3 days
7-stage May impactor	glass slides	20 LPM	indoors & outdoors	whole day (12 hours)	3 days
Filter holders	47-mm Mixed Cellulose Ester filters (0.4 μm pore size)	28.3 LPM	indoors & outdoors	1 hour, 3 times per day	3 replicates for 3 days
Biosampler impinger	Phosphate-buffered saline solution	12.5 LPM	Indoors	1 hour	3 days
Suspended passive collectors	Empty petri dishes	n/a	indoors	1 month	3 replicates

Decontamination of sampling equipment

A variety of sterilisation methods was used depending on material damaging limitations. Prior to sampling, all filters were wrapped in autoclaved foil and autoclaved at 121°C for 15 minutes. Filter holders, glass slides (ThermoFisher Scientific, UK) used as collection substrates for May impactor stages, glass petri dishes and PTFE disks used for Andersen impactor stages were all washed in 10% bleach solution (0.05% sodium hypochlorite) for at least 2 hours. Items were after rinsed with ultrapure water (Milli-Q, Millipore) and 70% (v/v) ethanol, placed in autoclavable bags and send for autoclaving. Following each day's sampling, the Andersen impactor was disassembled and wiped thoroughly with 70% ethanol and then each stage-part was wrapped in aluminium foil and sterilised in a dry heat oven at 200°C for 2 hours (impactor o-rings were replaced with silicone-made ones that can stand high temperatures required), in order to avoid staining or corrosion of the apparatus aluminium surfaces that might occur during repeated steam sterilization. The May impactor was also wiped with 70% (v/v) ethanol and then each stage was placed in an autoclavable pouch and sent for autoclaving (due to dissimilar metal parts, dry heat processing had to be

avoided). In addition, prior to moving sampling site, both impactors' parts were also sterilised from both sides under UV light for 20 minutes in UV crosslinker, in order to eliminate any DNA present. Assembling of impactors and loading of sampling substrates was carried out in a safety cabinet using aseptic techniques. All openings were sealed to prevent contamination from ambient air

Handling and storage of samples

After sampling, 47-mm filters were removed from the samplers using disposable sterile forceps (# WZ-06443-20, Cole-Parmer Instrument Co Ltd, UK) and placed in sterile 50-ml tubes (62.547.254, Sarstedt Ltd, UK). 85-mm filters were collected from the Andersen impactor using autoclaved aluminium tweezers, stored in sterile Petri-dishes and sealed with parafilm. Slides from the May impactor were removed and swabbed immediately using nylon flock swabs (#552C, COPAN Diagnostics Inc). Swabs were removed from the sterile dry transport tubes immediately prior to each stage swabbing and were moistened with sterile 1×PBS (10 mM $PO4^{3-}$, 137 mM NaCl and 2.7 mM KCl), pre-aliquoted in 1.5-ml microcentrifuge tubes (Axygen®). Once the swabbing was complete, swabs were returned to the transport tubes. At the end of each sampling month-period, the suspended petri dish - samplers were covered with sterile lids from new plates and sealed with parafilm. After collection, all samples were placed in the sampling flat's freezer and at the end of each day samples were transferred to the University of Essex -20°C storage for downstream processing.

Real-time particles concentration monitoring

Particle mass measurements in $\mu g/m^3$ for both PM_{10} and $PM_{2.5}$ were performed indoors and outdoors using an optical particle counter Grimm 1.108 (GRIMM Aerosol Technik GmbH & Co. KG, Germany) and an Aerocet 531 instrument (Met One Instruments Inc., USA). For indoor fine particulate matter ($PM_{2.5}$) recording, a Speck sensor (Airviz Inc, USA) air quality monitor was also utilised.

To accurately compare results obtained with the different instruments, the performance of PM monitors was assessed by intercomparison measurements against a Grimm 1.109 OPC (GRIMM Aerosol Technik GmbH & Co. KG, Germany), conducted in an additional experiment. Based on the pairwise plots of data collected from the different instruments, correction of the mass readings was performed by applying the adjustments factors derived

from linear regression between the Grimm 1.108, Aerocet 531 and Speck sensor data (independent variables) with the Grimm 1.109 OPC levels (see Appendix S4).

Environmental parameters monitoring

Parameters of the microclimate (temperature and relative humidity) in the study houses, as well as carbon dioxide concentration, were recorded continuously at one minute interval using Rotronic CP11 indoor air quality meter. Outdoor meteorological parameters, including temperature, relative humidity, wind speed and precipitation accumulation for the duration of the sampling periods were retrieved from WU Personal Weather Station Network (www.wunderground.com).

4.2.2 Sample processing

4.2.2.1 DNA extraction

MCE filters

For the processing of aerosol samples collected using MCE filters as sampling substrate, sterile 5-ml screw-cap tubes (Axygen Scientific Inc., USA), pre-filled with 0.75 g of sterile 0.1-mm zirconium/silica lysing beads (Biospec, Bartlesville, OK, USA) were used. 47-mm filter samples were inserted into tubes by rolling the filter into a cylinder with the top side facing inward. 85-mm filters were cut into 2 pieces and each half was crashed into smaller pieces in each tube using sterile tweezers. Cells were lysed by bead beating at Precellys Evolution tissue homogeniser (Bertin Instruments, France) for 3×60s at 7200 rpm in the presence of 1 ml, for the 47-mm filters, and 2 ml, for the 85-mm filter-halves, of extraction buffer [2.5% (v/v) sodium dodecyl sulfate (SDS), 10mM Tris-HCl pH 8, 25mM Na_2EDTA pH 8, 100mM NaCl and molecular biology grade water]. Samples were then incubated at 75°C for 30 minutes. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) pH 8.0 was added and the tubes were centrifuged at 2,000×g for 5 minutes in a swing bucket centrifuge. The supernatant fractions were placed in new 2-ml microcentrifuge tubes (2 tubes were used for each 85-mm filter-half) and subjected to another centrifugation at 11,337×g for 5 minutes in microcentrifuge. DNA was precipitated by the addition of equal volume of isopropanol and 1.5-µl co-precipitant GlycoBlue (15 mg/ml, Invitrogen Ambion) and incubation for 100 mins at room temperature, followed by centrifugation at 11,337×g for 30 minutes. The DNA pellets were washed with 80% (v/v) ice-cold ethanol, air-dried and finally resuspended in 35 µl of sterile water (PCR-Grade, Roche) [4 resulting pellets from 85-mm filter-halves were pooled into the same elution volume].

Passive collection samples

For nucleotide extraction from the petri-dish samplers, nylon flocked swabs (#552C, COPAN Diagnostics Inc.) moistened with sterile PBS buffer, were used to wipe thoroughly the surface. The tips of the swabs were cut into 2-ml zirconium/silica-bead filled (0.1-mm, 0.5 g) screw-cap tubes using sterile scissors. Cells were lysed as described before except a volume of 500 μl extraction buffer was used for each sample and heating step prior bead beating was omitted.

4.2.2.2 16S rRNA gene quantification

Total and size-resolved bacterial concentration for the filter samples and the bacterial load for the settle plates were determined by universal 16S rRNA gene qPCR using the Bakt_341F/Bakt_805R primer pair (Herlemann et al., 2011). All real-time PCR runs were performed in duplicate, on a CFX96 Real-Time System/C1000 Thermal cycler (BioRad, USA). Reaction mixture was prepared in a total volume of 10 μl using 5 μl (1×) SensiFAST™ SYBR® No-ROX Kit (Bioline), 0.2 μl of each primer (final concentration 200 nM), 3.6 μl microbial DNA-free water (Qiagen) and 1 μl DNA template. The thermal cycling protocol was as follows: 95°C for 3 min, followed by 40 cycles of 5 s of denaturation at 95°C and 30 s of annealing and extension at 60°C. A melt-curve analysis was also performed for every qPCR assay with a temperature gradient of 0.5°C from 65°C to 95°C to confirm primers specificity. Genomic DNA extracted from *Escherichia coli* K-12 was used to make a standard curve. The amplification efficiency of the standard curve ranged from 90.3% (slope = -3.580) to 98.5% (slope = -3.359), with the correlation coefficient being > 0.98 and the y-intercept value varying between 33.52 and 35.15, among runs. No-template controls included in each run either yielded no products or their Ct value varied between 36 and 39. Data were acquired by CFX Manager Software (BioRad). Comparisons with the standard curve gave the estimated 16S rRNA gene copy number, on the basis of genome size (4.64 Mbp) and the number of 16S rRNA gene copies (7) per *E. coli* genome, in each qPCR reaction. Elimination of the effects of PCR inhibitors was done by dilution of the template DNA. The concentration of diluted sample was then re-calculated by taking into consideration the dilution factor.

4.2.2.3 Illumina MiSeq sequencing of 16S rRNA gene

Bacterial diversity in size-resolved samples was assessed using high-throughput amplicon sequencing. In order to obtain a representative size-resolved aerosol microbial profile from each season, DNA samples from each one of the three days were pooled for each size

fraction. Therefore 20 samples corresponding to different size-fractions were analysed per each house and season (6 indoor samples obtained with the Andersen impactor, 7 indoor and 7 outdoor samples collected with the two May impactors operating simultaneously). The V3 and V4 region of the 16S rRNA gene was sequenced using primers described by Herlemman et al. (2011) with overhang adapter sequences for compatibility with Illumina indices, as described in the Illumina Library Preparation Workflow (16S Metagenomic Sequencing Library Preparation, Part # 15044223 Rev. B). Sequencing libraries were prepared following standard Illumina protocols and pooled in equimolar concentrations. Samples were sequenced as paired-end reads (2×300 bp) on Illumina MiSeq platform with v3-chemistry and a 20% PhiX spike-in on two separate runs, at Earlham Institute (formerly The Genome Analysis Center, Norwich, UK) and at School of Biological Sciences, University of Essex (UK). Full details of the library preparation protocol and sequence processing are provided in Chapter 2 Methods.

4.2.3 Data analysis

Data analysis was based on statistical tests described in previous chapters. In order to assess diurnal and seasonal variation, when normality (Shapiro-Wilk test) and equal variance (Levene's test) assumptions were met, one-way analysis of variance (ANOVA) was conducted. If the homogeneity of variance assumption (Levene's test) was violated, one-way Welch test was carried out, whereas non-parametric Kruskal-Wallis test was performed for non-normally distributed data. Significance reported for any analysis was defined as $p < 0.05$. The indoor to outdoor (I/O) ratio was calculated to determine the impact of outdoor sources on indoor air concentrations. Spearman's rank correlation analysis was also performed to assess the influence of environmental variables on bacterial abundance. Particle size distribution plotting and calculations of geometric means and geometric standard deviation of aerodynamic diameters was performed as described in Chapter 2. Sequencing data were analysed within QIIME (Caporaso et al., 2010), as described previously, with beta diversity analysis carried out using the abundance-based Bray-Curtis dissimilarity metric. In addition, Linear Discriminant Analysis Effect Size (LEfSe) was used (Segata et al., 2011), implemented in *MicrobiomeAnalyst* web-based tool (Dhariwal et al., 2017), in order to explore differential abundance in the taxa recovered by passive sampling between the two study houses.

4.3 Results

4.3.1 Overall description of seasonal characteristics and PM concentration

Table 4.4 summarises the weather parameters and the indoor microclimatic conditions (daily median ± standard deviation) for the two sites across all sampling periods. The daily outdoor temperature and relative humidity per each season for London and Colchester respectively ranged from 5.6 to 6.8°C (67 - 81% RH) and 6.0 to 7.1°C (59 - 66% RH) in winter, from 15.0 to 16.9°C (60 - 97% RH) and 11.7 to 20.8°C (59 - 95% RH) in spring and from 19.4 to 20.9°C (54 - 70% RH) and 23.8 to 29.2°C (44 - 59% RH) in summer. There were 3 rainy days for each site throughout the sampling campaign, 2 days in winter (02/03 and 04/03) and 1 day in spring (11/05) for the London site, and 1 day per each season (22/02, 31/05 and 22/8) for the Colchester site.

Table 4.4 Environmental parameters (median ± standard deviations) measured indoors and outdoors at the study residential flats.

	OUTDOORS								INDOORS					
Site	London				Colchester				London			Colchester		
	Temp. (°C)	RH %	Wind speed (kph)	Precip. accum. (mm)	Temp. (°C)	RH%	Wind speed (kph)	Precip. accum. (mm)	Temp. (°C)	RH%	CO_2 (ppm)	Temp. (°C)	RH%	CO_2 (ppm)
WINTER														
DAY 1	6.8 ± 1.7	68% ± 8%	3.7 ± 2.4	--	7.1 ± 1.2	66% ± 9%	2.5 ± 2.8	1.8	21.5 ± 0.5	54% ± 4%	1254 ± 299	21.0 ± 1.2	49% ± 3%	856 ± 221
DAY 2	5.6 ± 0.5	81% ± 6%	12.6 ± 4.5	3.0	6.7 ± 5.2	59% ± 20%	0.9 ± 1.1	--	20.9 ± 0.5	50% ± 3%	875 ± 268	21.2 ± 0.6	53% ± 4%	1265 ± 299
DAY 3	6.3 ± 2.4	67% ± 11%	7.9 ± 4.7	2.0	6.0 ± 3.3	57% ± 10%	2.8 ± 2.6	--	22.7 ± 0.5	47% ±4%	1034 ± 277	21.3 ± 1.1	52% ± 4%	1516 ± 311
SPRING														
DAY 1	16.9 ± 1.4	97% ± 3%	2.4 ± 1.2	8.1	16.3 ± 2.9	59% ± 10%	7.6 ± 1.6	--	26.3 ± 0.6	56% ± 2%	869 ± 542	25.2 ± 0.7	49% ± 6%	773 ± 160
DAY 2	15.0 ± 2.9	77% ± 6%	11.5 ± 2.2	--	20.8 ± 3.8	59% ± 11%	4.8 ± 1.6	--	27.5 ± 0.7	45% ± 4%	557 ± 167	25.1 ± 1.0	48% ± 2%	675 ± 178
DAY 3	16.0 ± 2.8	60% ± 14%	4.8 ± 2.4	--	11.7 ± 0.7	95% ± 3%	11.4 ± 2.8	36.8	26.9 ± 0.5	40% ± 4%	771 ± 207	24.5 ± 0.5	54% ± 2%	817 ± 148
SUMMER														
DAY 1	19.4 ± 1.7	54% ± 6%	8.9 ± 3.9	--	23.8 ± 3.0	59% ± 10%	6.8 ± 2.3	1.8	28.5 ± 1.0	31% ±4%	528 ± 107	27.3 ± 1.8	48% ± 4%	459 ± 45
DAY 2	20.9 ± 2.3	70% ± 7%	8.6 ± 3.1	--	29.2 ± 4.2	54% ± 13%	2.6 ± 0.7	--	27.9 ± 0.4	38% ± 3 %	498 ± 63	28.4 ± 2.2	54% ± 5%	466 ± 50
DAY 3	20.4 ± 2.7	68% ± 12%	5.2 ± 1.2	--	26.4 ± 4.4	44% ± 17%	5.3 ± 2.2	--	27.7 ± 0.9	39% ± 3 %	503 ± 192	27.9 ± 3.7	45% ± 7%	531 ± 80

Indoor temperature in the naturally ventilated flats followed the seasonal pattern of the meteorological conditions, exhibiting the highest temperature values during summer (28.5°C in London flat and 28.4°C in Colchester flat), whereas indoor relative humidity levels showed

moderate variability across the whole sampling period within the range of 31 - 54% in the urban flat and 45 - 54% in the semi-urban flat.

The CO_2 levels measured indoors (see Appendix S4.6-S4.19 for time-series of CO_2 concentration) varied significantly between the two sites (Mann Whitney U test, p-value < 0.05) per each season and across seasons (Kruskall Wallis test, p-value < 0.05) per each site (Fig. 4.3). The highest concentrations were observed during winter (median values 1052 ± 312 ppm and 1176 ± 341 ppm for the urban and semi-urban houses respectively), while lower levels were observed during spring (763 ± 385 ppm and 758 ± 171 ppm for the urban and semi-urban houses respectively) and summer (509 ± 135 ppm and 472 ± 68 ppm for the urban and semi-urban houses respectively).

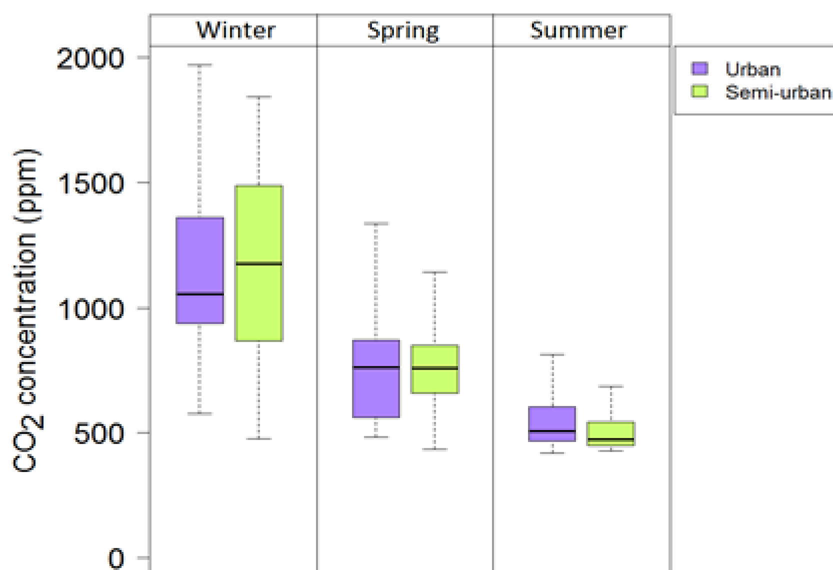


Figure 4.3 Carbon dioxide levels (ppm) measured in the two study houses across seasons. The line through the middle of each boxplot represents the median, the box is the 25th to 75th percentile, and the whiskers (lines) indicate the maximum and minimum values.

The seasonal pattern of PM_{10} and $PM_{2.5}$ particle mass measured during the campaign is summarised in Figure 4.4 (see Appendix S4.6-S4.19 for real-time profiles). PM levels varied greatly indoors and outdoors (Mann Whitney U test, p-value < 0.05) and among seasons (Kruskall Wallis test, p-value < 0.05). In particular, particle levels were notably higher indoors than outdoors in both houses during winter and summer. However, in spring outdoor particles exhibited higher levels compared to the indoor concentrations at the semi-urban flat (outdoor PM measurements were not obtained for the urban site). Overall, the average

outdoor $PM_{2.5}$ levels ranged from 6 μg/m^3 (both sites - summer) to 30 μg/m^3 (semi-urban - spring), with the levels at the urban site being substantially higher compared to the semi-urban site (Mann Whitney U test, p-value < 0.05) only during wintertime. Indoors, the highest PM_{10} and $PM_{2.5}$ concentrations across all seasons were measured during winter (median PM_{10} ± standard deviation = 330 ± 654 μg/m^3 and median $PM_{2.5}$ ± standard deviation = 321 ± 604 μg/m^3) at the urban apartment. For the rest of the campaign, the average indoor particle concentrations during different seasons ranged from 10 μg/m^3 (semi-urban - spring) to 50 μg/m^3 for $PM_{2.5}$ (semi-urban - summer) and from 32 μg/m^3 (semi-urban - winter) to 59 μg/m^3 (semi-urban - summer) for PM_{10}, with the levels being significantly lower at the urban house compared to the semi-urban flat (p-value < 0.05) only during summertime.

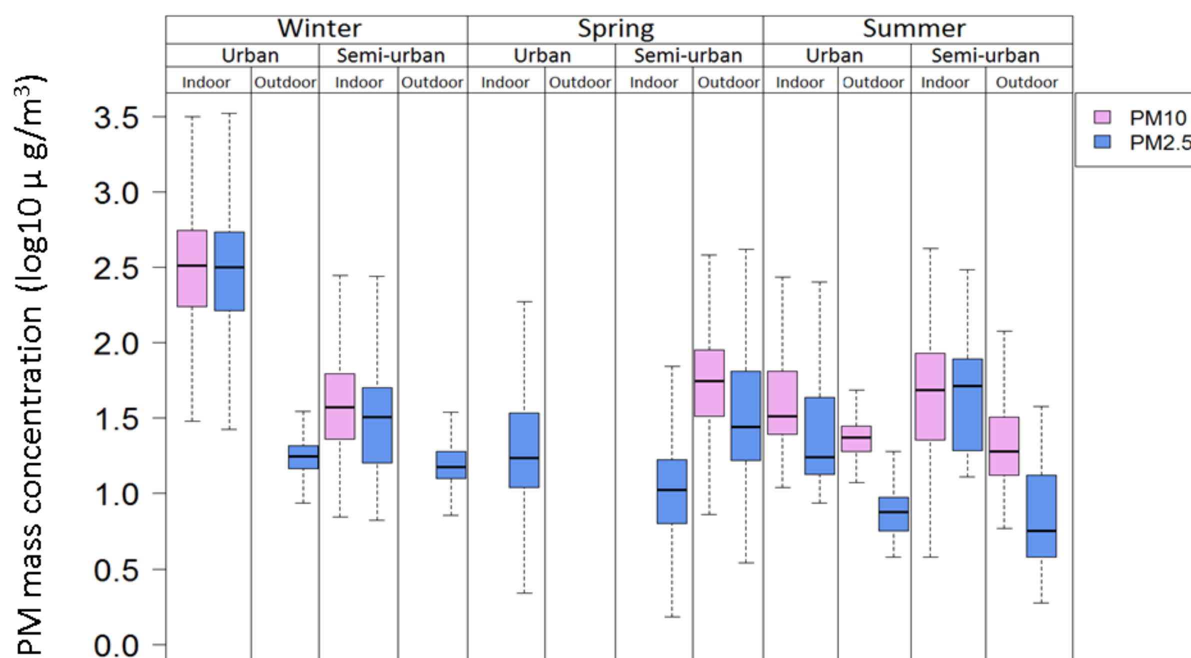


Figure 4.4 Boxplots summarising overall results for recorded $PM_{2.5}$ and PM_{10} mass concentration (μg/m^3) data over 3-day indoor and outdoor measurements per season and per house (outliers are not shown). Axis y is on log10 scale. Measurements for outdoor PM_{10} during winter, indoor PM_{10} as well as outdoor PM_{10} and $PM_{2.5}$ at the urban site during spring were not performed due to equipment limitations.

The potential relationship between the CO_2 levels and the indoor PM concentrations was investigated by Spearman's rank correlation test (Table 4.5). CO_2 levels measured during all

three seasons were found to be positively associated with PM mass, demonstrating stronger correlation ($\rho > 0.5$) in both sites during winter time.

Table 4.5 Spearman's rank correlations between CO_2 concentrations and indoor PM_{10} – $PM_{2.5}$ levels. PM_{10} mass was not measured during spring due to equipment limitations.

	Urban flat		**Semi-urban flat**	
CO_2 correlation (ρ) with	**PM_{10}**	**$PM_{2.5}$**	**PM_{10}**	**$PM_{2.5}$**
Winter	0.62*	0.60*	0.53*	0.61*
Spring	-	0.42*	-	0.1*
Summer	0.30*	0.29*	0.36*	0.36*

*p-value < 0.001

4.3.2 Diurnal variation of bacterial abundance and PM concentration

Overall results based on 1-hour time-integrated (non- size-resolved) samples showed that during winter bacterial levels were significantly higher indoors than outdoors in both sites (Mann-Whitney U test, p-value < 0.05), whereas during summer indoor abundance tended to follow the outdoor levels (p-value > 0.05). There was no statistical difference in the bacterial load between the two houses (p-value > 0.05), except summer that the outdoor concentrations reported were significantly higher at the urban site (Mann Whitney U test, p-value < 0.05).

In accordance with the overall particle results described in 4.3.1, $PM_{2.5}$ levels based on 1-hour time-averaged measurements were substantially higher indoors compared to outdoors (Mann-Whitney U test, p-value < 0.05) for both seasons. Mann-Whitney U test confirmed that there is no significant difference (p-value > 0.05) between both sites' indoor levels during summer. However, during winter, indoor concentrations were significantly higher at the urban residence (Mann-Whitney U, p-value < 0.05), as mentioned in 4.3.1 (see also Appendix S4.22).

Figures 4.5 and 4.6 show the diurnal variation of indoor/outdoor bacterial abundance and $PM_{2.5}$ mass concentration, per each day and each house, for winter and summer, respectively, whereas figures 4.7 and 4.8 summarise the overall results over the 3-days measurement period per each site and season.

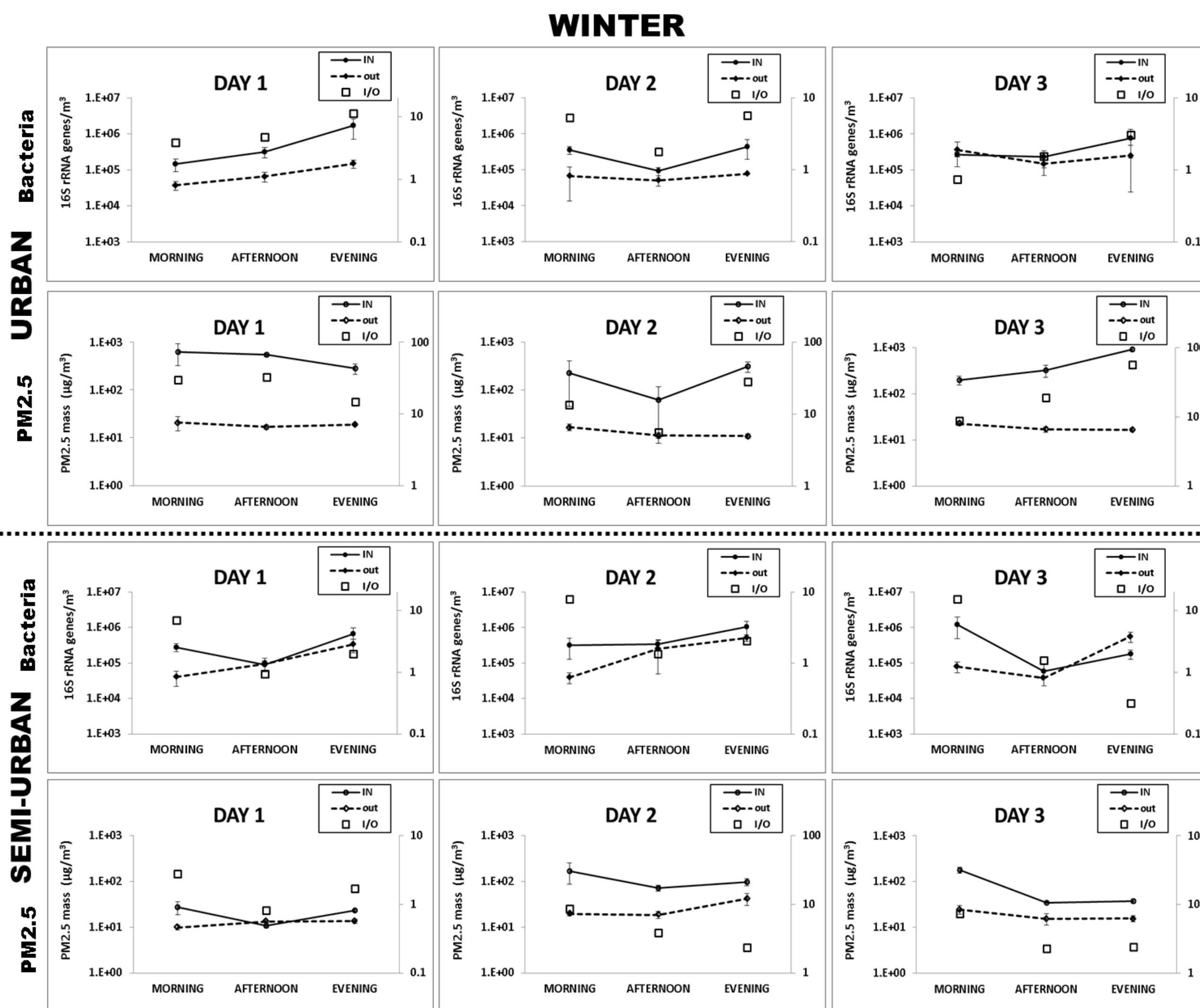


Figure 4.5 Diurnal variation of indoor and outdoor bacterial abundance (16S rRNA genes per m^3 of air), as quantified by qPCR, and $PM_{2.5}$ mass concentration per each day and house during winter sampling. Each time point represents 1-hour average of collected air between 9:00-10:00 (morning), 14:30-15:30 (afternoon) and 20:00-21:00 (evening). Error bars indicate standard deviation, while secondary y axis represents Indoor-to-Outdoor concentration ratios (I/O).

Winter

In particular, for the winter period (Fig. 4.5), the morning average bacterial concentrations ranged from 1.45×10^5 to 3.53×10^5 (3.73×10^4 - 3.65×10^5 outdoors) 16S rRNA gene copies per m^3 of air for the urban flat and from 2.80×10^5 to 1.23×10^6 (3.93×10^4 - 8.02×10^4 outdoors) gene copies/m^3 for the semi-urban site. In the afternoon, the mean bacterial levels ranged from 9.11×10^4 to 3.17×10^5 (5.07×10^4 - 1.47×10^5 outdoors) 16S rRNA genes/m^3 at the London residence and from 5.87×10^4 to 3.39×10^5 (3.80×10^4 - 2.53×10^5 outdoors) copies/m^3 at the Colchester site. In the evening the abundance was higher in both

sites, compared to morning and afternoon, ranging, on average, from 4.42×10^5 to 1.69×10^6 (7.70×10^4 - 2.53×10^5 outdoors) 16S rRNA genes/m^3 at the urban flat and from 1.78×10^5 to 1.05×10^6 (3.33×10^5 - 5.61×10^5 outdoors) copies/m^3 at the semi-urban apartment. Indoor mean $PM_{2.5}$ levels were in the range of 199 - 624 μg/m^3 and 28 - 179 μg/m^3 at the urban and semi-urban sites, respectively, in the morning, 62 - 540 μg/m^3 and 11 - 72 μg/m^3 in the afternoon and 279 - 942 μg/m^3 and 23 - 98 μg/m^3 in the evening with the outdoor levels ranging between 11 and 23 μg/m^3 at London and from 10 to 42 μg/m^3 at the Colchester site.

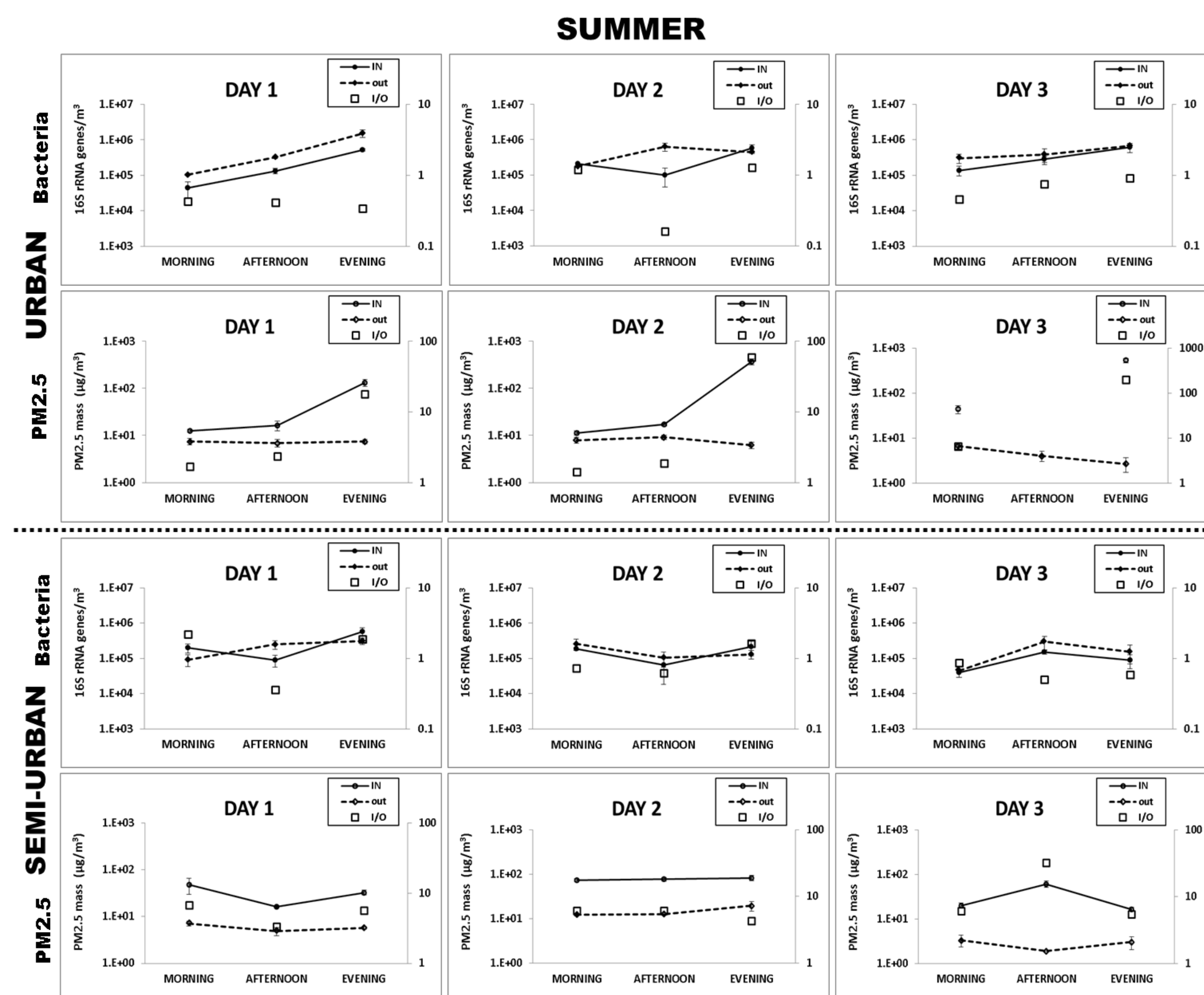


Figure 4.6 Diurnal variation of indoor and outdoor bacterial abundance (16S rRNA genes per m^3 of air), as quantified by qPCR, and $PM_{2.5}$ mass concentration per each day and house during summer sampling. Each time point represents 1-hour average of collected air between 9:00-10:00 (morning), 14:30-15:30 (afternoon) and 20:00-21:00 (evening). Error bars indicate standard deviation, while secondary y axis represents Indoor-to-Outdoor concentration ratios (I/O). Indoor $PM_{2.5}$ levels during day 3-afternoon at the urban site were not recorded.

Summer

During summer (Fig. 4.6), for the urban house, the mean indoor bacterial abundance ranged from 4.43×10^4 to 2.09×10^5 (1.04 - 3.02×10^5 outdoors) 16S rRNA genes/m^3 during morning, 1.01×10^5 - 2.86×10^5 (3.23 - 6.36×10^5 outdoors) gene copies/m^3 during afternoon and from 5.18×10^5 to 6.13×10^5 (4.45×10^5 - 1.53×10^6 outdoors) copies/m^3 during evening. The average $PM_{2.5}$ levels indoors ranged between 13 and 44 μg/m^3 in the morning, 16 - 17 μg/m^3 in the afternoon and 131 - 530 μg/m^3 in the evening, while variability outdoors was very low at the range 3 - 9 μg/m^3. For the semi-urban house, the average bacterial levels during summer varied from 3.91×10^4 to 2.01×10^5 16S rRNA genes/m^3 (4.50×10^4 - 2.58×10^5 outdoors) for the morning, from 6.55×10^4 to 1.52×10^5 (1.05 - 3.01×10^5 outdoors) copies/m^3 for the afternoon and from 9.03×10^4 to 5.87×10^5 (1.32 - 3.13×10^5 outdoors) copies/m^3 for the evening. The $PM_{2.5}$ concentrations were from 20 to 74 μg/m^3 in the morning, 16 - 76 μg/m^3 in the afternoon and 16 - 82 μg/m^3 in the evening, with outdoor levels ranging from 3 to 19 μg/m^3 throughout the day.

Indoor/outdoor relationship

The indoor to outdoor (I/O) ratio was also calculated for both airborne bacteria and $PM_{2.5}$ to determine the impact of outdoor sources on indoor air concentrations (Fig. 5 & 6). Bacterial abundance I/O ratios were significantly greater (Mann Whitney U test, p-value < 0.05) during winter (median I/O = 2.55) compared to summer (median I/O = 0.67) and they were higher under occupied conditions (i.e. morning, evening). No substantial differences were observed between the two houses. However, median $PM_{2.5}$ I/O ratios did not differ significantly between winter (7.98) and summer (6.03) and they were constantly greater than 1, suggesting that the indoor $PM_{2.5}$ concentrations were associated with in-house generated emissions. The $PM_{2.5}$ I/O was significantly higher at the urban flat compared to the semi-urban only during the winter season. When comparing the bacterial ratio I/O to the $PM_{2.5}$ ratio I/O, there was no significant correlation (Spearman's rank correlation, $\rho=0.309$, $p=0.071$), indicating that different sources affect the indoor bioaerosol and fine particle mass loads.

Summary

Both houses demonstrated similar diurnal variation patterns of bacterial abundance exhibiting the highest indoor concentrations typically during occupied periods (i.e. evening) and lower levels, similar to those outdoors, during vacant or low activity periods (i.e. afternoon). During winter, the time zone when sampling was carried out appeared to have a significant effect on

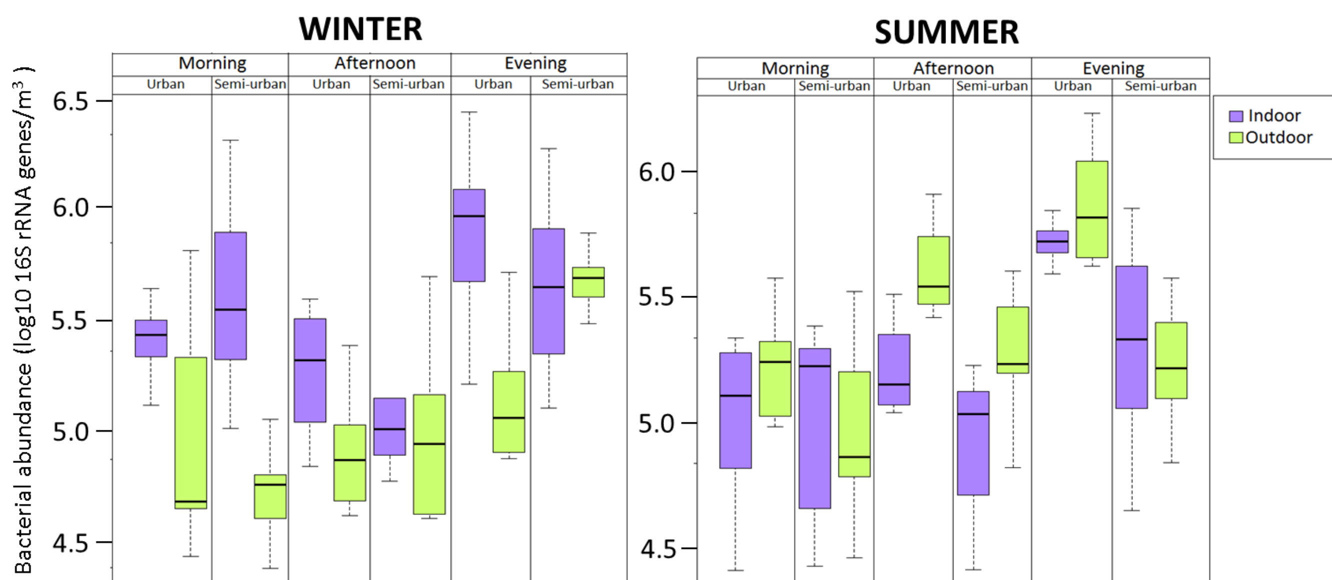


Figure 4.7 Summary boxplots of diurnal variation of indoor and outdoor airborne bacterial concentrations determined by qPCR, per house and season (outliers not shown). All measures of bacterial abundance are in 16S rRNA genes per m^3 of air.

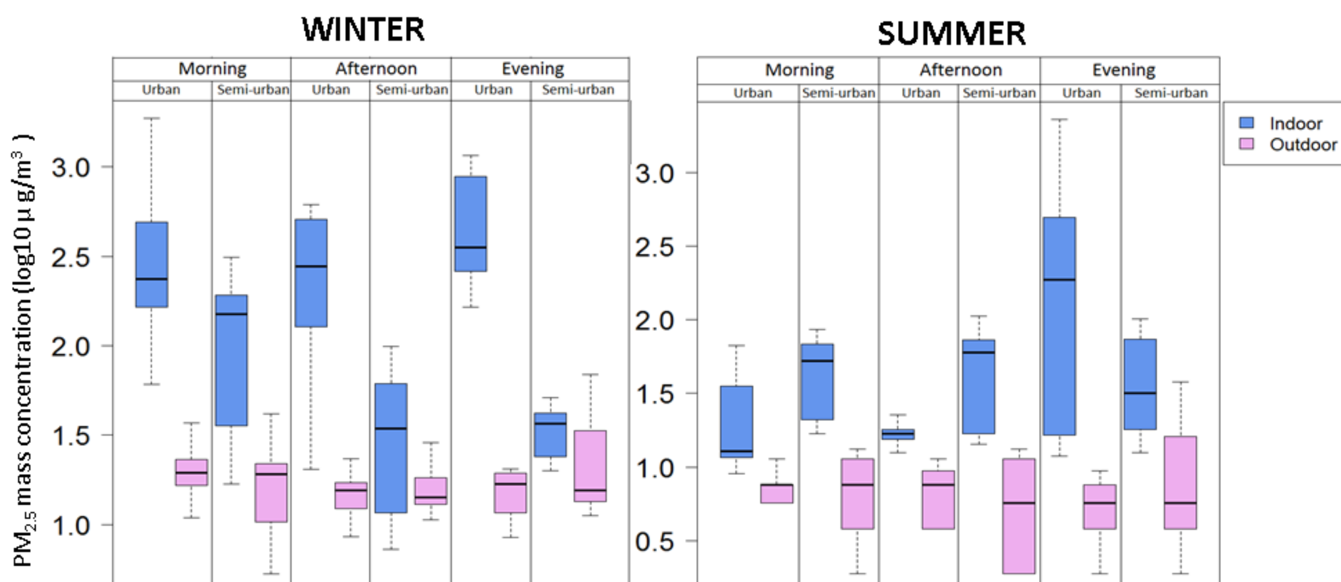


Figure 4.8 Summary boxplots of diurnal variation of indoor and outdoor $PM_{2.5}$ mass concentrations ($\mu g/m^3$) per house and season. The line through the middle of each boxplot represents the median, the box is the 25th to 75th percentile, and the whiskers (dashed lines) indicate the maximum and minimum values (outliers not shown).

the indoor aerosol bacterial abundance, for both the urban (Welch's $F_{2,\ 14.18} = 3.84$, $p = 0.046$) and the semi-urban (Kruskal-Wallis, $\chi^2(2) = 8.96$, $p = 0.011$) sites, whereas the impact on the outdoor concentrations was substantial only for the semi-urban (Kruskal-Wallis, $\chi^2(2)$ =

16.51, $p < 0.001$) site (urban site: Kruskal-Wallis, $p > 0.05$). During summer, the significance of the indoor intra-day variation appeared to be dependent on the outdoor variability, in both sites (urban indoors: ANOVA, $F_{2,24} = 50.92$, $p <0.001$; urban outdoors: Kruskal-Wallis, $\chi^2(2) = 17.17$, $p<0.001$; semi-urban indoors: Welch's one-way test, $p > 0.05$; semi-urban outdoors: ANOVA, $p > 0.05$). Indoor $PM_{2.5}$ concentrations exhibited significant differences among the different times of the day (Kruskal-Wallis, $p < 0.05$) for both houses and seasons, indoors and outdoors, except summer for the semi-urban house (Kruskal-Wallis, $p > 0.05$).

Influence of environmental parameters on bacterial abundance

The microbial (bacterial) abundance estimated for the 1-hour integrated samples of indoor air was compared with the 1-hour time-averaged values of the concurrently recorded environmental variables by Spearman's rank correlation analysis (Table 6). When considering the full dataset together, all parameters correlated significantly with indoor bacterial abundance, except the outdoor bacterial abundance and relative humidity. PM_{10} and $PM_{2.5}$ were the factors showing the strongest positive correlations ($\rho > 0.6$, p-value < 0.001) among the other parameters. These were the only two variables that correlated significantly with the bacterial levels at the urban residence, when separating the analyses by home. Moreover, the indoor relative humidity exhibited a significant positive correlation ($\rho = 0.473$, p-value < 0.05 only for the semi-urban flat. Interestingly, when separating the analysis by season, the outdoor bacterial abundance showed a statistically significant positive association ($\rho > 0.6$, p-value < 0.05) with the indoor levels only during summer. However, in winter the only factors that correlated significantly with the microbial abundance were CO_2 concentration ($\rho = 0.521$), outdoor temperature ($\rho = -0.505$) and relative humidity indoors ($\rho = 0.608$). Finally, when separating the analysis by time-zone, outdoor $PM_{2.5}$ ($\rho > 0.6$) and indoor and outdoor temperature ($\rho < -0.7$) seemed to have the strongest effect (p-value < 0.05) on indoor bacterial concentration during morning periods.

Table 4.6 Spearman's rho (ρ), with p-values shown in brackets, between indoor bacterial abundance (1-hour time integrated samples), as measured via qPCR, and environmental parameters. Bolded values indicate statistical significance, defined as $p < 0.05$.

	Outdoor Bacterial abundance	PM_{10} in	$PM_{2.5}$ in	$PM_{2.5}$ out	CO_2 in	Temp in	Temp out	RH% in	RH% out
Both houses & seasons	0.294 (.081)	**0.670** (<.001)	**0.610** (<.001)	**0.339** (.044)	**0.412** (.013)	**-0.402** (.015)	**-0.468** (.004)	0.299 (.076)	0.319 (.057)
Urban site (both seasons)	0.263 (.290)	**0.672** (.004)	**0.645** (.006)	0.020 (.941)	0.325 (.188)	-0.306 (.217)	-0.325 (.188)	0.344 (.162)	0.207 (.410)
Semi-urban site (both seasons)	0.315 (.203)	**0.628** (.006)	**0.498** (.037)	**0.550** (.019)	0.381 (.120)	**-0.491** (.038)	**-0.490** (.041)	**0.473** (.047)	0.368 (.133)
Both sites, Summer season	**0.616** (.008)	**0.696** (.003)	**0.571** (.018)	-0.098 (.699)	-0.026 (.921)	-0.342 (.164)	-0.180 (.476)	-0.117 (.644)	0.293 (.237)
Both sites, Winter season	0.395 (.105)	0.408 (.094)	0.325 (.188)	0.360 (.142)	**0.521** (.028)	-0.160 (.525)	**-0.505** (.035)	**0.608** (.007)	0.259 (.300)
Both sites, Morning	-0.175 (.588)	**0.622** (.035)	0.461 (.134)	**0.699** (.014)	0.475 (.121)	**-0.764** (.004)	**-0.766** (.005)	0.403 (.193)	0.291 (.359)
Both sites, Afternoon	0.406 (.193)	0.600 (.056)	0.445 (.173)	0.182 (.573)	0.133 (.683)	-0.364 (.246)	-0.154 (.635)	0.035 (.914)	0.095 (.770)
Both sites, Evening	0.091 (.783)	0.461 (.134)	0.399 (.201)	0.357 (.256)	0.538 (.075)	**-0.578** (.049)	-0.382 (.221)	0.169 (.600)	0.130 (.688)

4.3.3 Particle size distribution

Daily size-resolved bacterial concentration indoors and outdoors

Figures 4.9, 4.10 and 4.11 show the daily size-resolved bacterial concentration profiles of indoor and outdoor 16S rRNA gene copies/m^3 per each house across 3 seasons, obtained from the May impactor measurements, while Figure 4.12 shows the seasonally averaged particle-size distribution of bacterial gene copies and the indoor-to-outdoor (I/O) ratios for all particle sizes at the urban and the semi-urban study sites.

Particle size distribution figures showed daily variability and similar seasonal trends with respect to the particle size in both sites. Across all seasons, outdoor bacterial aerosols were dominated by the largest measured particles, that is those greater than 2 µm, with the highest concentrations occurring for particles greater than 16 µm in aerodynamic diameter in most cases. However, examination of the indoor profiles showed a strong peak on the stage corresponding to the 2 - 4 µm size range during wintertime and at 4 - 8 µm during summer at both sites. A mixed pattern was observed during spring for the two houses, where the most dominant particle size range for the airborne bacteria, on average, was 2 - 4 µm for the semi-urban house and 4 - 8 µm for the urban house.

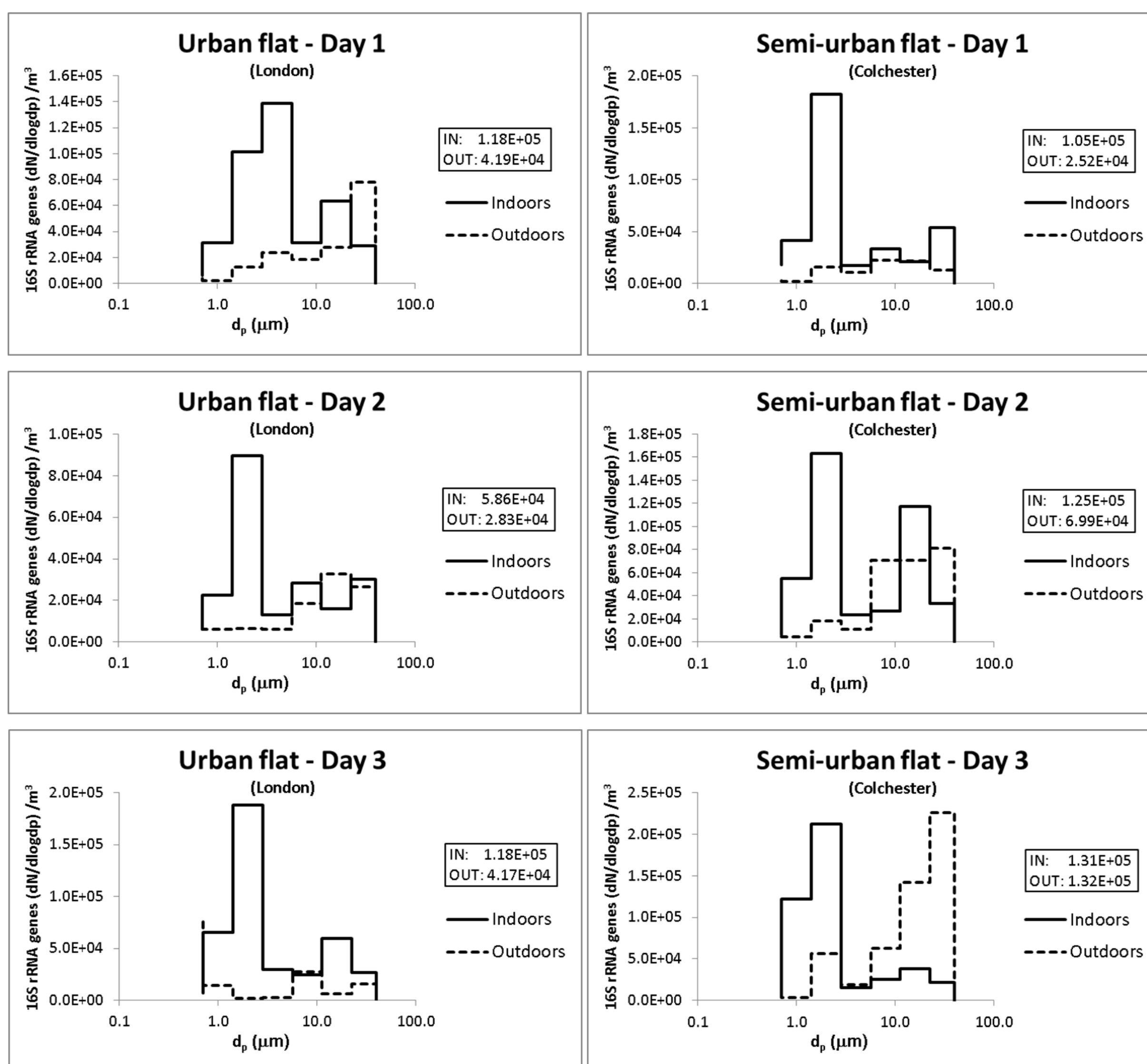


Figure 4.9 The particle-size distributions of airborne bacteria determined by qPCR (y axis, dN/dlogd$_p$ 16S rRNA genes per m^3 of air) during winter sampling. An upper limit of particle size for the stage of >32.0 µm is set at 40 µm. Solid lines represent indoor air and dashed lines represent outdoor air. The numerical values within each frame report the total concentrations for all sizes (16S rRNA gene copies/m^3).

SPRING

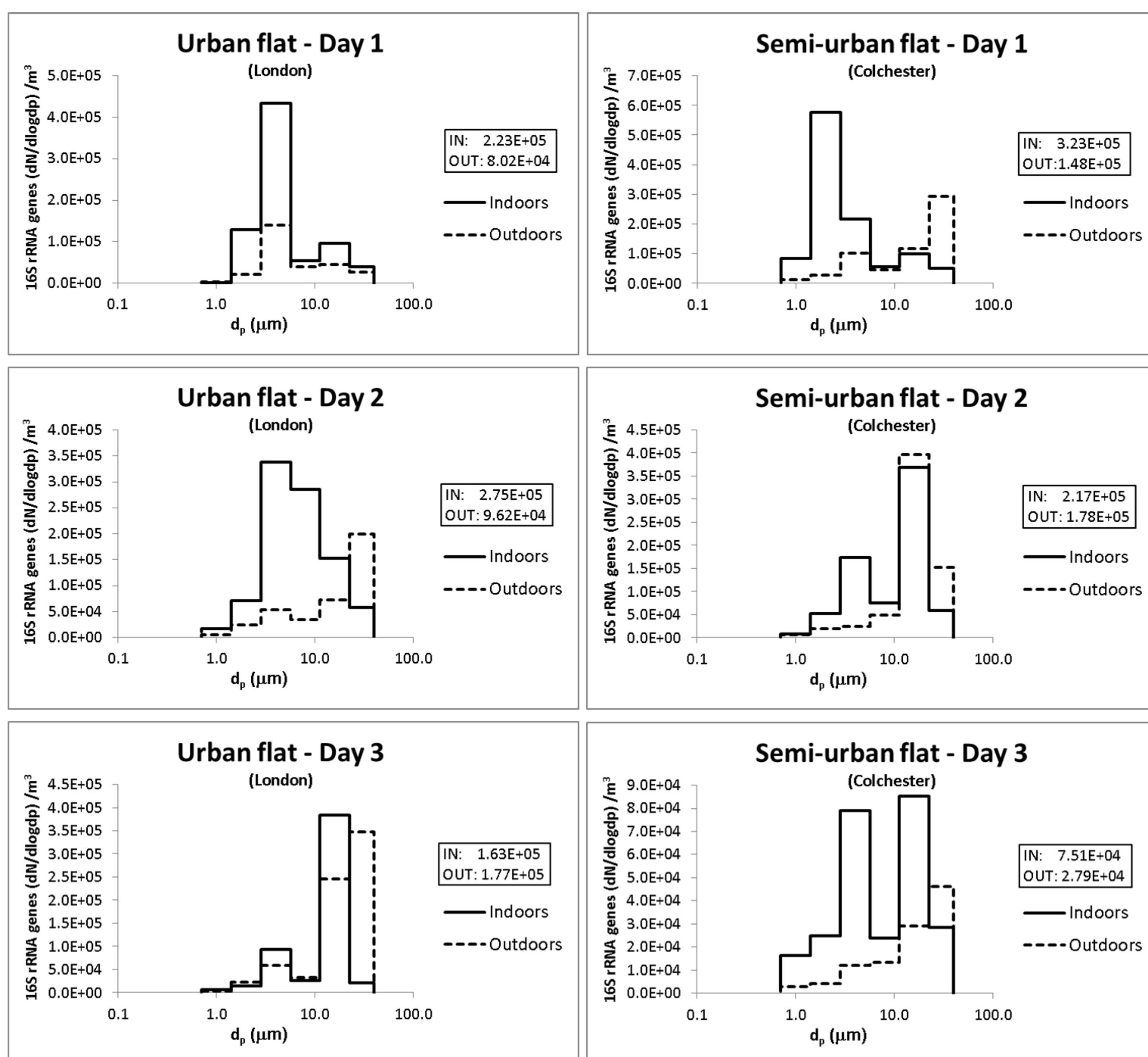


Figure 4.10 The particle-size distributions of airborne bacteria determined by qPCR (y axis, dN/dlogd$_p$ 16S rRNA genes per m^3 of air) during spring sampling. An upper limit of particle size for the stage of >32.0 μm is set at 40 μm. Solid lines represent indoor air and dashed lines represent outdoor air. The numerical values within each frame report the total concentrations for all sizes (16S rRNA gene copies/m^3).

SUMMER

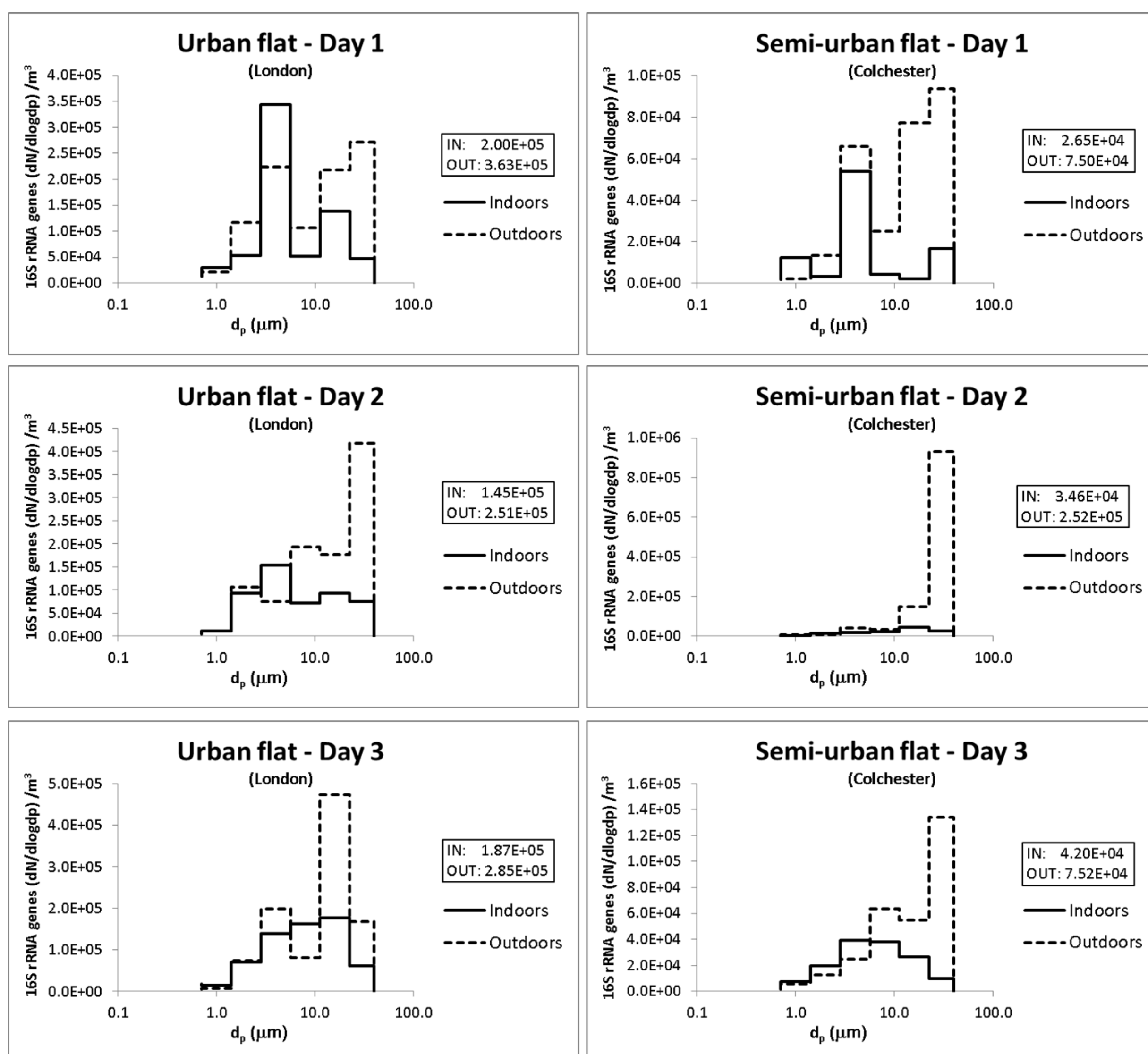


Figure 4.11 The particle-size distributions of airborne bacteria determined by qPCR (y axis, dN/dlogd$_p$ 16S rRNA genes per m^3 of air) during spring sampling. An upper limit of particle size for the stage of >32.0 µm is set at 40 µm. Solid lines represent indoor air and dashed lines represent outdoor air. The numerical values within each frame report the total concentrations for all sizes (16S rRNA gene copies/m^3).

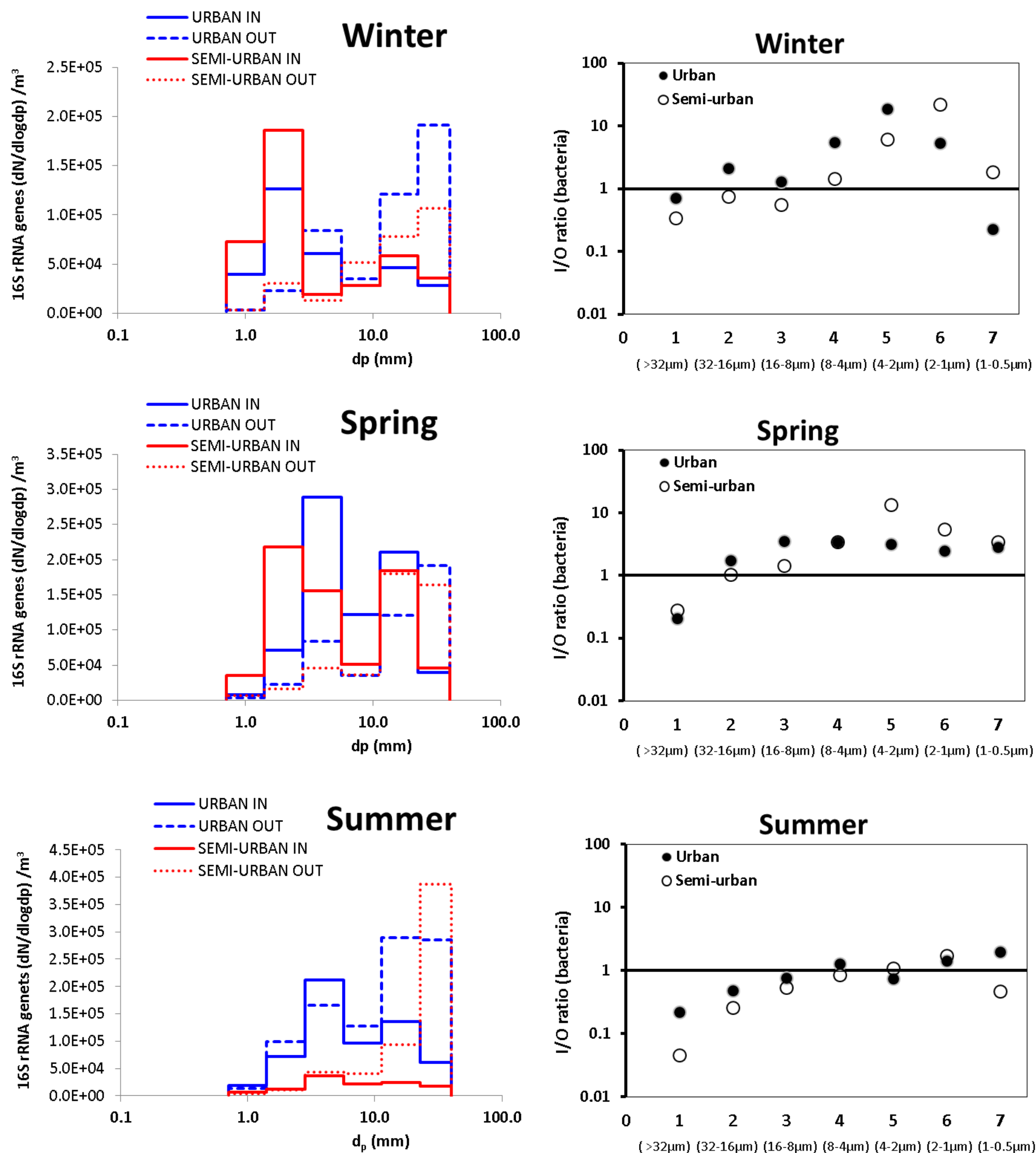


Figure 4.12 On the left panel: Seasonally averaged particle-size distributions (n = 3 days) of aerosol bacterial abundance determined by qPCR (y axis, dN/dlogd$_p$ 16S rRNA genes per m^3 of air) indoors and outdoors for each site. An upper limit of particle size for the stage of >32.0 µm is set at 40 µm. On the right panel: Size-resolved Indoor-to-Outdoor concentration ratios (I/O) for airborne bacteria in the urban (closed circles) and the semi-urban flats (open circles) across seasons. Each I/O ratio presented per season is an average over three days. Axis x represents the May impactor size bins 1 (> 32 µm), 2 (16 - 32 µm), 3 (8 - 16 µm), 4 (4 - 8 µm), 5 (2 - 4 µm), 6 (1 - 2 µm) and 7 (0.5 - 1 µm).

Indoor/outdoor relationship

When examining the different particle size fractions, the only particles that consistently showed I/O ratios lower than unity for both houses were the ones with sizes greater than 32 μm (Fig. 4.12). Apart from the specific size, during winter and spring most particle size-fractions demonstrated I/O ratios greater than unity, with the largest particles (size range 16 - 32 μm) being close to unity. A clear decrease of the I/O ratio was observed during summer for particles of all sizes, with only the ratio of particles within the size range 1 - 2 μm remaining greater than unity for both sites. This is the fraction that exhibited the highest I/O ratio across all seasons (22 for the urban house during winter).

The indoor to outdoor (I/O) ratio specifically for the fine fraction is also presented for both bacteria and particles ($PM_{2.5}$) measured (Fig. 4.13). Spearman's rank correlation analysis showed that there is no association between the bacterial and PM I/O ($\rho = -0.097$, $p > 0.05$).

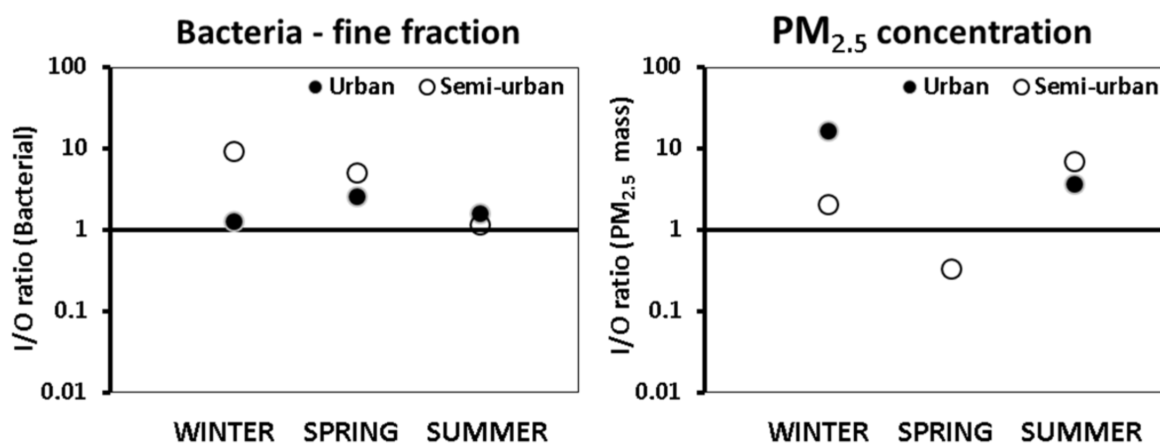


Figure 4.13 Indoor-to-Outdoor concentration ratios (I/O) for the fine fraction (May impactor size bins 6 & 7) of aerosol bacterial abundance determined by qPCR (left panel) and $PM_{2.5}$ mass concentration (right panel in the urban (closed circles) and the semi-urban (open circles) flats across seasons. Each bacterial I/O ratio presented per season is an average over three days, whereas each $PM_{2.5}$ I/O ratio is an average over three daily medians.$PM_{2.5}$ measurements for the urban site were mot obtained during spring.

Comparing particle size distribution obtained with two different impactors

Indoor particle size distribution data based on bacterial concentrations were also obtained by sampling with the Andersen impactor which provides an additional size resolution at the range 2.1 - 4.7 μm (size bins 2.1 - 3.3 μm and 3.3 - 4.7 μm). Size-resolved profiles (Fig. 4.14) showed that during summer bacteria were highly enriched in the 4.7 - 7 μm stage in both

houses, whereas in spring concentrations peaked within the size-range 3.3 - 4.7 at the urban flat and within the ranges 3.3 - 4.7 and 4.7 - 7 for the semi-urban flat. Wintertime profiles demonstrated high variation among days and between the two sites, with peaks observed on several different size ranges, i.e. 1.1 - 2.1 μm, 3.3 - 4.7 μm and 4.7 - 7 μm.

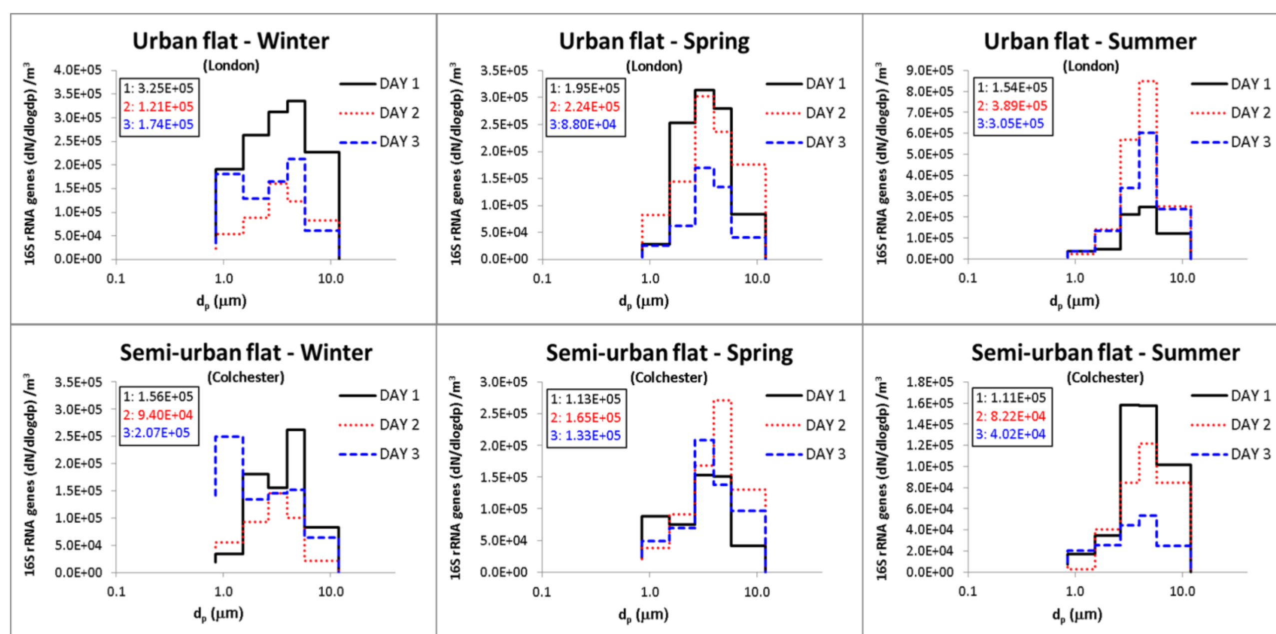


Figure 4.14 The particle-size distributions of indoor airborne bacteria determined by qPCR (y axis, $dN/dlogd_p$ 16S rRNA genes per m^3 of air) for each house across three seasons. An upper limit of particle size for the stage of >7.0 μm is set at 20 μm. Black solid, red dotted and dashed blue lines correspond to different sampling days. Numerical values within each frame report the total concentrations for all sizes (16S rRNA gene copies/m^3) per each day.

Table 4.7 provides geometric means (GM) of aerodynamic diameters and geometric standard deviation (GSD) size distribution data for the two sites, obtained with the two impactors. Based on the data obtained with the May impactor, the average GM particle diameters for the outdoor bacterial aerosols were larger than for the indoor aerosols for all seasons, ranging from 13.68 to 28.65 μm. Consistent with the seasonal trends previously described, the geometric mean particle sizes indoors varied seasonally with the smallest average GM values observed in winter (3.40 μm at the urban flat and 2.94 μm at the semi-urban site) and the biggest during summer (8.07 μm and 9.93 μm at the urban and semi-urban houses respectively) and spring (11.95 μm and 11.32 μm for the urban and semi-urban flats respectively). Average GM acquired based on the Andersen impactor size-resolved data, exhibited slightly higher values for winter, compared to the May impactor. In contrast, the

average GM for spring and summer were smaller compared to the ones derived from the May impactor, with the biggest differences occurred when the May-acquired geometric mean diameters were at the range over 10 μm. However, when comparing all the GM obtained between the two impactors, Mann Whitney U test confirmed that there was no significant difference ($p > 0.05$). Moreover, Spearman rank coefficient analysis demonstrated a strong positive correlation ($\rho = 0.674$, $p = 0.002$).

Table 4.7 Geometric mean particle sizes (d_g) and geometric standard deviation (σ_g), as calculated for each impactor (May, Andersen), site (urban, semi-urban) and season. Note that the upper detection limit of particle size for the two impactors is different.

		May impactor	**Andersen impactor**	**May impactor**	**May impactor**	**Andersen impactor**	**May Impactor**
		Urban IN		**Urban OUT**	**Semi-Urban IN**		**Semi-urban OUT**
Winter	**DAY 1**	4.82 (2.33)	4.50 (2.49)	21.25 (3.00)	2.69 (1.50)	4.72 (1.81)	10.79 (2.79)
	DAY 2	2.76 (1.67)	4.69 (2.25)	19.20 (2.09)	3.82 (3.00)	3.35 (1.98)	18.36 (2.17)
	DAY 3	2.63 (1.59)	3.04 (2.34)	0.59 (1.53)	2.32 (1.54)	2.03 (2.60)	24.06 (2.35)
Average GM		**3.40**	**4.08**	**13.68**	**2.94**	**3.37**	**17.74**
Spring	**DAY 1**	5.27 (1.46)	4.36 (1.69)	6.02 (1.54)	3.12 (1.52)	3.61 (2.14)	23.12 (3.00)
	DAY 2	8.39 (1.99)	5.28 (2.18)	24.38 (3.00)	21.26 (1.52)	5.78 (1.80)	24.04 (1.39)
	DAY 3	22.18 (1.32)	4.71 (1.64)	28.95 (1.67)	9.58 (2.88)	5.11 (2.11)	21.56 (2.65)
Average GM		**11.95**	**4.78**	**19.78**	**11.32**	**4.83**	**22.91**
Summer	**DAY 1**	5.70 (1.53)	5.89 (1.69)	11.93 (3.00)	5.70 (1.39)	6.23 (1.77)	15.45 (3.00)
	DAY 2	7.43 (2.85)	5.68 (1.49)	18.99 (3.00)	15.70 (2.35)	6.68 (1.80)	50.00 (2.14)
	DAY 3	11.07 (2.40)	6.00 (1.58)	22.49 (1.55)	8.40 (2.42)	4.56 (2.20)	20.51 (3.00)
Average GM		**8.07**	**5.86**	**17.80**	**9.93**	**5.82**	**28.65**

In order to further compare the performance of the two impactors used in the study, the size bins of the Andersen impactor and the May impactor were made similar by grouping the 2.1 - 3.3 (stage 4) and 3.3 - 4.7 μm (stage 3) stages of the Andersen impactor to compare against the 2.0 - 4.0 μm channel (stage 5) of the May impactor, and by grouping the 8.0 - 16.0 (stage 3), 16.0 - 32.0 (stage 2) and > 32.0 μm (stage 1) size ranges of the May model against the >7.0 μm stage (1) of the Andersen impactor (Table 4.8). Bacterial abundance obtained per equivalent stages with the two impactors, per season, is illustrated in Fig. 4.15. Average percentages of bacterial abundance in different size stages per site and season is also presented in Appendix (S4.23).

Table 4.8 Size ranges of the 7-stage May and 6-stage Andersen impactors. Shaded with different colours are the channels grouped for the purpose of comparison.

May impactor		Andersen impactor	
Stage	**Size range (µm)**	**Stage**	**Size range (µm)**
1	> 32		
2	16 – 32	1	> 7
3	8 – 16	2	4.7 – 7
4	4 – 8	3	3.3 - 4.7
5	2 – 4	4	2.1 - 3.3
6	1 – 2	5	1.1 - 2.1
7	0.5 – 1	6	0.65 - 1.1

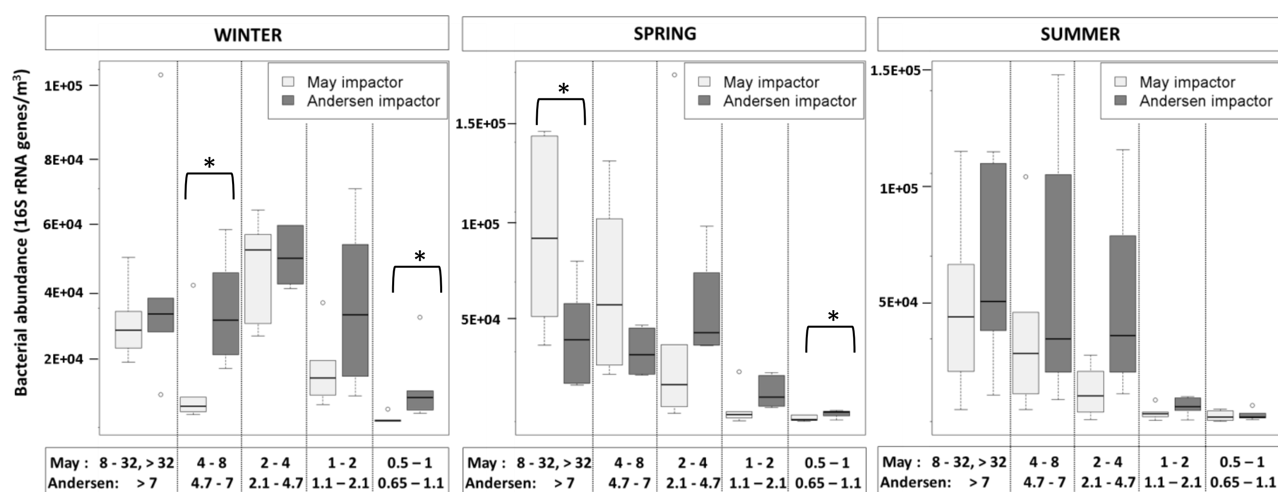


Figure 4.15 Bacterial abundance (16S rRNA genes /m^3) recovered for each size fraction for both May and Andersen impactors across three seasons. Axis x size bins ranges are in µm units. Dissimilar impactors size bin ranges have been grouped for the purpose of comparison. Each box and whisker includes values from both sampling sites (n = 6 days) per each season. Asterisks denote statistical significance (p-value < 0.05). Note that axis y has a different range for each seasonal plot.

Based on normality of data (Shapiro-Wilk test), pair-wise independent samples t-test or Mann-Whitney U test was used for comparison of bacterial abundance between the two impactors per each stage. Statistically significant differences were found during winter sampling for the size range 4 - 8 µm (4.7 - 7 µm) (p-value = 0.026, Mann-Whitney U test), and for size bin 0.5 - 1 µm (0.65 - 1 µm) (p-value = 0.009, Mann-Whitney U test), with the concentrations being higher for the Andersen impactor across all stages. Similarly, in summer all stages exhibited higher concentrations for the Andersen impactor compared to the levels shown for the May impactor, with only the difference found at the size range 2 - 4 µm (2.1 - 4.7 µm) being at the limit of significance (p-value = 0.050, t-test). In spring, the airborne

bacteria levels were higher for the May impactor for the stages corresponding to particles larger than 8 μm (>7 μm) (p-value = 0.049, t-test) and within size range 4 - 8 μm (4.7 - 7 μm) (p-value > 0.05). The rest of the stages demonstrated lower values compared to the abundance recovered by the Andersen impactor, with only the difference found at the size range 0.5 - 1 μm (0.65 - 1 μm) being significant (p-value = 0.025, t-test). When comparing the total bacterial concentrations obtained with the two impactors, Andersen six-stage and May seven-stage (Fig. 4.16), no statistically significant difference was found (Independent two samples t-test, p-value > 0.05). However, no strong association was found between the concentrations recovered by the two impactors (Pearson's correlation, r = 0.277, p > 0.05).

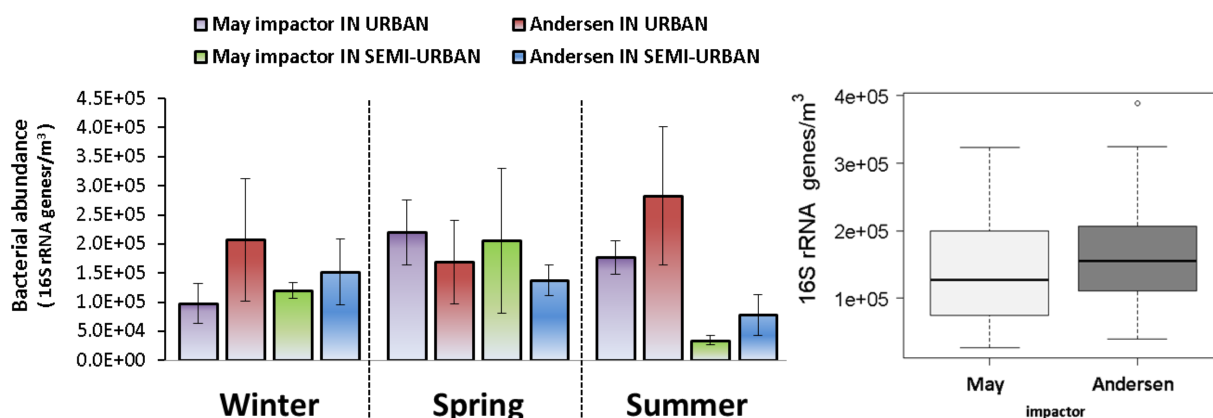


Figure 4.16 Total average indoor concentration of airborne bacteria (16S rRNA genes /m^3) as recovered from each impactor (boxplot on the right: each box and whisker includes values from both sampling sites and all three seasons) and per each site (n = 3 days) and season (barplot on the left, error bars represent standard deviation).

4.3.4 Daily total bacterial concentration indoors and outdoors

The seasonal variation was further evaluated based on the daily non size-resolved total concentrations. Mean bacterial abundance per site and season are presented in Fig. 4.17. Overall, there was no significant difference in the May impactor-based total airborne concentrations between the two sites (see also Appendix S4.24), indoors and outdoors (two samples independent t-test, p > 0.05), except for summer that the urban indoor concentrations were significantly higher than the semi-urban ones (t-test, p < 0.05). Similarly, the Andersen-based indoor aerosol bacterial abundance at the urban site was also found to be significantly greater than the concentration in the semi-urban only during summer (t-test, p < 0.05). When comparing the bacterial load in the air between indoors and outdoors, the indoor

concentrations were lower than outdoors only in summer, with the difference being significant only for the urban site (t-test, $p < 0.05$).

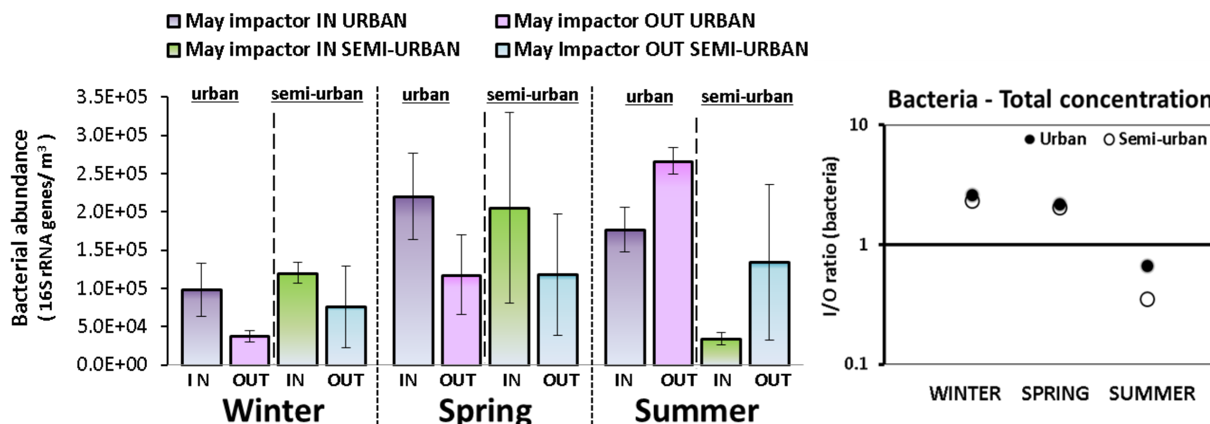


Figure 4.17 On the left panel: Seasonally averaged total concentrations (n = 3 days) of aerosol bacterial abundance determined by qPCR (16S rRNA genes per m^3 of air), indoors and outdoors, for each site. On the right panel: Indoor-to-Outdoor concentration ratios (I/O) for airborne bacteria in the urban (closed circles) and the semi-urban flats (open circles) across seasons. Each I/O ratio presented per season is an average over three days.

In both houses, the highest bacterial load indoors was observed during spring and outdoors during summer. The urban site exhibited significant differences across seasons indoors (ANOVA, $F_{2,6} = 6.70$, $p = 0.029$) and outdoors (ANOVA, $F_{2,6} = 39.82$, $p < 0.001$). However, the season was not found to have any significant effect on the aerosol bacterial abundance indoors ($F_{2,6} = 4.17$, $p = 0.073$) or outdoors ($F_{2,6} = 0.42$, $p = 0.677$) for the semi-urban site.

Indoor/outdoor relationship

Bacterial abundance mean I/O ratios based on non size-resolved concentrations were for both houses greater than unity during winter (urban: 2.57, semi-urban: 2.31) and spring (urban: 2.18, semi-urban: 2.03) and lower than unity during summer (urban: 0.66, semi-urban: 0.35).

Influence of environmental parameters on bacterial abundance

The indoor air bacterial abundance estimated for the total of May impactor stages was compared with the median values of the 12-hour concurrently recorded environmental variables by Spearman's rank correlation analysis (Table 4.9). When considering the full dataset together, the only parameter that correlated significantly with the indoor bacterial

levels was the outdoor bacterial abundance (ρ = 0.48). When separating the analysis by season, the outdoor bacterial concentration exhibited a significant positive association (ρ = 0.94) with the indoor levels only during winter. A high rho correlation coefficient between indoor and outdoor bacterial abundance was found for summer (ρ = 0.77), too. However the relationship between the two variables was not significant (p = 0.103). CO_2 concentration and relative humidity were also found to be strongly correlated with the bacterial levels in both houses during winter (ρ = 0.94) and summer (ρ = -0.89), respectively. No significant association was found between bacterial and particle mass median levels.

Table 4.9 Spearman's rho (ρ), with p-values shown in brackets, between indoor bacterial abundance (May impactor-based 12-hour samples), as measured via qPCR, and environmental parameters. Bolded values indicate statistical significance, defined as $p < 0.05$.

	Outdoor Bacterial abundance	PM_{10} in	$PM_{2.5}$ in	$PM_{2.5}$ out	CO_2 in	Temp in	Temp out	RH% In	RH% out
Both houses & all seasons	**0.478** (.047)	-0.021 (.956)	-0.337 (.171)	0.236 (.397)	0.077 (.761)	0.139 (.581)	-0.036 (.888)	-0.254 (.310)	0.162 (.519)
Urban site (all seasons)	0.566 (.121)	-0.714 (.136)	-0.650 (.066)	-0.714 (.136)	-0.483 (.194)	0.600 (.097)	0.517 (.162)	-0.200 (.613)	0.066 (.880)
Semi-urban site (all seasons)	0.200 (.613)	0.143 (.803)	-0.217 (.581)	0.633 (.076)	0.600 (.097)	-0.466 (.212)	-0.550 (.133)	-0.025 (.948)	0.033 (.948)
Both sites, Summer season	0.771 (.103)	0.028 (.999)	-0.429 (.419)	-0.086 (.919)	0.657 (.175)	0.464 (.354)	-0.829 (.058)	**-0.886** (.033)	0.145 (.784)
Both sites, Winter season	**0.943** (.017)	-0.086 (.919)	-0.086 (.919)	0.429 (.419)	**0.943** (.017)	0.486 (.356)	0.029 (.999)	0.543 (.297)	-0.771 (.103)
Both sites, Spring season	0.143 (.803)	*	0.486 (.356)	-0.500** (.999)	-0.200 (.714)	0.429 (.419)	0.314 (.564)	0.029 (.999)	-0.290 (.577)

*PM_{10} measurements were not obtained during spring.

**Outdoor $PM_{2.5}$ measurements during spring were only obtained for the semi-urban site.

4.3.5 Bacterial abundance on settle plates

Figure 4.18 presents the seasonal variation of airborne bacteria concentration levels per each site, as determined by 1-month period passive settling. The bacterial abundance recovered on the suspended passive collectors (i.e. empty petri dishes) did not differ significantly between the two sites, except winter that the semi-urban flat exhibited higher levels (Welch's t-test, p = 0.021). Across seasons, the highest bacterial load was observed during spring for the urban flat, in accordance with the aforementioned results (see 4.3.4). However the highest concentration for the semi-urban flat was found during winter. Overall bacterial quantification results based on passive sampling were positively but not significantly associated with the total bacterial abundance based on active sampling with the May impactor (average over 3 days) for both sites (Pearson's correlation, p > 0.05). Finally, both houses

exhibited significant variation across seasons (ANOVA, $p < 0.05$), with the effect of seasonality on the settle-plate bacterial load being larger for the semi-urban site ($F_{2,6} = 14.06$, $p = 0.005$) compared to the urban site ($F_{2,6} = 7.02$, $p = 0.027$).

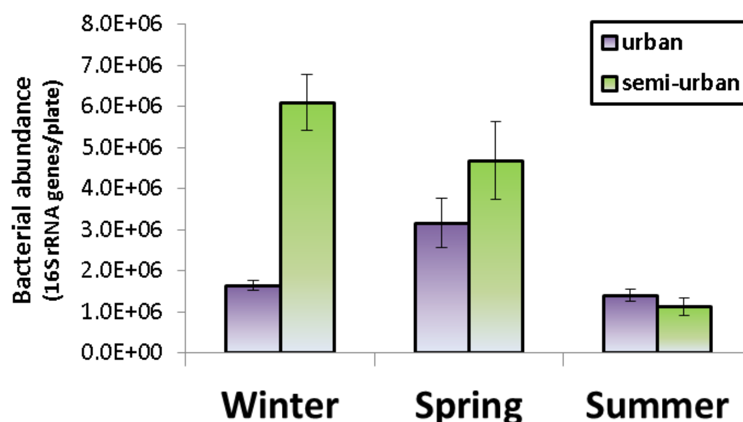


Figure 4.18 Indoor bacterial abundance in 16S rRNA gene copies as determined by passive collection on petri dish samplers left for whole-month duration per each house and each season. Results are presented as average over three replicates. Error bars represent standard error.

Influence of environmental parameters on bacterial abundance

The possible relationship between the bacterial load recovered by the settle plates for both houses per each season and the environmental parameters (monthly average over 3 daily medians) was examined but there was no significant association found (Spearman's rank coefficient analysis) with any of the factors, apart from the positive correlation with CO_2, which was at the limit of significance ($\rho = 0.83$, $p = 0.058$).

4.3.5 Size-resolved bacterial diversity and composition

The MiSeq run generated a total of 6,087,724 bacterial sequencing reads for the total of size-resolved samples recovered from both impactors (Andersen: n=36 indoors, May: n=42 indoors, 42 outdoors) and negative controls. After quality trimming, chimera filtering and removal of singletons, a total of 316,153 and 1,287,812 16S rRNA gene sequences were obtained for the Andersen and May impactor, respectively. These sequences clustered into 9,715 and 29,602 OTUs, for the two impactors, respectively, at the 97% similarity threshold. Subtraction of sequences resulting from the blanks/control samples and removal of sequences belonging to non-bacterial domains (chloroplasts, Archaea) and unassigned reads reduced the

numbers to 304,001 sequences (median/sample = 8,729 ± 2,032 read counts), comprising 8,533 OTUs, for the Andersen, and 1,181,434 sequences (median/sample = 14,529 ± 5,598 read counts), corresponding to 22,606 OTUs, for the May impactor. The numbers of sequences per samples were normalised based on the number of sequences obtained from the smallest library (4,604 for the Andersen and 3,599 sequences for the May-samples). The totals of remaining bacterial sequences were 165,744 for the Andersen and 302,316 for the May. Abundance-based filtering carried out to remove OTUs with 5 or fewer counts further reduced the numbers to 100,814 sequences for the Andersen and 140,511 sequences for the May impactor, represented by 149 and 156 OTUs, respectively.

Andersen impactor

Figure 4.19 summarises the numbers of bacterial OTUs recovered by the samples collected indoors with the Andersen impactor per sampling site and season. Shannon indices were also calculated (Appendix S4.26-S4.27). For the urban site, the number of OTUs shared among all six stages (i.e. core OTUs) was 95 for winter, 54 for spring and 89 during summer. For the semi-urban house, the OTUs shared across all stages were 70 during winter, 54 during spring and 59 during summer. The unique numbers of OTUs recovered per each size-specific sample are presented in Appendix S4.25. Except winter, both sites exhibited the lowest level of bacterial diversity for the smaller particles (size range: 0.65 - 1.1 μm), which were also the ones with the lowest levels of 16S rRNA gene abundance.

The OTU richness of bacterial communities showed significant variation across seasons only for the urban (ANOVA, $F_{2,15} = 5.68$, $p = 0.015$) site (semi-urban: ANOVA, $F_{2,15} = 2.65$, $p = 0.103 > 0.05$), with both sites exhibiting the lowest numbers of OTUs during spring. Moreover, the numbers of OTUs varied significantly between the two sites (Mann-Whitney, $p = 0.004$). During winter, 53 core OTUs were found to be present in both the urban and the semi-urban flats, whereas only 34 and 41 were common for the two sites during spring and summer, respectively. Overall, only 20 OTUs were commonly detected across all size-resolved samples and seasons in both sites.

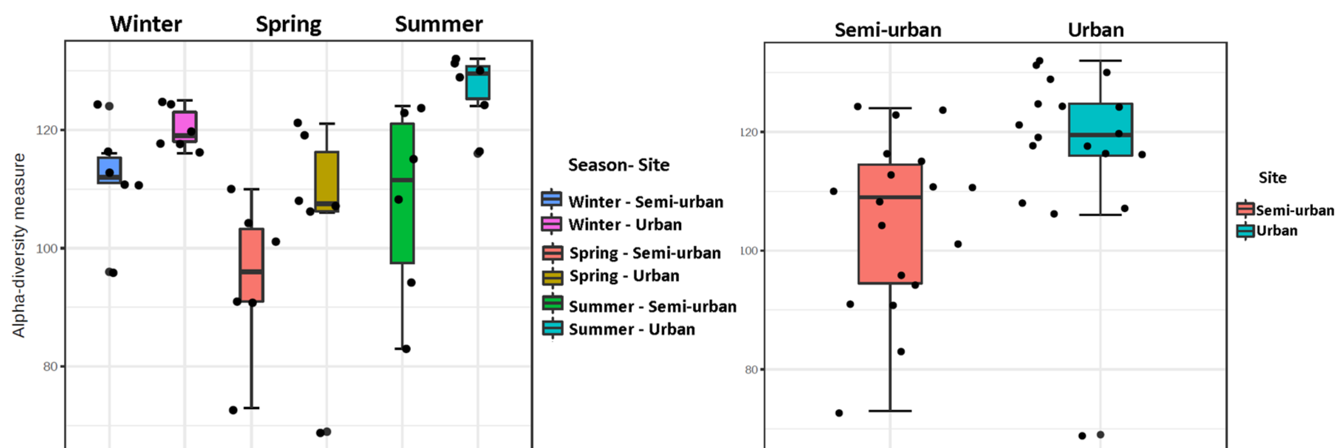


Figure 4.19 Boxplots of numbers of observed bacterial OTUs per sampling site and season (panel on the left) and per site (panel on the right) recovered from 12-h day-time size-resolved aerosol samples collected indoors with the six-stage Andersen impactor.

In order to assess compositional variation differences among samples, principal coordinate analysis plots based on Bray-Curtis dissimilarity matrices were constructed (Fig. 4.20). Results showed that samples were grouped broadly in an urban and a semi-urban cluster, and within each cluster most samples were also grouped by season (Fig. 4.20 - panel on the left). A tighter clustering was observed for the size-resolved samples representing the coarse particles (i.e. stages 1, 2, 3 & 4) for each site and within each season, whereas the samples associated with the smaller particles (i.e. stages 5 & 6) were scattered (Fig 4.20 - panel on the right). The effects of seasonality and location on bacterial community composition were tested with permutational multivariate analysis of variance (PERMANOVA) using Bray-Curtis dissimilarity matrices. The analysis revealed that both site (pseudo$F_{1, 34}$ = 11.44, R^2 = 0.25, $p < 0.001$) and season (urban: pseudo$F_{2, 15}$ = 9.64, R^2 = 0.56, $p < 0.001$; semi-urban: pseudo$F_{2,15}$ = 8.45, R^2 = 0.54, $p < 0.001$) were in fact significant factors for the indoor aerosol bacterial composition in both study houses. Based on PCoA plots, PERMANOVA analysis accounting for season was repeated only for the samples representing the coarse particles and results demonstrated an increased effect on community composition variation for both sites (urban: pseudo$F_{2, 9}$ = 13.73, R^2 = 0.75, $p < 0.001$; semi-urban: pseudo$F_{2,9}$ = 13.05, R^2 = 0.74, $p < 0.001$). Overall, the particle size was not found to be a strong predictor of the bacterial community structure (pseudo$F_{1, 34}$ = 2.60, R^2 = 0.07, $p = 0.015$).

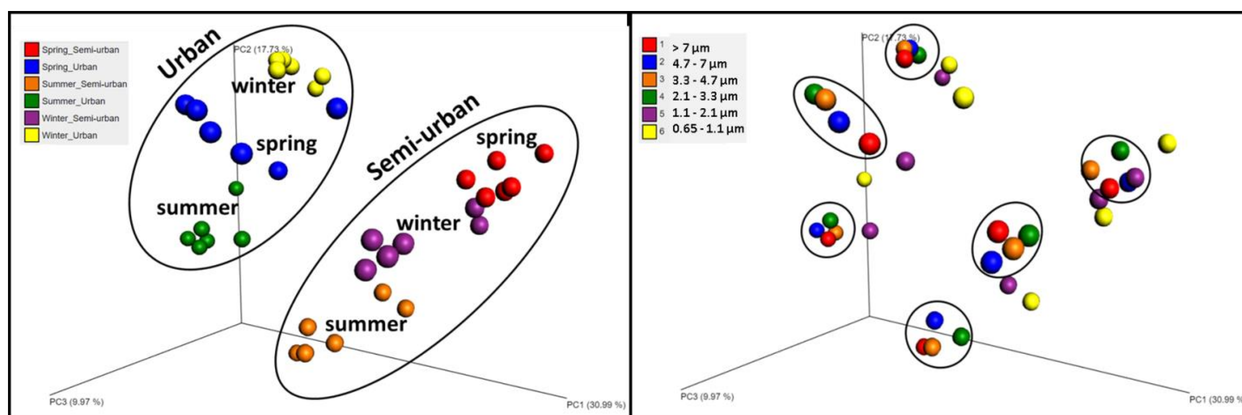


Figure 4.20 Principal coordinate analysis 3D-plots of bacterial beta diversity based on Bray-Curtis dissimilarity matrix with grouped samples based on season-site (panel on the left) and particle-size (panel on the right). Size-resolved aerosol samples were obtained indoors with the six-stage Andersen impactor. Circles were drawn manually to denote groups of interest.

Taxonomic composition of size-resolved samples collected with the Andersen sample at the study houses per season is illustrated in Fig. 4.21. Looking broadly at the composition of the identified taxa in aerosol particles, the most abundant bacterial phyla across samples were Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes with some low proportions (< 3%) of Tenericutes (*Mollicutes*) and Verrucomicrobia (*Verrucomicrobiae*). The relative abundance of Actinobacteria was higher at the semi-urban flat compared to the urban house during all seasons and across all particle size ranges, while Proteobacteria was the predominant phylum at the urban site. Bacteroidetes was also detected at higher abundance at the urban flat compared to the semi-urban site. In terms of season, two different seasonal patterns were observed at each site. At the urban house, Firmicutes exhibited the highest relative abundance during winter and the lowest during summer, while Bacteroidetes were more abundant during summer compared to the other seasons. Proteobacteria were present at increased abundance during spring and summer. At the semi-urban house, the main seasonal difference was observed for Actinobacteria and Firmicutes that were more highly enriched across all stages during spring-time, whereas the abundance of Proteobacteria was decreased compared to winter and summer. In terms of particle size, there were no consistent tendencies observed, apart from stage 6, which demonstrated slightly higher relative proportions of Actinobacteria, compared to the other stages in both sites.

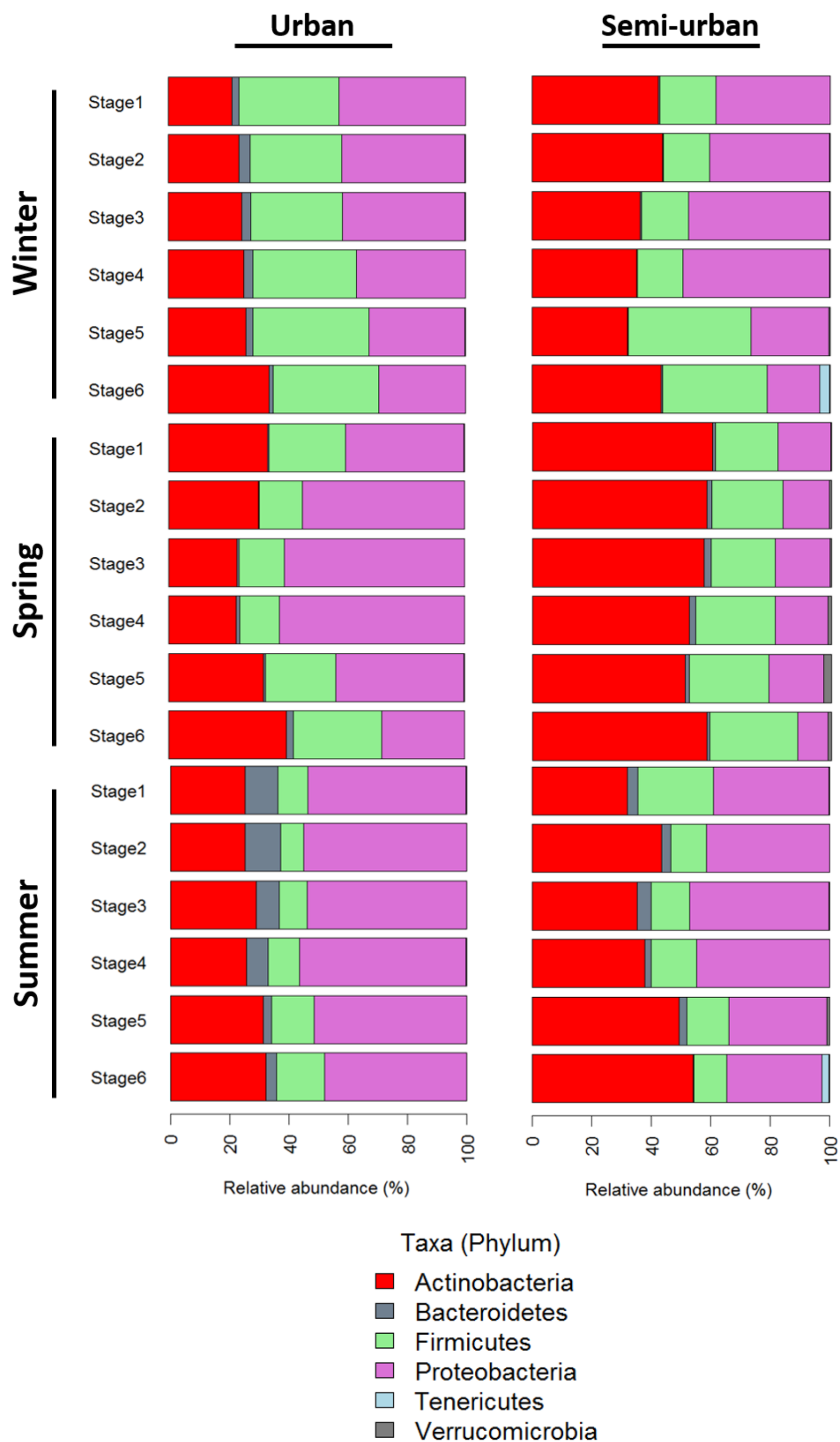


Figure 4.21 Relative abundance of indoor air bacterial OTUs at the phylum level per each sampling site (urban, semi-urban) and across seasons (winter, summer, spring). Size-resolved samples were obtained with the Andersen impactor. Stages 1, 2, 3, 4, 5 and 6 correspond to particle size ranges: > 7 µm, 7 - 4.7 µm, 4.7 - 3.3 µm, 3.3 - 2.1 µm, 2.1 - 1.1 µm and 1.1 - 0.65 µm.

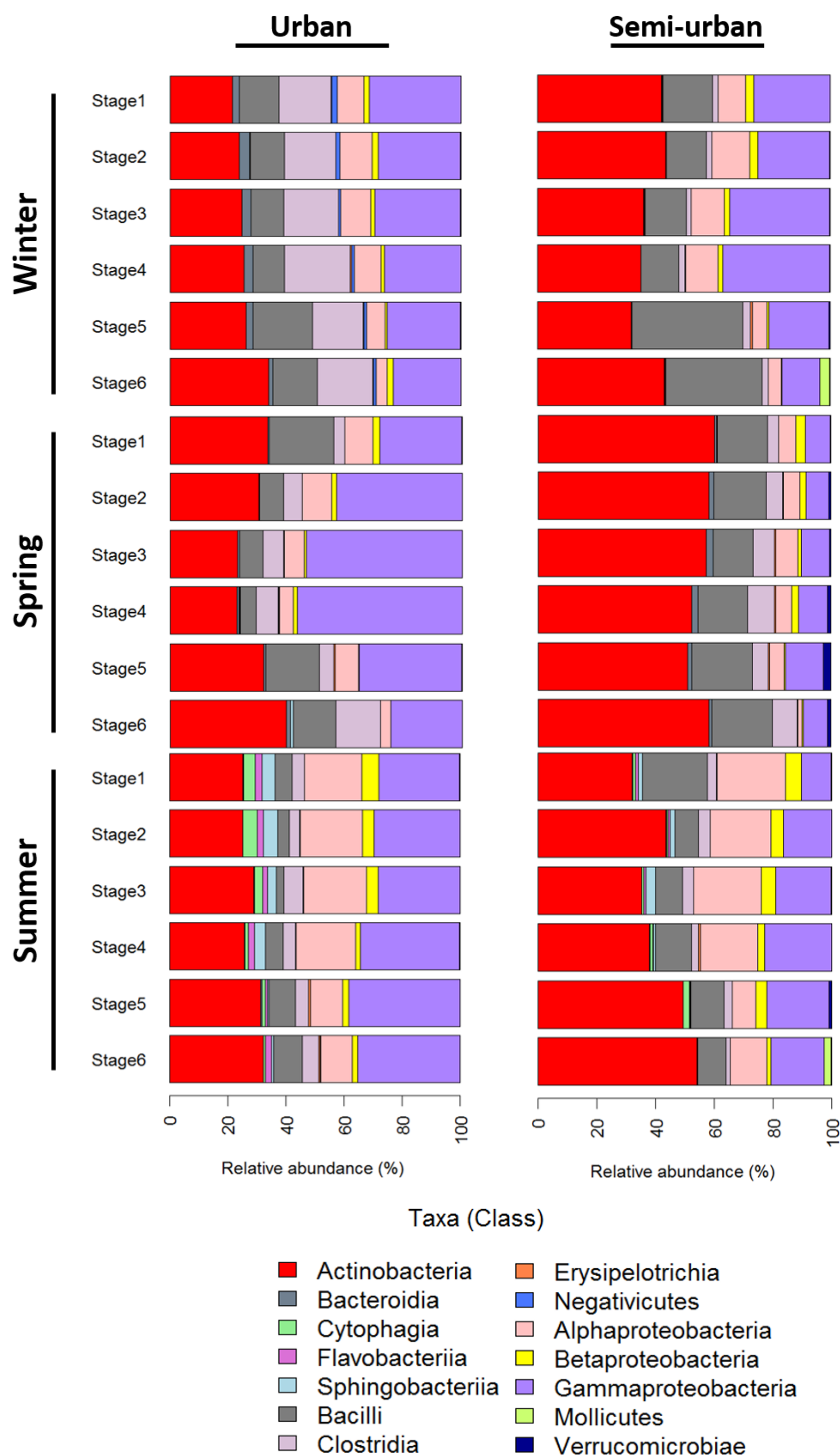


Figure 4.22 Relative abundance of indoor air bacterial OTUs at the class level per each sampling site (urban, semi-urban) and across seasons (winter, summer, spring). Size-resolved samples were obtained with the Andersen impactor. Stages 1, 2, 3, 4, 5 and 6 correspond to particle size ranges: > 7 µm, 7 - 4.7 µm, 4.7 - 3.3 µm, 3.3 - 2.1 µm, 2.1 - 1.1 µm and 1.1 - 0.65 µm.

Taxonomic classification at the class level (Fig. 4.22) revealed that Actinobacteria was represented by the *Actinobacteria* class, while Proteobacteria was dominated by *Gammaproteobacteria* and lower proportions of *Alphaproteobacteria* and *Betaproteobacteria.* The proportions of both *Alphaprotebacteria* and *Betaproteobacteria* were more enriched at the larger particle sizes and more increased during summer for both sites. The predominant class within Firmicutes at the semi-urban house was *Bacilli*, while at the urban site *Bacilli* seemed to be almost equally abundant with *Clostridia*. Bacteroidetes phylum, which was more enriched at the urban site, was composed by *Bacteroidia*, *Cytophagia*, *Flavobacteriia* and *Sphingobacteriia*.

May impactor

The numbers of observed OTUs identified for the samples collected with the May impactor are summarised in Fig. 4.23. The unique numbers of OTUs recovered per each size-specific sample indoors and outdoors are presented in Appendix (S4.28-S4.29). For the urban site, the number of common indoor air OTUs found in all seven stages (i.e. core OTUs) was 44 for winter, 33 for spring and 40 during summer. The corresponding numbers of OTUs found outdoors at the urban location were 19, 14 and 41, respectively. For the semi-urban house, the OTUs shared across all stages were 17 during winter, 17 during spring and 16 during summer. Outdoors, the core OTUs present in all size-specific stages were 9, 23 and 2 for winter, spring and summer, respectively.

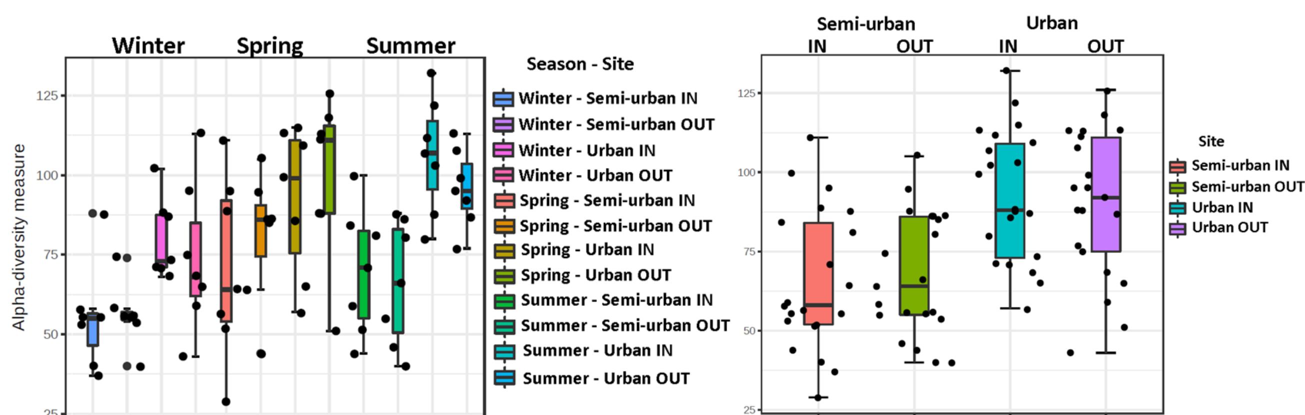


Figure 4.23 Boxplots of numbers of observed bacterial OTUs per sampling site and season (panel on the left) and per site (panel on the right) recovered from 12-h day-time size-resolved aerosol samples collected indoors and outdoors with the seven-stage May impactor.

The variation in indoor air bacterial richness across seasons was at the limit of significance only for the urban house (ANOVA, urban: $F_{2,18} = 3.50$, p = 0.052; semi-urban: $F_{2,18} = 1.10$, p

= 0.354), whereas outdoor air OTU richness revealed strong seasonality only for the semi-urban location (ANOVA, urban: $F_{2,18} = 2.91$, $p = 0.080$; semi-urban $F_{2,18} = 4.00$, $p = 0.048$). The two sampling sites exhibited statistically significant differences, with the urban site demonstrating a higher level of diversity both indoors (t-test, $p < 0.001$) and outdoors (t-test, $p = 0.002$). In contrast, there were no substantial differences found between indoor and outdoor air species richness in both sites (t-test, $p > 0.05$).

In terms of presence/absence of OTUs, 12 core OTUs were found to be commonly present in the indoor air of both the urban and the semi-urban flats during winter, whereas only 8 OTUs were shared between the two sites for both spring and summer. The outdoor air core OTUs which were common for both sites were 5, 6 and 1 during winter, spring and summer, respectively. Overall, only 4 core OTUs were commonly detected indoors across all samples and seasons in both sites.

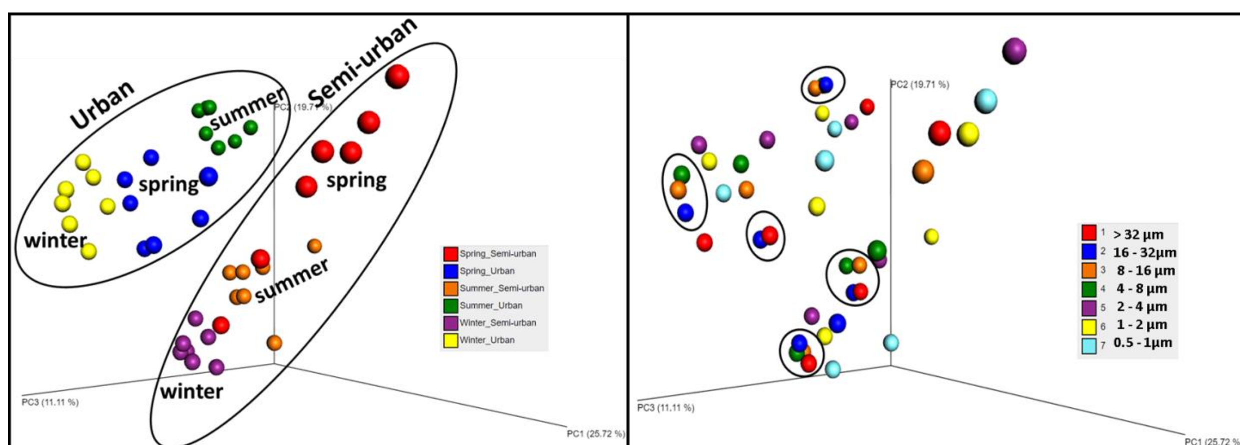


Figure 4.24 Principal coordinate analysis 3D-plots of bacterial beta diversity based on Bray-Curtis dissimilarity matrix with grouped samples based on season-site (panel on the left) and particle size (panel on the right). Size-resolved aerosol samples were obtained indoors with the seven-stage May impactor. Circles were drawn manually to denote groups of interest.

PCoA analysis of indoor air bacterial communities based on Bray-Curtis dissimilarity metric revealed the presence of two broad groups representing the aerosol samples collected in each house and showed that the aerosol samples appeared to cluster together according to season, with the spring samples being relatively scattered (Fig 4.24 - panel on the left). In terms of particle size (Fig. 4.24 - panel on the right), samples associated with the bigger sizes tended to be more closely clustered, whilst most samples of the finer fractions were less concentrated compared to the other within-season samples.

PERMANOVA analysis showed that indoor air bacterial assemblages exhibited significant differences between samples collected with the May impactor in the urban and the semi-urban flat ($pseudoF_{1, 40} = 8.99$, $R^2 = 0.18$, $p < 0.001$). In accordance with the aforementioned results based on the Andersen impactor, PERMANOVA also revealed strong seasonal patterns in bacterial community composition indoors for both sites (urban: $pseudoF_{2, 18} = 7.41$, $R^2 = 0.45$, $p < 0.001$; semi-urban: $pseudoF_{2, 18} = 10.42$, $R^2 = 0.54$, $p < 0.001$). When analysis was performed excluding the smaller size fractions (0.5 - 1 μm, 1 - 2 μm & 2-4 μm), the percentage of variation explained by season was increased (urban: $pseudoF_{2, 9} = 6.49$, $R^2 = 0.59$, $p < 0.001$; semi-urban: $pseudoF_{2, 9} = 6.40$, $R^2 = 0.59$, $p < 0.001$).

The aerosol bacterial assemblages encountered outdoors at the urban and at the semi-urban sites exhibited a mixed pattern, as demonstrated in Fig. 4.25, forming distinct groupings based on season rather than location, with many samples collected during spring, mostly from the urban location, being more similar to samples obtained during winter (Fig. 4.25 - panel on the left). In addition, samples associated with the smaller particles appeared to harbour more distinct airborne bacterial communities compared to the bigger particles (Fig. 4.25 - panel on the right).

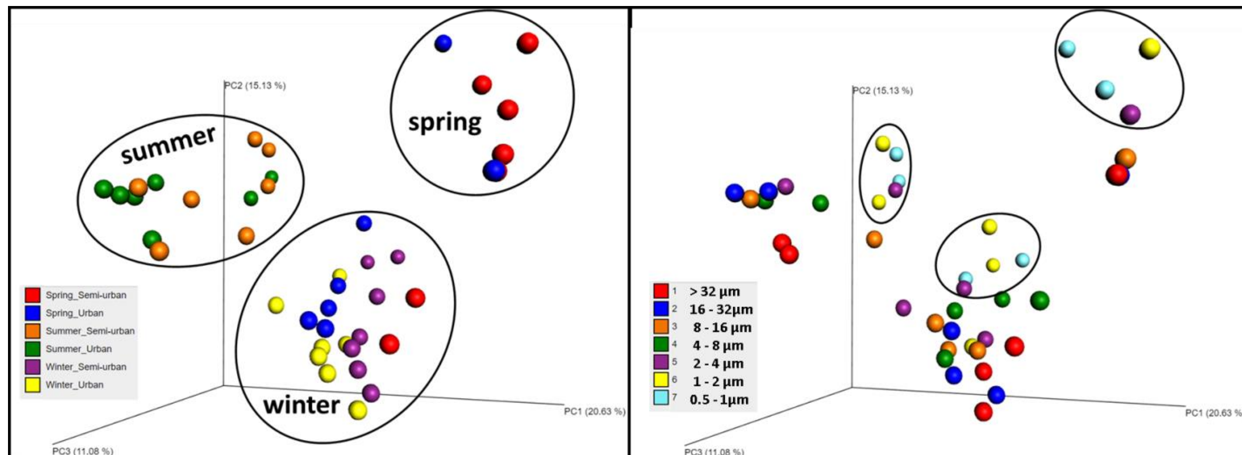


Figure 4.25 Principal coordinate analysis 3D-plots of bacterial beta diversity based on Bray-Curtis dissimilarity matrix with grouped samples based on season-site (panel on the left) and particle size (panel on the right). Size-resolved aerosol samples were obtained outdoors with the seven-stage May impactor. Circles were drawn manually to denote groups of interest.

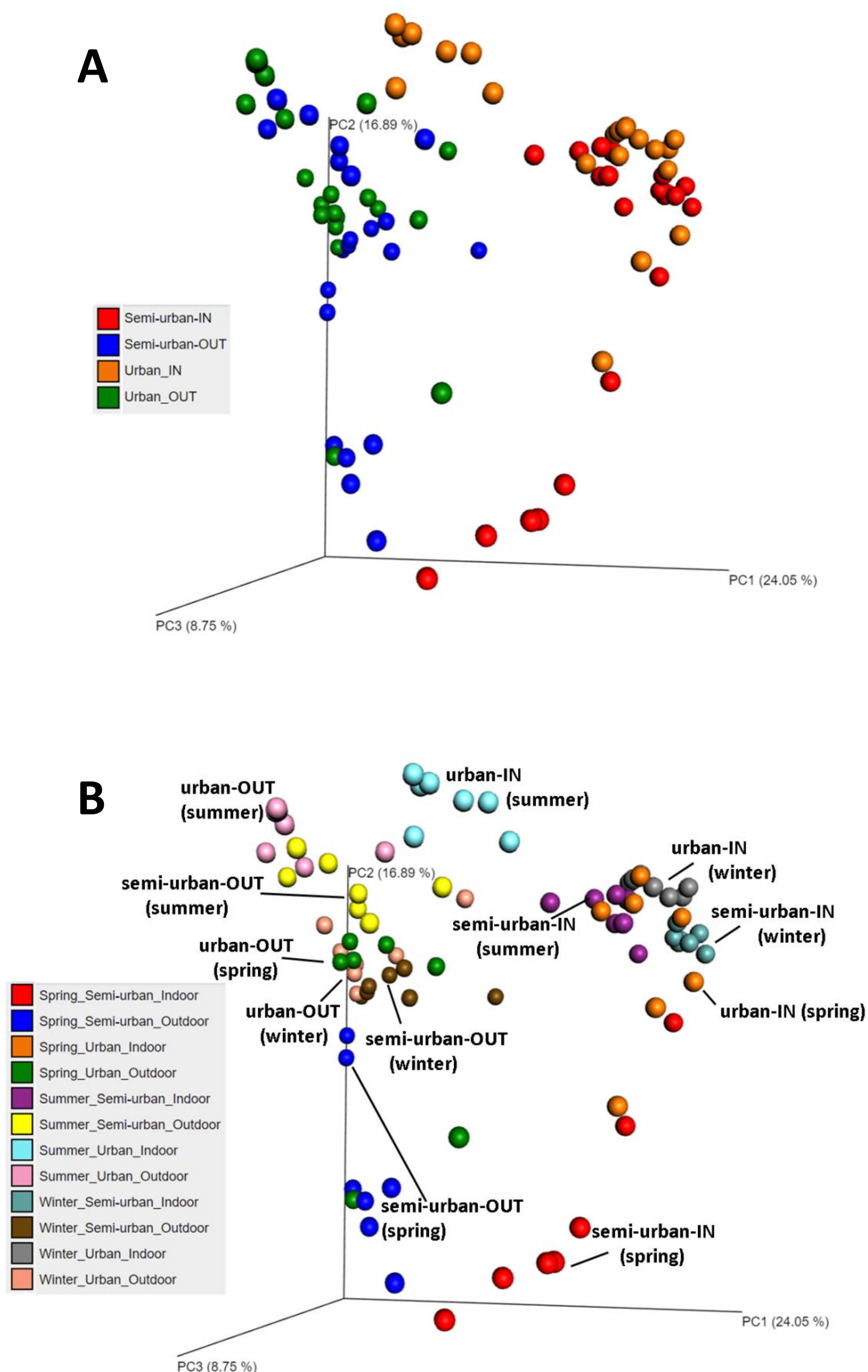


Figure 4.26 Principal coordinate analysis 3D-plots of bacterial beta diversity based on Bray-Curtis dissimilarity matrix with grouped samples based on sampling site (A) and season/sampling site (B). Size-resolved aerosol samples were obtained indoors and outdoors with the seven-stage May impactor.

The location, in fact, had a significant but almost negligible effect on the outdoor air community composition variation between the urban and the semi-urban sites (PERMANOVA, $pseudoF_{1, 40} = 2.64$, $R^2 = 0.06$, $p = 0.003$), whereas the season had a larger effect both when considering sites separately (urban: $pseudoF_{2, 18} = 4.80$, $R^2 = 0.35$, $p < 0.001$; semi-urban: $pseudoF_{2, 18} = 5.16$, $R^2 = 0.36$, $p < 0.001$) and together ($pseudoF_{2, 39} = 7.28$, $R^2 = 0.27$, $p < 0.001$).

The variation of bacterial communities collected indoors and outdoors, at the two sites, during different seasons, were also visualised together using principal coordinate analysis (Fig. 4.26). Results showed that samples representing airborne bacterial communities indoors and outdoors did not cluster (Fig. 4.26A). PERMANOVA analysis confirmed that the aerosol bacterial assemblages exhibited significant differences between indoor and outdoor air in both locations (urban: $pseudoF_{1, 40} = 15.01$, $R^2 = 0.27$, $p < 0.001$; semi-urban: $pseudoF_{1, 40} = 11.92$, $R^2 = 0.23$, $p < 0.001$). The only groups of indoor/outdoor samples that were found at closer proximity (i.e. less dissimilar) were the ones obtained during summer for the urban site and the ones obtained during spring for the semi-urban site (Fig. 4.26B). The particular pattern is also reflected at the Bray-Curtis dissimilarity values (Fig. 4.27).

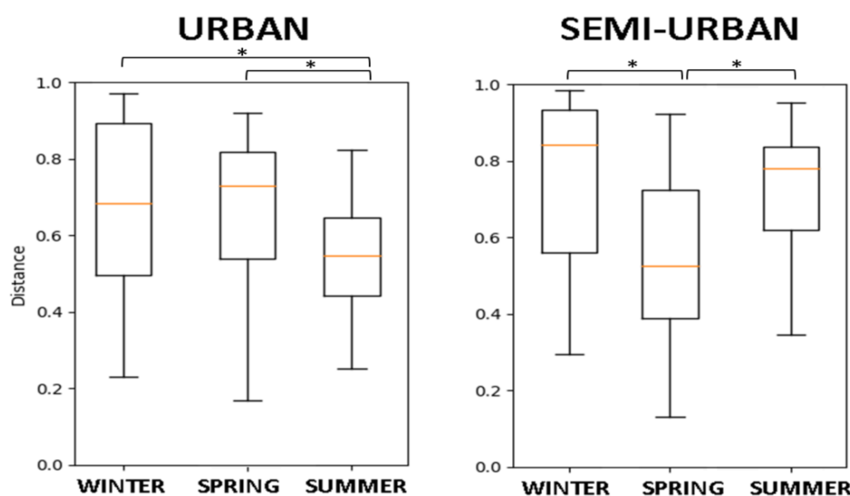


Figure 4.27 Boxplots of pairwise Bray-Curtis dissimilarity of bioaerosol bacterial composition based on both indoor and outdoor air samples within each season and per each site. The Bray-Curtis dissimilarity metric assumes values between 0 and 1, with two samples being identical when the value is 0 and completely dissimilar if the value is 1. The statistical significance of the difference between the distributions of distances was calculated using non-parametric two-sample t-test with Bonferroni correction and 9999 Monte Carlo permutations (* p-value < 0.001).

When examining the outdoor bacterial composition at the two sites, as resulted from the samples collected with the May impactor (Fig. 4.28), two more phyla associated with environmental sources were detected, Cyanobacteria and Deinococcus-Thermus (also detected indoors at very low proportions). The main differences observed between the two sites outdoors were the higher relative abundance that Firmicutes exhibited at the semi-urban site compared to the urban during winter, and their decreased presence during spring compared to the proportions that the urban site demonstrated. Both sites were more enriched for Bacteroidetes outdoors, with larger proportions detected for the urban site during spring and summer. From the other hand, Proteobacteria were more abundant at the semi-urban site outdoors, compared to the urban location, during the same seasons.

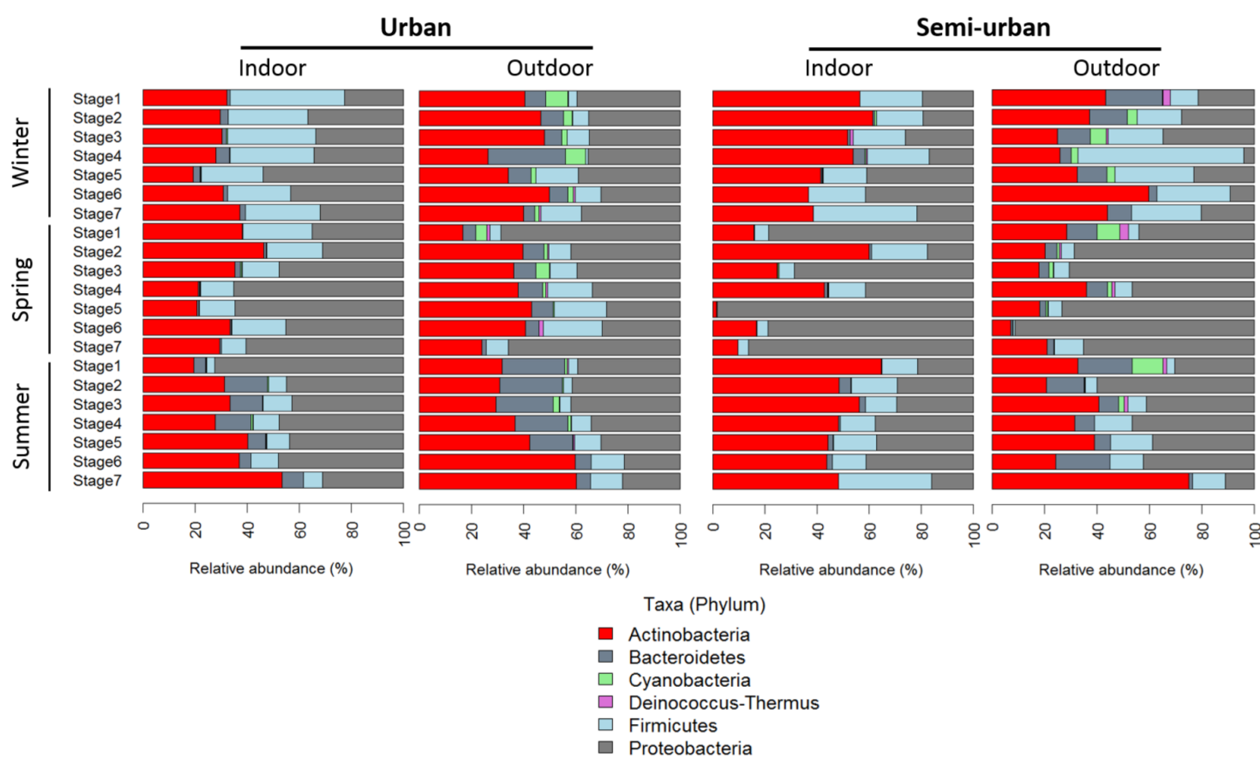


Figure 4.28 Relative abundance of indoor and outdoor air bacterial OTUs at the phylum level per each sampling site (urban, semi-urban) and across seasons (winter, summer, spring). Size-resolved samples were obtained with the May impactor. Stages 1, 2, 3, 4, 5, 6 & 7 correspond to particle size ranges: >32 μm, 32 - 16 μm, 16 - 8 μm, 8 - 4 μm, 4 - 2 μm, 2 - 1 μm and 1 - 0.5 μm.

Looking at the indoor composition profile for the urban site, a similar pattern to the one obtained with the Andersen samples was observed, with higher representation of Proteobacteria compared to Actinobacteria across all seasons, larger proportions of Firmicutes during winter and increased abundance of Bacteroidetes during summer. When

considering the outdoor air bacterial composition, it was clear that the increased indoor occurrence of Firmicutes during winter was not associated with outdoor sources, since outdoor samples were less enriched for bacterial OTUs affiliated with Firmicutes. On the other hand, the increased relative abundance of Bacteroidetes indoors during summer, seemed to be related with the larger proportions of Bacteroidetes detected outdoors during summer, compared to the other seasons.

For the semi-urban house, even though Actinobacteria were present at higher relative abundance compared to Proteobacteria and in relation with the urban site's proportions, as observed with the Andersen taxonomic profile, the increased abundance that the Andersen samples exhibited during spring was not encountered across all stages for the May impactor. However, a similar pattern was also observed outdoors suggesting that the relative abundances at the phylum level indoors during spring were most likely influenced by the outdoor air.

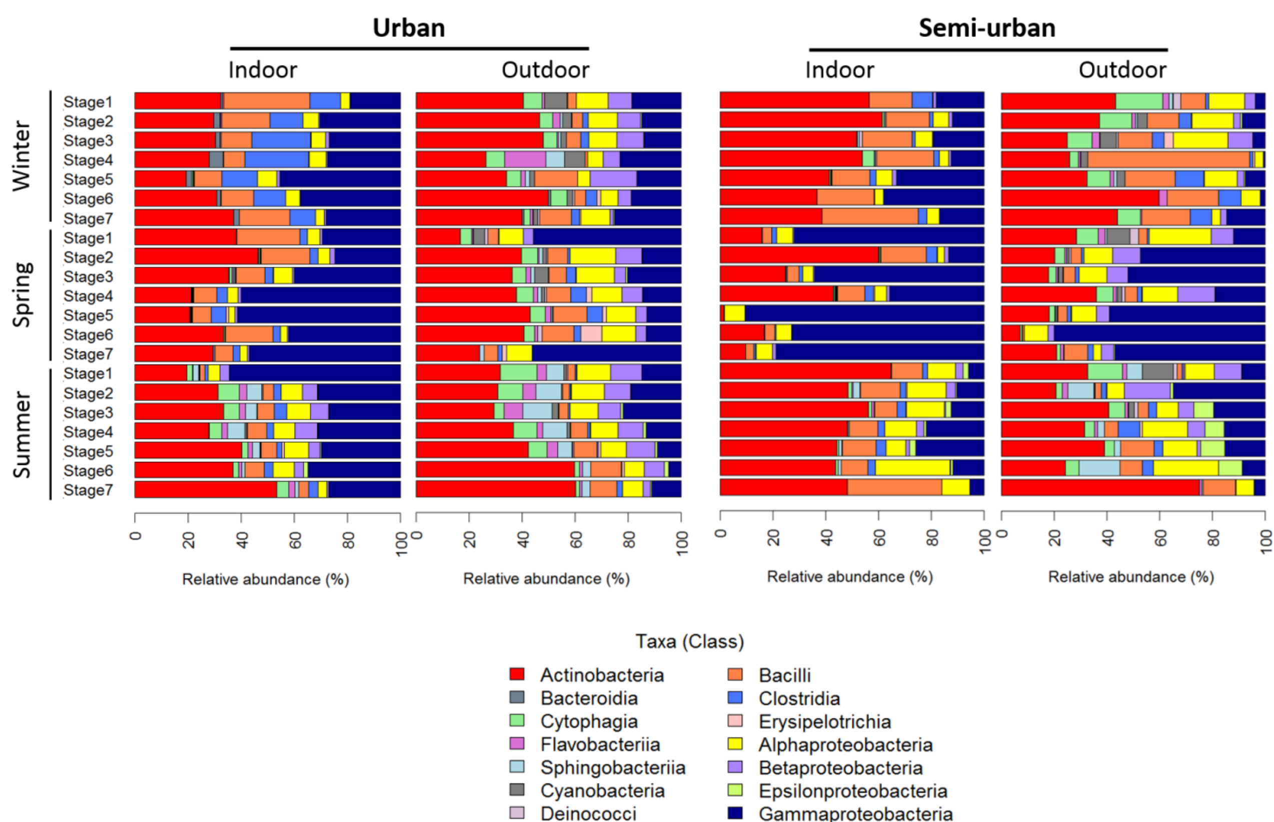


Figure 4.29 Relative abundance of indoor and outdoor air bacterial OTUs at the class level per each sampling site (urban, semi-urban) and across seasons (winter, summer, spring). Size-resolved samples were obtained with the May impactor. Stages 1, 2, 3, 4, 5, 6 & 7 correspond to particle size ranges: >32 μm, 32 - 16 μm, 16 - 8 μm, 8 - 4 μm, 4 - 2 μm, 2 - 1 μm and 1 - 0.5 μm.

The phyla of Tenericutes and Verrucomicrobia which were detected at low proportions (< 3%) within the samples collected with the Andersen impactor, were not among the phyla recovered with the samples obtained with the May impactor.

At the class level (Fig. 4.29), in general, indoor and outdoor profiles seemed more dissimilar during winter and spring and more similar during summer. Similar seasonal trends to the ones obtained with the Andersen impactor were observed indoors, with no specific particle size-related pattern. However, there was one more class, *Epsilonproteobacteria*, detected, assigned to phylum Proteobacteria. *Epsilonproteobacteria* were more abundant outdoors, with the highest relative abundance occurring for the semi-urban site during summer. Moreover, *Negativicutes* class (Firmicutes) that was mainly detected at low proportions at the urban site with the samples obtained with the Andersen, was absent from the May impactor's dataset. Among the main compositional differences between the two sites, as demonstrated by the Andersen samples, was the dominant presence of *Clostridia* (Firmicutes) at the urban house, mainly during winter. Examination of the outdoor profiles (May impactor) revealed that *Clostridia* at the urban site were more abundant indoors compared to outdoors and thus their presence was attributed to indoor-generated sources. Among Bacteroidetes, taxa affiliated with *Cytophagia* class were highly enriched outdoors, mainly at the urban site, justifying the higher levels encountered indoors during summer at the urban flat. *Gammaproteobacteria* at both sites were, with few exceptions, more enriched indoors than outdoors, while *Alphaproteobacteria* and *Betaproteobacteria* were present at higher relative abundance outdoors across all seasons.

4.3.6 Passive sampling-based bacterial diversity and composition

Passive sampling employing suspended (empty) petri dishes for a whole-month duration per each season was also performed in the study houses. A total of 3,412,535 bacterial sequencing reads were generated by the MiSeq run for the suspended passive collectors (n = 18). This total was reduced to 620,857 sequences following quality trimming, chimera filtering and removal of singletons. These sequences clustered into 17,236 OTUs, at the 97% similarity threshold. Subtraction of non-bacterial OTUs, unassigned reads and sequences resulting from the blanks/control samples further reduced the total to 596,865 sequences (median/sample = 34,629 ± 15,058 read counts), corresponding to 15,982 OTUs. The OTU table was normalised to the sample with the lowest number of total sequence reads (7,172)

and a total of 125,096 sequences were retained. Supplementary filtering of low-abundant OTUs ($n \leq 5$ sequences) reduced the number to 85,775 sequences, which were represented by 213 OTUs.

Figure 4.30 summarises the numbers of observed OTUs for the bacterial communities recovered per each site. Overall, the OTU richness was found significantly higher at the urban house (Welch t-test, $p < 0.001$), in agreement with results obtained for the impactors. In particular, the core OTUs found in the air of the urban flat were 165, 167 and 169 during winter, spring and summer, respectively. The corresponding levels of bacterial richness for the semi-urban flat across seasons were 124, 124 and 145, respectively (the unique numbers of OTUs identified per each replicate, $n = 3$, as well as Shannon indices are also presented in Appendix S4.32-S4.33). The richness variation among seasons was found to be significant only for the semi-urban house (ANOVA, $F_{2,6} = 20$, $p = 0.002$) due to the higher levels observed during summer compared to spring and winter. In terms of presence/absence of common OTUs between the two sites, the OTUs being present in the interior of both houses were 104 for winter, 98 for spring and 112 for summer. In total, 56 OTUs were shared between the two sites across all three seasons.

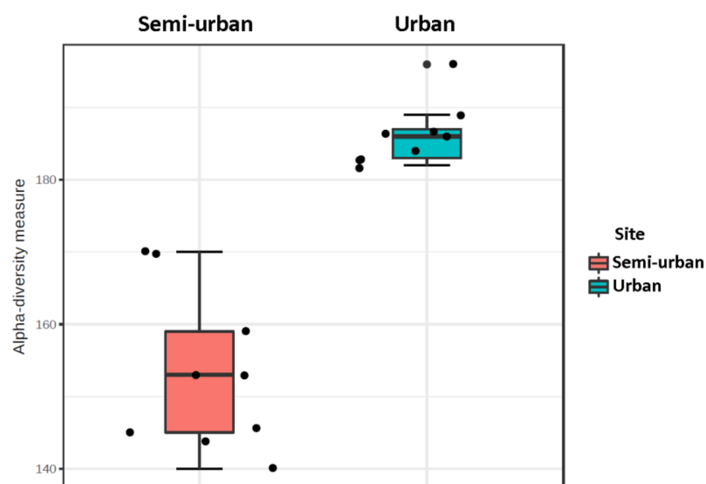


Figure 4.30 Boxplots of numbers of observed bacterial OTUs per sampling site recovered from whole-month passive collection using suspended (empty) petri dishes indoors.

Whole-month passive settling samples collected in each house were also analysed in terms of bacterial beta diversity based on Bray-Curtis dissimilarity and results exhibited significant seasonal differences, in accordance with impaction-based samples, with an even stronger

grouping based on season (PERMANOVA, urban: pseudo-$F_{2,6}$ = 17.81, R^2 = 0.86, p = 0.004; semi-urban: pseudo-$F_{2,6}$ = 17.89, R^2 = 0.86, p = 0.004).

In terms of taxonomic composition (see Appendix S4.34), the classification at the phylum level was in agreement with the one presented for the aerosol samples obtained with the May impactor. The mean relative proportions of Actinobacteria were substantially higher at the semi-urban flat across all seasons (winter: 70.40%, spring: 78.0%, summer: 66.54%) compared to the urban residence (winter: 26.16%, spring: 34.23%, summer: 41.88%). In contrast, Proteobacteria (urban: winter 29.14%, spring 38.21%, summer 32.39%; semi-urban: winter 11.66%, spring 9.49%, summer 22.80%) and Firmicutes (urban: winter 41.56%, spring 25.33%, summer 14.89%; semi-urban: winter 17.33%, spring 12.24%, summer 8.44%) were more enriched in the air of the urban house. Members of Bacteroidetes were also found at increased proportions at the urban site.

Taxonomic classification at the class level revealed that the big difference for Firmicutes and Proteobacteria between the two sites was mainly attributed to the increased proportions of *Clostridia* (urban: winter 27.30%, spring 12.23%, summer 7.51%, semi-urban: winter 0.83%, spring 0.41%, summer 1.25%) and *Gammaproteobacteria* (urban: winter 15.86%, spring 21.81%, summer 15.55%; semi-urban winter 5.71%, spring 3.15%, summer 4.62%), respectively, at the urban flat. The classes of *Epsilonproteobacteria*, which was only detected with the May impactor at the semi-urban site, and *Negativicutes*, which was mainly detected in the samples obtained with the Andersen at the urban house, were both present in the aerosol bacterial composition recovered with the petri-samples.

According to results at the genus level, the most abundant taxon identified in both sites and across all seasons was *Corynebacterium*, exhibiting higher relative abundance at the semi-urban flat. Few other genera were consistently detected among the top 15 most enriched bacterial taxa in both sites including *Staphylococcus*, *Paracoccus*, *Pseudomonas* (all seasons), *Acinetobacter* (winter), *Kocuria* (winter & spring), *Micrococcus* (spring), *Microbacteriaceae*, *Massilia* and *Sphingomonas* (summer). In addition, *Brevibacterum*, *Brachybacterium*, *Roseomonas* and *Moraxellaceae* (unidentified genus) were abundantly detected in the air of the semi-urban flat across all seasons, while *Dietzia* and *Brevundimonas* were among the 15 most enriched taxa during winter and spring only. The unique taxa recovered at high proportions for the urban site were *Roseburia*, *Lactobacillus*, *Anaerococcus* and *Ruminococcaceae* (unidentified genus), which were recovered across winter and spring.

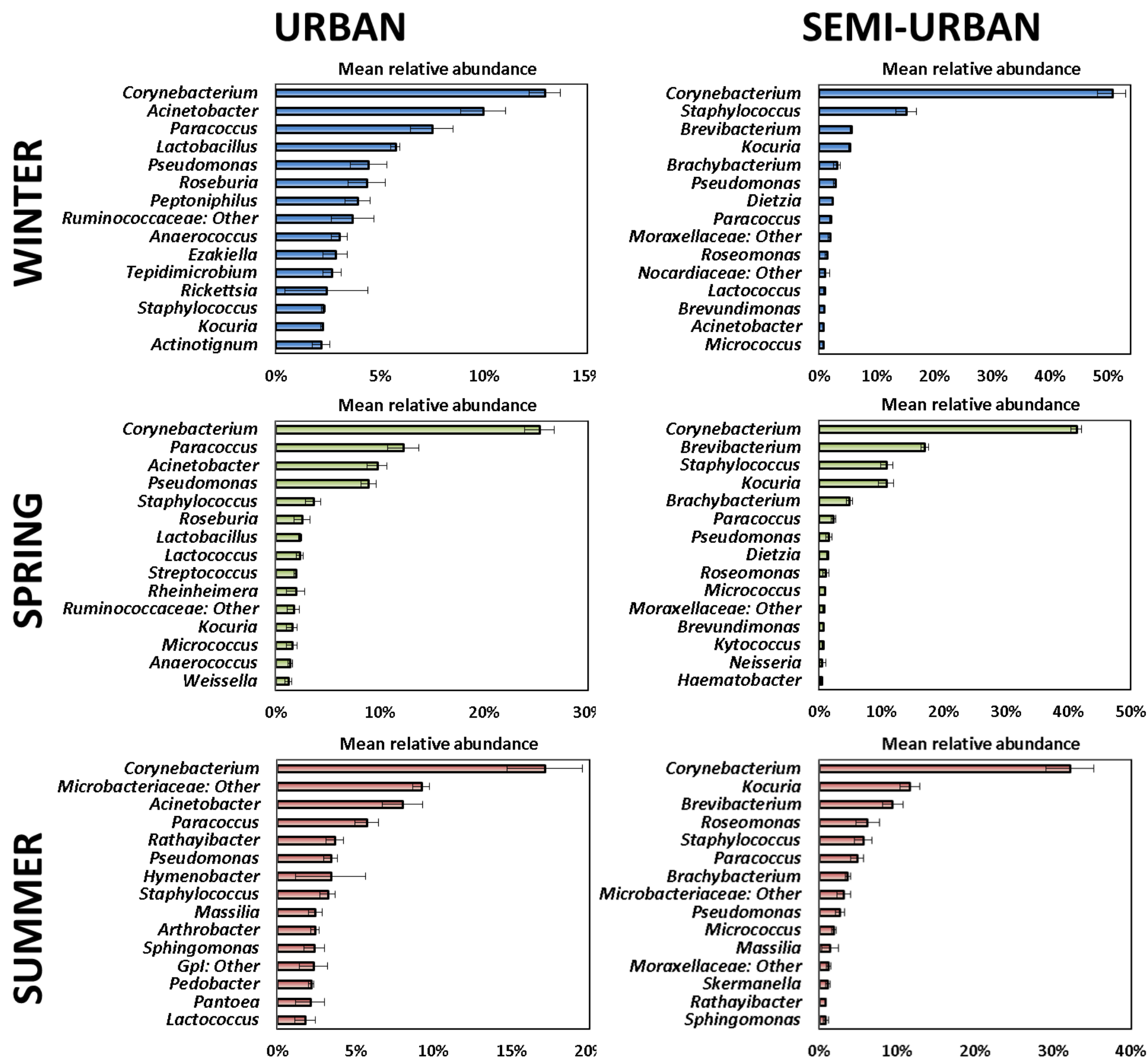


Figure 4.31. Mean relative abundance of the top 15 most enriched indoor air bacterial genera found per each house and season. Samples were obtained with whole-month passive collection using suspended (empty) petri dishes. Error bars represent standard deviation across replicates (n = 3).

Although these highly abundant bacterial genera could be detected in both flats, the relative abundance of each taxon varied quite widely between them. To further identify which airborne genera exhibited significantly differential abundance between the two houses, LEfSe (Linear Discriminant Analysis Effect Size) analysis was applied. Fig. 4.32 shows the 25 most differentially abundant genera in relation to the sampling site. From those, *Acinetobacter* (*Gammaproteobacteria*), *Lactobacillus* (*Bacilli*), *Roseburia* (*Clostridia*), *Peptoniphilus* (*Clostridia*) and *Tepidimicrobium* (*Clostridia*) genera were identified as significantly more abundant in the urban site and *Brevibacterium* (*Actinobacteria*), *Staphylococcus* (*Bacilli*), *Kocuria* (*Actinobacteria*), *Brachybacterium* (*Actinobacteria*) and *Roseomonas*

(*Alphaprotebacteria*) genera were significantly more abundant in the semi-urban house. The variability in bacterial community composition between the two sites was driven not only by abundant but also by various less frequent taxa, such as *Bacteroides* and *Porphyromonas* (*Bacteroidia*), *Negativicoccus* (*Negativicutes*) and members of *Clostridia: Romboutsia*, *Clostridium* XVIII, *Ruminococcus*2, *Blautia*, *Anaerostipes*, which were more overrepresented in the urban flat.

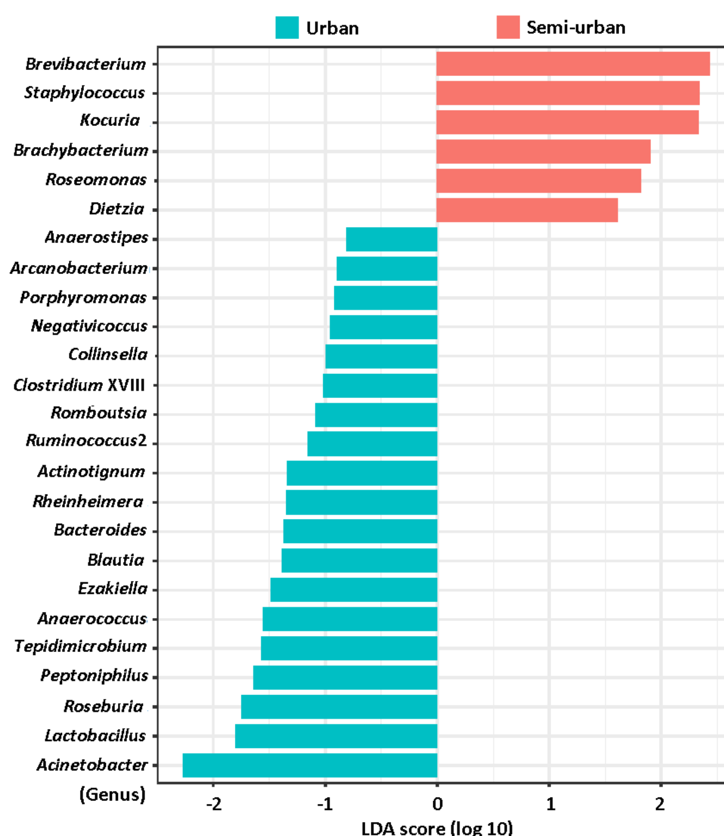


Figure 4.32 LEfSe (Linear Discriminant Analysis Effect Size) analysis showing the most significantly differentially abundant taxa between the two study houses (LDA score > 1) at the genus level.

Comparing passive and active sampling methods

The aerosol bacterial assemblages obtained indoors using different sampling methods, active (i.e May & Andersen impactors) and passive ("petri") were reanalysed together and visualised using PCoA (Fig. 4.33). Different methods employed, i.e. active (May & Andersen impactor) and passive (petri dishes), seemed to be in good agreement as demonstrated by the close proximity of the samples obtained during winter and summer but not for spring, mainly due to the large dispersal among the samples obtained with the May impactor.

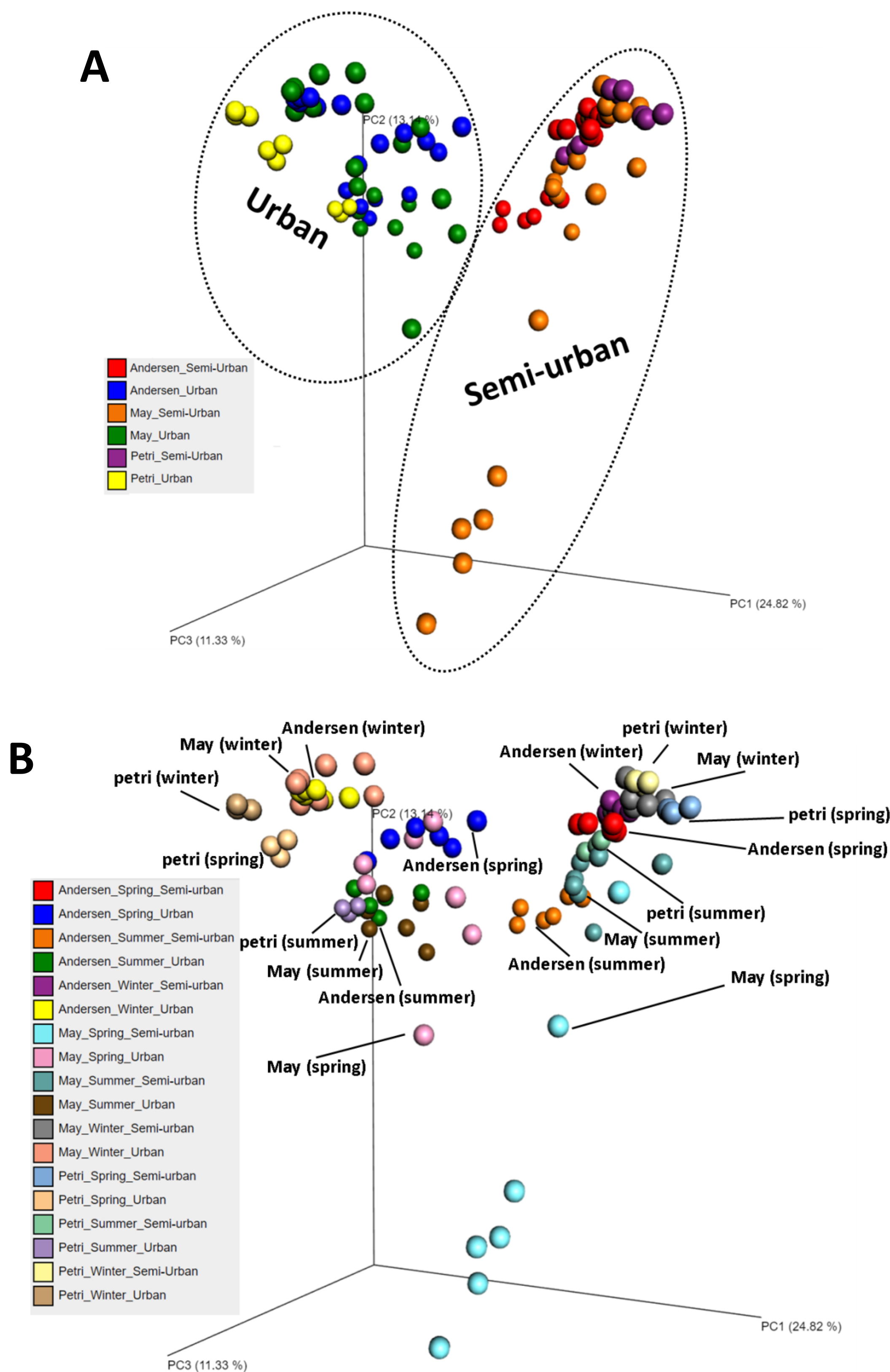


Figure 4.33 Principal coordinate analysis 3D-plots of bacterial beta diversity based on Bray-Curtis dissimilarity matrix with grouped samples based on sampling method (Andersen impactor, May impactor and petri dishes-passive collection) and site (A) and sampling method/season/site (B). All samples were collected indoors. Circles were drawn manually to denote groups of interest.

Although the grouping of samples by collection method was found to be statistically significant, the effect size was negligible (PERMANOVA, pseudo-$F_{2,93} = 4.46$, $R^2 = 0.08$, $p < 0.001$). Analysis of similarities further confirmed that that there was no strong dissimilarity between groups of samples (ANOSIM, $R = 0.11$, $p < 0.001$).

4.4 Discussion

Although it has been estimated that about 48% (Kornartit et al., 2010) to 69% (Lai et al., 2004; Klepeis et al., 2001) of the time spent indoors (~90%) is in the residential environment, the amount of time people spend in their homes among other types of enclosed spaces varies and depends on the lifestyle, occupation as well as age and health status of the occupants. Yet, the fact that people spend about 1/3rd of their lives in the sleep microenvironment (Boor et al., 2017) underlies the importance of the domestic environment and its significant contribution to the daily exposure to air pollutants, including bioaerosols, as well as the significance of assessing the risks associated with air quality and microbial exposure in the house environment.

This study aimed to investigate the bacterial aerosols in the residential environment using approaches that take into account both the time-resolved as well as particle-size resolved dynamics of bioaerosols in relation with the variability of the outdoor bioaerosols. At the same time, the current work aimed to explore the spatial variation of the aerosol bacterial composition while accounting for temporal variability by sampling in two different types of locations, including an urban and a semi-urban site. To best of our knowledge, this is the first study to present size-resolved profiles of the bacterial composition of bioaerosols in residential environments, in parallel with the outdoor airborne particles, as well as the first one to present seasonal profiles of size-segregated bacterial aerosols based on high throughput sequencing data.

The effect of seasonality

Results showed that the aerosol bacterial assemblages in the two residential flats investigated exhibited significant compositional variation among seasons, both indoors and outdoors. At the same time the airborne bacterial communities differed substantially between indoors and outdoors, indicating that the key determinants shaping the indoor and outdoor air bacterial microbiomes were different.

Long-term monitoring surveys of the outdoor air bacterial aerosols have been common and seasonal changes have been well described. Large variations in bacterial diversity among seasons have been observed by several sequencing-based investigations (e.g. Du et al., 2018; Zhen et al., 2017; Xu et al., 2017; Gandolfi et al., 2015; Tanaka et al., 2015; Barberán et al., 2014; Woo et al., 2013; Bowers er al., 2012; 2011; Franzetti et al., 2011; Fahlgren et al., 2010), whereas few studies concluded that the composition of the airborne bacterial

assemblages remained relatively steady throughout the year, despite changes in season (Genitsaris et al., 2017; Park et al., 2016). Longitudinal HTS-based studies of bioaerosols in the indoor environment have been less common (Emerson et al., 2017; Prussin et al., 2016; Gaüzère et al., 2014; Adams et al., 2014) and paired indoor and outdoor air samples have been rarely considered together (Shin et al., 2015; Adams et al., 2014; Meadow et al., 2014, Kembel et al., 2012). Indoor bioaerosols are a complex mixture of micro-organisms originating from both the various indoor sources and the outdoor air transferred to the inside via air exchange-induced supply, which is again dependent on the various sources located outdoors (e.g. vegetation, soil, water reservoirs etc.), and therefore when studying the indoor aerosol microbiome both indoor and outdoor bioaerosols should be taken into account (Meadow et al., 2014) and both indoor and outdoor conditions should be considered.

Results to date have pointed out that the seasonal dynamics of the outdoor airborne bacteria might not necessarily be reflected in the indoor bioaerosol microbiome. Gaüzère et al. (2014) explored the indoor air diversity in the Louvre museum during a 6-month campaign (Gaüzère et al., 2014) and results based on samples collected at the beginning (day 1) and towards the end of the campaign (days 157 and 164) revealed that the composition remained stable over time. Moreover, qPCR-based abundance throughout the campaign did not exhibit any significant variations. Prussin et al. (2016) studied the intra-annual variation of the airborne bacterial community in a mechanically ventilated day-care center by collecting samples from the settled dust recovered from the HVAC filter every two weeks for a year. However, they were not able to notice any substantial compositional variation, except for an unoccupied period of 13 days, and they suggested that the lack of any observed seasonality was the result of the strong impact of human occupancy on the indoor air microbiome. Another longitudinal study of the indoor air diversity was conducted in a university-housing complex based on one-month duration passive settling of bioaerosols in empty petri dishes, both indoors (flats) and outdoors (balcony), for summer and winter (Adams et al., 2014). In accordance with our results, heir findings showed that indoor and outdoor samples harboured distinct bacterial populations. Despite the outdoor air community variation observed, the indoor air bacterial composition varied only among residential units but not between seasons, which was mainly attributed to the dominance of human-associated bacteria indoors. It should be mentioned that the apartments in the housing complex had a forced-air ventilation system (Adams et al., 2013).

A few studies have examined the seasonal bioaerosol dynamics using the time-integrated sampling approach of collecting settled dust, a proxy for the airborne material. Rintala et al. (2008) examined the indoor bacterial diversity by taking swab samples from interior surfaces in two buildings over a period of a year and found that the variation occurring between the two buildings was more pronounced than the variation among seasons, with the most dominant taxa largely sourced from humans. The strong influence of the occupants living in the houses on the indoor settled dust bacteria has been previously demonstrated (Barberán et al., 2015; Lax et al., 2014; Dunn et al., 2013). Weikl et al. (2016) sampled dust (once) in multiple households in an urban area over the course of a year and observed that the main indoor bacterial community shifts occurred during the full spring (plant) phenological period. The authors noticed, though, that they were able to discover the particular association due to the large time-point number of samples collected.

In consistence with the aforementioned studies as well as most indoor air studies, a distinct human signal, which is the main factor causing the homogenising effect on bacterial bioaeorosols typically observed over time in enclosed spaces, was detected in the recovered bacterial composition for both sites in the present investigation. In particular, highly abundant human-associated taxa widespread across seasons in both residential flats included *Corynebacterium*, *Staphylococcus*, *Acinetobacter*, *Pseudomonas*, *Kocuria, Micrococcus* and *Kytococcus*, which are known to be part of the skin microbiome (Bouslimani et al, 2015; Grice & Segre, 2011; Costello et al., 2009; Szczerba, 2003; Roth & James, 1989; Kloos et al., 1974), *Streptococcus*, which is a member of the oral microbiota (Zaura et al., 2009), and genera such as *Lactobacillus*, *Anaerococcus*, *Lactococcus*, *Peptoniphillus*, *Ruminococcaceae*, *Ezakiella* and *Roseburia* which are members of the vaginal and gut microbiota (Diop et al., 2018; Yatsunenko et al., 2012; Ravel et al., 2011; Duncan et al., 2006). Moreover, the genus *Brevibacterium*, which was one of the dominant taxa detected in the semi-urban dwelling across all seasons studied and that typically exists in various different habitats, also includes one species which is human skin commensal (Collins et al., 2006). The strong contribution of the home residents as a source for bacteria was further supported by the fact that *Corynebacterium* remained the most abundant genus across all three seasons, with relative proportions ranging from 51% (winter) to 32% (summer) at the semi-urban flat and from 13% (winter) to 25% (spring) at the urban flat. On the other hand, it should be noted that even though the occurrence of the aforementioned genera in the indoor environment has been linked to the human presence (Adams et al., 2015; Gaüzère et al., 2014b; Meadow et al.,

2015; 2014; Hospodsky et al., 2012), many of those encompass species that are associated with various environmental sources. For instance, even though *Kocuria* could originate from human skin and found in indoor air due to skin shedding (Stackebrandt et al., 1995), several *Kocuria* spp. have also been found in environments such as soil, plants, aquatic habitats, cyanobacterial mats as well as foodstuff including meat and milk (Seo et al., 2009; Takarada et al., 2008; Tremonte et al., 2007; Reddy et al., 2003; Kovács et al., 1999). Another example is *Pseudomonas,* which is a widely distributed genus and can be found in all natural habitats, including terrestrial, freshwater and marine environments (Spiers et al., 2000). However, the examination of the compositional profiles of the indoor air broader taxonomic groups in parallel with the outdoor air profiles provided further evidence that most of the aforementioned taxa were associated with in-house sources. For instance, *Clostridia* (*Anaerococcus*, *Roseburia*, *Peptoniphillus*, *Ruminococcaceae*, *Ezakiella)* were found to be more enriched indoors compared to outdoors at the urban flat. The relative proportions of *Bacilli* (e.g. *Staphylococcus*, *Lactobacillus*, *Lactococcus*, *Streptococcus*) were higher indoors than outdoors during summer at the semi-urban house. *Gammaproteobacteria* (e.g. *Acinetobacter*, *Pseudomonas*) were also present at increased relative abundance in indoor air.

Despite the detection of various human commensal bacteria, one major parameter that contributed to the distinct seasonal differences observed for the bacterial composition of the indoor bioaerosols in the current study was the type of ventilation, which is one of the most important building characteristics driving the indoor microbiome. In our study both flats were naturally ventilated throughout the year with air entering the houses directly through open windows and short-term opening of windows even during wintertime. Previous surveys have demonstrated that in naturally ventilated buildings, the indoor air bacterial composition tends to resemble the outdoor air community composition when the occupants are absent whilst still maintaining a distinct human signal (Meadow et al., 2014). In contrast, the effect of outdoor air is less pronounced in mechanically ventilated spaces due to filtration which limits to an extend the influx of bioaerosols through ventilation. Kembel et al. (2012) compared naturally ventilated and mechanically ventilated rooms in the same building and found that the indoor air bacterial community composition was significantly different from the composition in outdoor air when the type of ventilation was mechanical, whereas window-ventilated rooms contained a combination of bacterial taxa found in both the mechanically ventilated rooms and in outdoor air. Kembel et al. (2014) also found that the ventilation type

was the most significant predictor of microbial community variation among offices within the same building.

Moreover, the effect of the ventilation mechanism on the recovered microbiome was evident from the fact that both indoor and outdoor air bacterial assemblages in both sampled flats in our study exhibited similar levels of bacterial diversity, in consistence with previous investigations conducted in environments where ventilation was provided naturally through windows (e.g. Shin et al., 2015; Meadow et al., 2014). On the other hand, bioaerosol surveys in mechanically ventilated buildings usually find higher bacterial OTU-richness outdoors than indoors (e.g. Adams et al., 2014) and indoor microbial communities in mechanically ventilated rooms have been reported to be less diverse compared to naturally ventilated spaces (Kembel et al., 2012).

As the dispersal of bacterial taxa brought into the flats from outdoors is expected to be higher, when natural ventilation rates are highest, the compositional dissimilarity between indoor and outdoor air bacteria was lower during spring and summer compared to winter that windows mostly remain shut. Thus, a pronounced increase of environmental bacteria in indoor air was observed during the warmer periods, especially during summer, which was particularly illustrated by the increased proportions of bacterial groups that were observed at their greatest relative abundance outdoors, such as *Alphaproteobacteria*, *Betaproteobacteria* and *Cytophagia.* A higher occurrence of members of *Alphaproteobacteria* and *Betaproteobacteria* towards summer reflecting the stronger influence of outdoor sources was also observed by Rintala et al. (2008). The highly abundant environmental bacteria associated with various outdoor sources that were detected during spring or summertime either in the urban or the semi-urban flat included taxa such as *Microbacteriaceae,* which can be found in terrestrial and aquatic habitats with several species associated with plant pathogenicity (Evtushenko & Takeuchi, 2006), such as *Rathayibacter* spp. (Dorofeeva et al., 2002; Zgurskaya et al., 1993), the plant-associated *Sphingomonas* (Shen et al., 2015; Kim et al., 1998), *Massilia*, which has been isolated from soil environments including riverbank soil and the rhizosphere (Embarcadero-Jiménez et al., 2016; Ofek et al., 2012; Wang et al., 2012; Weon et al., 2010; 2009; 2008; Zhang et al., 2006), *Skermanella,* which has been found in freshwater and desert soil (An et al., 2009; Weon et al., 2007; Sly & Stackebrandt, 1999), members of the widespread cyanobacterial group *GpI*, which are capable of living in both terrestrial and water-related places (Mur et al., 1999), *Arthrobacter*, which are common inhabitants of soil and rhizosphere (Zhang et al., 2012; Vaishampayan et al., 2007; Cacciari

& Lippi, 1987), *Hymenobacter*, which have been recovered from soil (Kim et al., 2008) and various aqueous sources such as lakes, rivers, estuaries, wetlands, seawater and snow (Ten et al., 2017; Kang et al., 2013; Joung et al., 2011; Baik et al., 2006; Hirsch et al., 1998) and *Pedobacter*, also related with soil and sediments (Zhou et al., 2012; Luo et al., 2010; Gordon et al., 2009; Roh et al., 2008). However, there were few non-human related genera which were among the most dominant taxa steadily throughout the year (e.g. *Paracoccus*). The particular bacteria were most likely related to local land sources and their presence could have been either associated with the natural ventilation or could have also been the result of indirect transfer via the occupants' footwear or clothes followed by subsequent resuspension.

It should be noted that the seasonal patterns observed at the two sites exhibited few differences. For the urban site, the higher compositional similarity observed outdoors between winter and spring was also reflected indoors, whereas the semi-urban site exhibited a more distinct profile outdoors for spring in relation to the other seasons, which resulted in summer and winter exhibiting more similar indoor bacterial communities. Moreover, the highest degree of similarity between indoor and outdoor aerosol bacterial assemblages was observed during summer for the urban site and during spring for the semi-urban site. This could be just the result of a stronger impact of in-house sources in the urban house, compared to the semi-urban site, in combination with reduced ventilation compared to summertime, that could have resulted in more homogeneous communities between winter and spring. However, the fact that a similar pattern was also observed outdoors for the two locations suggests that this might be the result of the different dates within each season that the sampling was conducted for each residence and possible within-season variability. For spring, the sampling at the urban flat took place between 11 and 16/05, whereas the sampling at the semi-urban flat was performed between 21 and 31/05. The within-season conditions appeared to be a lot more variable during the second sampling period (semi-urban) with a heavy rainfall (36.8 mm precipitation accumulation) occurring and outdoor air temperature ranging from 11.7 to 20.8^{o}C, compared to a more steady temperature profile (15 - 16.9^{o}C) observed during the first sampling period (urban). Possible within-season community variabilty could be also related to the local plant phenological periods, as noticed by Weikl et al. (2016), which could be easily overlooked due to limited time-point samples per season. Inter-day variability in community structure has been reported previously for outdoor air bacteria showing that they can change within few days (Polymenakou et al., 2013; Fierer et al., 2008). Emerson et al. (2017) studied the bioaerosol temporal variability within residential environments and they

found that shifts in indoor air bacterial communities could be as large at closer time points as they can be over longer periods and they suggested that multiple sample-collection time points are required for characterising the indoor air microbiome. In any case, caution should be taken when drawing conclusions on seasonality based on single time-point samples as one day might not necessarily represent accurately a whole season.

The influence of temporal variability

Despite season being a significant predictor of the bioaerosol microbial community structure in both homes studied in the current work, based on all sampling collection methods, different results were found when considering bacterial richness. The effect of season on bacterial diversity was found to be significant only for the urban site, based on active sampling methods, despite outdoor seasonal diversity being significant only at the semi-urban location. On the other hand, the richness variation among seasons was found to be substantial only at the semi-urban flat according to the passive collection method. This discrepancy in results across sample types was also reflected in the recovered bacterial abundance, as measured by qPCR of 16S rRNA gene. The season was found to be a significant factor for the aerosol bacterial abundance, both indoors and outdoors, only at the urban location, based on active sampling, whereas both houses exhibited strong seasonality based on the gravity settled air samples, with the effect being larger for the semi-urban flat, though. Given, that passive-collection samples represent the accumulation of particles over a whole month per season, and not just three individual 12-h daytime periods, suggests that inter-day variability in terms of diversity and abundance is high and might affect the outcome of a study if the goal is to provide an assessment of seasonality. Notwithstanding, establishing a clear seasonal pattern for concentrations of airborne bacteria is not always possible (Reponen et al., 1992) and it has been reported to be residence-dependent (Moschandreas et al., 2003).

Furthermore, the seasonal pattern was found to be different when examined based on 1-hour duration filter samples. Based on 12-hour active sampling, the urban house exhibited lower mean bacterial abundance in winter, both indoors and outdoors, compared to summer, whereas the semi-urban residence demonstrated higher average bacterial concentration in winter compared to summer, indoors but not outdoors. However, the indoor air average abundance estimated based on one-hour time integrated samples collected on 3 different time points per day was found to be higher in the urban flat during winter compared to summer (the outdoor bacterial load in winter was still lower than the one for summer). This result shows the dynamic and highly variable nature of bioaerosols and shows the strong impact

that the sampling strategy can have on the outcome of an investigation. The fact that bacterial abundance varies diurnally and can demonstrate high within-day temporal variation has been pointed out by several culture-based studies outdoors (Fang et al., 2007; Shaffer & Lighthart, 1997; Lighthart & Shaffer, 1995) and indoors (Zhu et al., 2003). The current study presented the diurnal bacterial concentration variation based on qPCR of 16S rRNA gene and results showed that the abundance varied substantially between different times of the day mainly in winter, for both houses, despite the outdoor bacterial concentration variability being significant only in one of the two locations (semi-urban), whereas in summer the intra-day variation was significant only at the site that the variation appeared to be also significant outdoors (i.e. at the urban flat).

At the same time, the bacterial abundance was monitored in parallel with the $PM_{2.5}$ mass concentration levels, both indoors and outdoors. Although microbial aerosols constitute part of the airborne particulate matter, PM levels are rarely included in bioaerosol studies (e.g. Gou et al., 2016; Gandolfi et al., 2015; Hospodsky et al., 2014; Qian et al., 2012). Moreover, the indoor-to-outdoor ratio (I/O) was estimated for both bacterial bioaerosols and $PM_{2.5.}$ The fine PM fraction was particularly chosen due to its significance in terms of human exposure and because the indoor PM_{10} mass was generally found to be dominated by $PM_{2.5}$ particles. Relationships between indoor and outdoor (non-biological) particles have been commonly studied (e.g. Hoek et al., 2008; Morawska et al., 2001; Jones et al., 2000; Monn et al., 1997; Li, 1994; Kim & Stock, 1986). However, indoor-to-outdoor ratios estimated based on qPCR data have been rarely reported (e.g. Clements et al., 2018, Deng et al., 2016; Sippula et al., 2013) as sampling and determination of indoor bioaeorosols is not always coupled with collection of outdoor bioaerosols. Elevation in indoor air concentrations compared with outdoors (i.e. I/O greater than 1) is an indication of the potential importance of in-house sources. Having documented the presence and household activities of inhabitants during the sampling periods it was evident that the diurnal variation pattern for both bacteria and $PM_{2.5}$ was strongly affected by human occupancy and associated with the typical activities taking place in the study houses. The lowest concentrations as well as lower I/O ratios were observed during the afternoon that was characterised by low activity level and most usually by complete absence of the occupants, whereas the highest levels and I/O ratios were most commonly occurring during the evening that was the busiest period of the day, with the occupants being present and activities such as meal preparation, cooking, sitting in the living room, watching television and conversing taking place. In addition, the bacterial abundance

I/O ratios were significantly greater during winter (median I/O = 2.55) compared to summer (median I/O = 0.67), when the outdoor bacterial concentrations were found to be significantly associated with the indoor levels. However, the $PM_{2.5}$ ratios did not exhibit and substantial difference between the two seasons (winter: 7.98, summer: 6.03). Despite the higher air exchange rates typically occurring during summer in naturally ventilated spaces which result in a less pronounced impact of indoor sources, as the indoor concentrations tend to track the outdoor levels more closely, the $PM_{2.5}$ I/O ratio still remained greater than 1. In addition, the lack of significant correlation between the bacterial and the $PM_{2.5}$ I/O ratios made evident that indoor bioaerosols and fine particles are affected by different sources. Although the presence of occupants mainly affects the bacterial levels in the form of direct emissions or through physical movements causing resuspension, as discussed previously, the main sources that were identified to lead to elevated $PM_{2.5}$ concentration levels, as indicated by the time-activity logs, were ordinary household activities such as cooking, toasting, smoking and use of personal care aerosol sprays, in accordance with previous findings (Hussein et al., 2006; He et al. 2004; Luoma & Batterman, 2001; Abt et al., 2000; Long et al., 2000; Thatcher & Layton, 1995; Owen et al., 1992; Kamens et al., 1991). The influence of the human presence on both biological and non-biological particles, even though in two different ways, was further supported by the significant positive association of both bacterial abundance and PM with the CO_2 levels, which was stronger during winter (the main source of CO_2 is human respiration).

Although, the $PM_{2.5}$ and PM_{10} mass concentrations were found to be strongly correlated with the indoor airborne bacterial levels based on the 1-hour samples, the particular associations were not observed based on the 12-hour daytime samples when compared with the 12-hour concurrently recorded PM median levels. This is probably related to the fact that a whole daytime sampling includes both occupied and vacant periods, as well as high-level and low-level activity periods and therefore the dynamics over short timescale samples cannot be captured by prolonged sampling. However the average bacterial I/O ratios estimated for winter and summer based on daily sampling were in agreement with those estimated based on short-term sampling (i.e. I/O >1 for winter and <1 for summer). Interestingly, when examining only the fine fraction of bacteria, as determined by daily size-resolved sampling, the average bacterial I/O ratios were greater than unity across all seasons, in accordance with the $PM_{2.5}$ I/O ratios, even though there was no correlation found with the $PM_{2.5}$ mass, suggesting that the fine bacterial bioaerosols are in fact associated with in-house sources.

Size-resolved bioaerosol dynamics

However the particle size distribution results demonstrated that the bacteria-laden particles were most abundant in the coarse fraction (> 2 μm), both indoors and outdoors, and they were dispersed across multiple sizes ranges including the range over 7 μm, which might explain the lack of strong correlations with the PM_{10} and $PM_{2.5}$ mass concentration. Our results are in agreement with previous findings based on culture-dependent and culture-independent methods, showing that for bacteria-associated particles the size distribution in the air is mostly not represented by the size ranges of their cells/spores and airborne bacteria tend to distribute into large-sized particles (Clauβ, 2015). Airborne bacteria occur mainly in the form of agglomerated colonies (i.e. aggregates) with surrounding matrix (Wittmaack et al., 2005; Lidwell et al., 1959) and the size of bacterial aerosols mostly depends on their sources as well as release mechanisms. For example, in environments with a high load of dust it is most likely that the micro-organisms are attached to dust particles (Gorny et al., 1999) and get into the airborne state by resuspension. Therefore, even though their small sizes do not justify their detection at the larger size fractions, their attachment on big particles is the main factor that drives their dissemination in the air. In the present study, indoor air concentrations peaked for bacteria associated with the particles in the size ranges 2 - 4 μm and 4 - 8 μm, based on the May impactor or 3.3 - 4.7 μm and 4.7 - 7 μm based on the Andersen impactor. Previous studies presenting size-resolved data of airborne particles carrying bacteria in indoor environments based on qPCR of 16S rRNA gene also reported similar findings. Sippula et al. (2013) measured the size distribution of bacterial aerosols in a family house in a suburban area in Finland using a modified version of the Harvard high-volume cascade impactor and found that the highest concentrations were measured in the coarse fraction ($PM_{2.5\text{-}10}$), both indoors and outdoors. Hospodsky et al. (2014) characterised the size-distributed bacterial concentrations in classrooms in four different countries based on sampling with a six-stage non-viable Andersen impactor and found the maximum levels at the 3.3 - 4.7 μm and 4.7 - 7 μm stages. Qian et al. (2012) studied the size-resolved bioaerosol concentrations in a university classroom (U.S.A) using an eight-stage non-viable impactor and observed that the bacterial abundance peaked on the stage corresponding to the 3.3 - 4.7 μm size range.

Although various bioaerosol investigations, mostly culture-based, have studied the size distribution of airborne micro-organisms, particle sizes over 12 μm or 20 μm have not been measured previously as most size-selective sampling instruments are not capable of

collecting particles > 20 µm (Clauβ, 2015). Particle size distribution for bioaerosols has been mostly studied with the Andersen six-stage viable impactor (Andersen, 1958), which is probably the most commonly used worldwide sampling system for size-selective collection of airborne micro-organisms and it has been mainly designed for the purposes of culture-based sampling covering the size spectrum from 0.65 µm up to >7 µm. Various culture-dependent studies have studied the size-resolved bacterial aerosols using the 6-stage system in various indoor environments (e.g. Brągoszewska et al., 2018; Li et al., 2015; Nasir et al., 2015; Dybwad et al., 2014; Xu & Yao, 2013; Gołofit-Szymczak & Górny, 2010; Kim & Kim, 2007; Meklin et al., 2002) and more specifically in residential environments (Balasubramanian et al., 2012; Nasir & Colbeck, 2012; 2010; Moschandreas et al., 2003; Górny, 1999). However in our investigation, instead of using nutrient plates, the impactor was modified by placing solid surfaces to support filters as collection substrates, instead of nutrient medium, in order to allow flexibility in downstream analysis using culture-independent methods. Various modifications of the specific sampling system reported in the past, such as spreading a mineral oil layer onto the agar-filled petri dishes (Xu et al., 2013), placing a membrane onto the agar (Chatterjee & Sigler, 2015) or using sterile water instead of an agar medium (Moschandreas et al., 1996), were mainly aimed for optimised recovery of culturable bioaerosols or for microscopy-based counting of both culturable and non-culturable bacteria.

Nonetheless, there have been limited reports of unique sampling systems with increased size range detection capability. For instance, Claub (2011) was able to detect bacteria in the particle size fraction 80 to 100 µm in exhaust air of animal houses, whereas a sampler which can determine biological aerosol particles with sizes up to 150 µm has been also employed in few outdoor air studies (Matthias-Maser & Jaenicke, 2000; 1995; 1994). In our study, apart from the modified version of the six-stage Andersen impactor, a seven-stage May impactor (May, 1975) which covers sizes from 0.5 µm to over 7 µm, including the size bins 8 - 16, 16 - 32 and > 32 µm, was deployed. The May impactor traps aerosols directly on microscopic slides and it has not been previously utilised for bioaerosol collection aimed for molecular analysis. The performance of the May sampler was evaluated in relation with the modified six-stage Andersen impactor and overall results based on the two impactors in the current study exhibited a relatively good agreement in terms of recovered abundance and composition. However, as the upper size limit for the two impactors is different, differences such as inflated geometric mean diameters estimated based on the May impactor as well as

non-identical compositional profiles are expected. Moreover, particle bounce in the impactors could also potentially bias the reported size distributions. Bacterial aerosols in the size ranges over 16 μm can still be inhaled through the mouth and nose and even though they may not have a direct critical impact on occupants' health in terms of lung deposition, it has to be considered that many of these big particles may be fragmented upon deposition (e.g. by desiccation and disintegration) and get re-aerosolised as smaller aerosols which might be respirable (i.e. capable of entering the deepest parts of the human respiratory system). Therefore the detection of large bioaerosols and the determination of their microbial composition is indeed important. To our knowledge, this is the first time that aerosol samples associated with the particular large size fractions are analysed with high throughput sequencing and therefore this study provides novel information on the microbiome of the large-sized particles.

Particle size distributions of airborne bacterial concentrations, determined for the first time for particles over 10 μm based on qPCR of 16S rRNA gene, allowed us to observe that the highest bacterial concentrations in outdoor air mostly occurred at the size fractions over 16 μm. The occurrence of bacteria aggregated or adhered to large particles in outdoor air has been reported to provide a protection to the micro-organisms from harsh environmental conditions (e.g. radiation) and therefore the geometric mean diameters of airborne bacteria tend to be higher during the warmer periods (Lighthart & Shaffer, 1997). In addition, the predominance of large-sized bacteria-ladden particles outdoors during spring and summer might be also related to the presence of pollen in the air (Matthias-Maser & Jaenicke, 1995), which is emitted in large quantities by plants during spring to autumn. Recent studies revealed that pollen grains are carriers of highly diverse bacterial communities (Ambika Manirajan et al., 2016; Obersteiner et al., 2016; Junker & Keller, 2015).

I/O ratios based on size-resolved qPCR-determined bacterial concentrations of the particular size spectrum were also reported indicating the size-dependent and season-related nature of the relationship between indoor and outdoor particles. The I/O rations being greater than unity for the size ranges that bacteria peaked indoors during winter (i.e. 2 - 4 μm for both sites) and spring (2 - 4 μm for the semi-urban and 4 - 8 μm for the urban site) indicated that the origin of those particles was most likely associated with indoor sources, whereas in summer the indoor sources for the predominant size range of bacterial aerosols (i.e. 4 - 8 μm for both sites) became less influential (I/O ratio close to unity) by the mixing with outdoor

air. Moreover, the season change affected all particle-size ranges as all ratios shifted towards values closer to or lower than unity during summertime. It should be noted, though, that the I/O ratios cannot always denote the origin of particles (Chen & Zhao, 2011) as airborne concentrations might appear elevated indoors due to resuspension of previously deposited material originating from outdoors. However, overall, the bacterial aerosols that appeared to be mostly associated with in-house sources and therefore less affected by the outdoor atmospheric changes were the fine ones, whereas the majority of large-sized bacteria-ladden particles were most likely of outdoor origin. It has been previously reported that large bioparticles in the atmosphere show strong seasonal variations due to their dependence on the plant phenology, whereas the abundance of smaller bioaerosol particles (< 2 um) exhibit minimal variation (Matthias-Maser & Jaenicke, 2000; 1995).

The particular observation might explain the higher compositional similarity observed among the coarse particles compared to the fine ones during each season. Although the season was found to be, overall, a strong predictor of the community structure of the airborne bacterial assemblages indoors (and outdoors) in both houses, the community composition variation was found to be even more significant for the coarse particles. Unlike mechanically ventilated spaces, naturally ventilated buildings cannot limit the influx of outdoor coarse particles and considering that the majority of the outdoor-originating bacterial aerosols were generally dominated by the larger-sized particles, suggests that the coarse particles found indoors carry a more distinct seasonal signal which gets more pronounced during warmer periods due to the more intense window-opening ventilation. Overall, the particle size was not found to be a strong predictor of the bacterial community structure. Bacterial taxa identified were dispersed across particles of various different sizes with no specific size-related pattern observed and even though compositional differences were found between the two examined residential flats, these were not associated with the particle size.

The effect of urbanisation

Results based on quantitative (abundance-based) measure of bacterial community dissimilarity showed that the sampling site location was not a strong predictor of the bacterial community structure outdoors. However, the indoor aerosol bacterial composition varied significantly by residential unit. The fact that the airborne microbiota found in the outdoor air of the two houses did not exhibit location-specific bacterial composition is most likely related to the fact that the spatial scale was not large enough to provide a biogeographic pattern.

Brodie et al. (2007) examined the outdoor aerosol bacterial composition in two geographically proximate U.S cities (~130 km apart) and their results also showed that the effect of the site location on the bacterial community variability was weaker than the effect of meteorological/temporal parameters. Chase et al. (2016) showed that the bacterial communities in offices differed more across different cities than between offices in the same city, suggesting that the location was an important factor for the indoor bacterial community composition. However, the urban locations of the offices were in three different climatically geographic regions, in contrast to our study that the distance between the two samplings sites was only 80 km.

However, as the two house locations in the present study were characterised by different levels of urbanisation, our main objective was to investigate the effect of the environment type on the bioaerosol bacterial composition. Various studies have explored the bioaerosol dynamics in the near-surface atmosphere across different land-use types (Bowers et al., 2011b; Kaarakainen et al., 2008; Després et al. 2007; Boreson et al., 2004; Shaffer & Lighthart, 1997; Matthias-Maser & Jaenicke, 1995) and have demonstrated that the microbial concentrations and composition vary with the type of environment. Bowers et al. (2011b) compared aerosol samples collected from agricultural fields, suburban areas and forests and were able to establish that the land-use type is a key determinant of the airborne bacterial communities in outdoor air. In contrast, Woo et al. (2013) did not find any significant differences among the airborne bacterial assemblages obtained from roadside-urban, urban, sub-urban or rural locations. Rather, similar to the current work, they found that the seasonal factor was a greater driver of the bacterial composition. Moreover, Barberán et al. (2015b) compared urban and rural areas based on continental-scale settled dust data and concluded that people living in more rural areas are not exposed to bacteria derived from different source environments than those living in more urbanized areas. Apart from the geographic factor, one possible reason for the lack of distinct airborne bacterial communities between the two sites in the present investigation could be the similar environmental characteristics between the two regions. Colchester ("semi-urban") is an urban settlement surrounded by many countryside areas in the predominantly rural Essex, where 72% of land area is mainly used for agriculture. However, Stratford ("urban") is a district of London, which is one of the greenest cities globally, with almost 47% of its land being vegetated public or private green space (http://www.gigl.org.uk/keyfigures/). It has been demonstrated that the urban greenness is a significant predictor of the aerosol bacterial composition (Mhuireach et al., 2016).

Furthermore, it is also possible that long-range transport of bacteria occurring in the atmosphrere could have potentially masked a location-specific composition of the near-surface aerosol microbiome.

Nevertheless, it has to be considered that microbial geographic patterns observed outdoors are not necessarily reflected indoors. Barberán et al. (2015b) studied the regional differences of airborne microbial communities on a large spatial scale based on settled dust collected from the external door trim of households across the United States and they revealed that the bacterial composition exhibited geographic patterns. On the contrary, when they examined the indoor settled dust composition in the same houses they found that the bacterial communities, which were significantly distinct from those outdoors, did not vary by location and they were more influenced by the home occupants (Barberán et al., 2015). Similarly, in our study the airborne bacterial communities for both sites differed substantially between indoors and outdoors, as already discussed. Considering that according to the study design implemented, the two flats chosen to be sampled were as similar to each other as possible in terms of various parameters known to structure the indoor microbiome including house features such as building age, ventilation system, occupancy patterns, female:male ratio as well as lack of pets (Barberán, et al., 2015; Dunn et al., 2013; Kembel et al., 2012; Fujimura et al., 2010), and main environmental characteristics, such as climate, as well the external environment in the immediate vicinity of the housing buildings, suggests that the factors contributing to the variation in aerosol microbiomes between the two residential environments are driven by in-house sources.

Previous studies have demonstrated that humans have personalised microbiomes (Califf et al., 2014) and therefore people leave a distinct microbial signal in the environments they occupy on both surfaces and in the surrounding indoor air, which can be used to track the identity of the occupants (Meadow et al., 2015; Fierer et al., 2010). In fact, many of the bacterial taxa which were identified as differentially abundant either in the urban or the semi-urban house were human-associated. *Acinetobacter*, *Lactobacillus* and members of *Clostridia*, *Roseburia*, and *Peptoniphilus*, were significantly enriched in the urban flat, whereas *Staphylococcus* and *Kocuria* were mostly associated with the semi-urban flat. The alphaproteobacterial genus *Roseomonas*, which was also an indicator taxon of the bacterial aerosols in the semi-urban flat, apart from encompassing species widely distributed in nature (soil, aquatic environments etc.) (Kim et al., 2013; Zhang et al., 2008; Yoo et al., 2008; Yoon

et al., 2007; Gallego et al., 2006; Jiang et al., 2006), it also includes few species which are human-associated (Romano-Bertrand et al., 2016; Rihs et al., 1993). Moreover, some other key bacterial genera that differentiated the two houses could also originate from common foodstuffs found in a house. In particular, bacteria found significantly abundant at the semi-urban flat included *Brevibacterium*, which exists in various different habitats, including those characterised by high salt concentration (e.g. cheese) (Collins, 2006) and *Brachybacterium*, which apart from being associated with various environments, such as lake sediments, soil, faeces it can also be found in foodstuffs (Singh et al., 2016; Hoang et al., 2014; Liu et al., 2014; Park et al., 2011; Schubert et al., 1996; Collins et al., 1988).

Apart from the human-associated taxa, it is also possible that local-specific environmental bacteria carried inside the houses by the occupants (Parajuli et al. 2018) and not through the incoming outdoor air could have contributed to the differentiation of the two indoor microbiomes. Also, it has to be considered that in densely populated areas highly dependent on public transport, such as London, the attachment of bacteria from environmental litter of various land-use types and sources on the footwear might be the result of indirect contact through the interaction of people with the numerous citizens rather than the result of direct contact with the source. This outdoors-originating material could then be subsequently resuspended in the air of the residential environment. The main indoor air compositional differences between the two houses, which were more clearly represented by the non size-resolved passively collected samples, were the larger proportions of Proteobacteria (*Gammaproteobacteria)*, Firmicutes (*Clostridia*) as well as Bacteroidetes across all seasons at the urban flat as opposed to the increased presence of Actinobacteria (*Actinobacteria* class) in the semi-urban house. These broad taxonomic groups include several environmental taxa, mostly related with soil and vegetation, which are some of the main sources of airborne bacteria. *Actinobacteria* are dominant taxa in surface soils and their increased presence in less urbanised areas compared to predominantly urban settings has been previously reported (Parajuli et al., 2018; Bowers et al., 2011b). On the other hand, the increased abundance of *Gammaproteobacteria* in the urban site is similar to earlier observations for urbanised locations (Parajuli et al., 2018; Genitsaris et al., 2017). Moreover, Bowers et al. (2011) presented evidence that the occurrence of members of Bacteroidetes and *Clostridia,* which are common mammalian gut-associated bacteria, in outdoor air samples in cities can be associated with dog faecal material. In addition, several taxa identified in both houses, belonging to various taxonomic groups (e.g. *Alphaproteobacteria*, *Betaproteobacteria*), have

been detected in river water and freshwater systems in general (e.g. Jordaan & Bezuidenhout, 2016; Boden et al., 2008). Since the study flats were both situated in riverside neighbourhoods with the blocks directly facing the riverside, it is possible that the origin of some bacteria could be related to the local rivers. Clearly, further investigation including sampling of source environments would be required to evaluate possible links between potential sources and the detected bacterial aerosols.

Interestingly, the bacterial diversity (how many types of bacteria are present) was consistently found higher both indoors and outdoors at the urban location compared to the semi-urban site suggesting that the location was in fact a driver of richness for the bacterial aerosols. Although it has been reported that more urbanised areas harbour generally less diverse microbiota compared to less urbanised/rural areas (Parajuli et al., 2018; Després et al. 2007) due to lower natural biodiversity which has been linked to reduced exposure of humans to beneficial microbes (Hanski et al., 2012; Heederik & von Mutius, 2012;; Von Hertzen & Haahtela, 2006), other studies have shown that urbanisation does not have any effect on the microbial diversity levels (Mhuireach et al., 2016; Barberán et al., 2015b; Bowers et al., 2011). A higher microbial richness in urbanised areas could be also related to the higher biodiversity in the urban landscape due to non-native species, such as plants used for ornamental purposes (Kowarik, 2011; Smith et al., 2006).

Comparing active and passive sampling methods

Apart from the influence of the temporal variability of bioaerosol concentrations which was reflected in the aerosol bacterial abundance levels found with the different sample collection strategies, the effect of the sampling approach was further investigated in terms of recovered aerosol bacterial composition. Even though both active and passive collection methods exhibited similar results, the microbial community composition recovered from 12-hour daytime impaction-based samples was more variable compared to the composition recovered by the whole-month passive collection on suspended empty petri dishes. The particular findings are in agreement with previous work comparing active and passive sampling methods. Mhuireach et al. (2016) compared bacterial aerosol samples collected outdoors with passive settling dishes and filter-based samplers in terms of composition and concluded that both methods give comparable results. Emerson et al. (2017) demonstrated that the composition of airborne bacteria collected by 24-hour filter-based active sampling exhibited more heterogeneity compared to settled dust samples collected from the door trim and the

HVAC filters. Moreover, the present study also revealed that the effect size of seasonality on the community structure was even larger based on the passive-collection samples.

Passive sampling is a convenient, cost-effective and non-intrusive collection method which is based on gravitational setting of particles and does not require the use of any mechanical equipment. It is worth noting that one of the major difficulties in the particular study was the simultaneous and prolonged use (including occupancy periods) of several pumps, characterised by high levels of noise, for the purposes of active sampling. The main advantage of passive sampling is that it can be used over extended periods of time and therefore the samples represent what has been in the air averaged over a long period of time, overcoming the issues associated with the variable nature of short-timescale samples. It has to be taken into account, though, that passive sampling is considered a semi-quantitative method as the collected sample cannot be expressed in volumetric units (m^3) of air. Several HTS-based studies have adopted the particular sampling approach of suspended petri dishes, for periods between 1 to 2.5 months, as it is more indicative of the actual airborne microbial material compared to settle-dust samples typically collected from surfaces at lower heights (Luongo et al., 2017; Emerson et al., 2015; Adams et al., 2015; 2014; 2013). Adams et al. (2014; 2013) also placed passive collectors in the balcony for whole-month parallel sampling of outdoor air. However, in the present study it was not possible to place a passive sampler outdoors for the extended period of one month due to the rainy/windy conditions that could have resulted in re-aerosolisation of the settled particles.

Limitations

Although the two residences selected for the current study were as similar as possible in terms of building characteristics as well as outdoor environmental characteristics in the vicinity of the buildings, the two flats were at different floors. Several studies have investigated the effect of height on the airborne particulate matter levels of the outdoor air by conducting height-resolved measurements at the immediate proximity of roadside buildings and have found that the concentrations of PM_{10} (Zhang et al., 2011; Wu et al., 2002; Chan & Kwok, 2000; Lee et al., 1999; Chen & Mao, 1998; Rubino et al., 1998), $PM_{2.5}$ (Quang et al., 2012; Wu et al., 2002; Chan & Kwok, 2000), PM_1 (Wu et al., 2002) and submicron particles (Väkevä et al., 1999) decrease, as distance above ground level increases as a consequence of the bigger distance from the street-level emitting sources (mainly traffic-related) as well as due to dilution and dispersion, even though no differences are found after a certain level, e.g.

between the 7th and 14th floor (Chen & Mao, 1998). In addition, Mendell et al. (2008) found associations between lower-level outdoor air intakes and increased occurrence of pollution-related health symptoms. On the other hand, few studies have exhibited contradictory results. For instance, Pakkanen et al. (2003) found no difference in the PM_1 average concentrations between 3.5 and 20m heights. However, the concentrations for particles larger than 4 μm were, in fact, higher at the street level. Moreover, Kalaiarasan et al. (2009) measured higher $PM_{2.5}$ levels at the balconies of the building's midfloors, compared to lower and upper floors. Although the two investigated flats in the present study were in different buildings/regions and therefore various parameters could have potentially contributed to differences in the outdoor aerosol concentrations between the two sites, the lack of significant differences during most of the year between the two sites do not support the hypothesis that the different height might have biased the results reported in the current work. In fact the only substantial difference, which was found during winter, was due to higher outdoor $PM_{2.5}$ levels at the urban site (6th floor) and not at the semi-urban flat (2nd floor).

In terms of bacterial abundance, literature information on the vertical distribution of bioaerosols at various heights is in fact limited. Li et al. (2010) found that the outdoor levels of culturable bacteria and fungi were significantly higher at the ground level compared to those obtained at a height of 238 m. Even though viable micro-organisms have been detected even at altitudes of 20,000 m in Earth's upper atmosphere (Griffin, 2004), viability and culturability are expected to be affected by the height due to various environmental stresses. However, when qPCR was applied, both ground and high-level (238 m) aerosol samples demonstrated similar levels of total bacterial concentrations. In addition, DGGE analysis exhibited similar bacterial diversity for both heights. Maki et al. (2008) also observed a similar composition in bioaerosol samples collected at 10 and 800 m. Lee & Jo (2006) examined the viable bacterial concentrations at residential buildings, indoors and outdoors, and found lower levels at the high-floor flats (between 10th and 15th floor) compared to the low-floor apartments (1st or 2nd) only outdoors. No significant differences were found in the interior of the flats between the different floors. It is worth noting that the indoor bacterial levels were in fact substantially higher than outdoors. In our study the outdoor air 16S rRNA gene abundance varied by season, and even though during winter the semi-urban site (2nd floor) exhibited higher levels compared to the urban site (6th floor), during summer the urban site exhibited the highest concentrations (based on active sampling with the May impactor). Furthermore, in our study the outdoor air composition was found to be similar in both sites

and therefore the different floor of the study houses should not be considered as a limiting factor for the comparison performed.

4.5 Conclusions

The present study aimed to investigate the seasonal size-resolved dynamics of the airborne bacterial assemblages in two naturally ventilated residential environments located in two areas characterised by different levels of urbanisation. Results showed that the indoor and outdoor aerosols harboured distinct bacterial populations, due to the dominance of human-associated taxa in indoor air, with the season being a strong predictor of the aerosol bacterial community structure both indoors and outdoors. Particle-size distribution analysis revealed that seasonality was mostly driven by the coarse particles. However, the particle size was not found to be a determinant of the bacterial composition variability. Although the outdoor air bacterial composition was not found to vary substantially by the location/type of environment, the indoor air bacterial composition was found to vary significantly by the residential unit, indicating that the differences between the two houses are driven by indoor occupancy-associated sources. In terms of abundance, overall results showed that bacterial concentrations exhibited high temporal variability and therefore short-term samples might not be able to provide a representative assessment of the airborne microbial load. Last, both active and passive sampling methods employed in the present investigation provided comparable results in terms of bacterial composition, with the community composition recovered by the passive collectors exhibiting less variation compared to the impaction-based sampling.

Chapter 5

Bioaresols in the transportation environment: Bioaerosols at an Athens Metro subway station

5.1 Introduction

"Metro", also known as subway, underground, rapid transit, tube or heavy rail, is an electric railway system, operating mainly or partially in tunnel passageways, used for public transport in cities worldwide. The metro system is used extensively by commuters in urban areas and it constitutes a unique, confined and typically heavily occupied type of public indoor micro-environment that many people spend considerable amount of their time in on a daily basis.

The assessment of indoor air quality and the exposure of commuters to air pollution in the metro system have drawn the attention of many researchers, globally, due to its public health importance (Moreno et al., 2018; Xu & Hao, 2017; Nyström et al., 2010; Nieuwenhuijsen et al., 2007). The focus of the majority of scientific articles has been on particulate matter (PM) exposure, investigating concentration levels as well as the aerosol chemical composition, on station platforms and inside trains. Even though airborne particles have been identified as the major metro air pollutant, only few studies have examined the biological fraction of the PM, the so-called- bioaerosols, in subway networks (Table 5.1). Most of the microbial investigations have used culture-based approaches, i.e. traditional plate counts and identification of micro-organisms based on their morphological characteristics for elucidation of airborne bacteria and fungi and thus, there is limited information on the metro air microbiota.

Despite the advent of molecular techniques and the increasing application of high throughput sequencing (HTS) on the built environment microbiome, only a few studies have utilised molecular approaches for characterising the aerosol microbial diversity in metropolitan underground transport networks (Dybwad et al., 2017; 2014; 2012; Zhou & Wang, 2013; Birenzvige et al., 2003), with only three studies to date having applied HTS methods, mainly for characterising the bacterial composition. In particular, Robertson et al. (2013) conducted the first extensive investigation of the bacterial bioaerosols in the New York subway using 454 pyrosequencing of the 16S rRNA gene. Leung et al. (2014) determined the bacterial air microbiome within the metro network of Hong Kong utilising the Illumina MiSeq sequencing technology targeting the 16S rRNA gene, whereas in 2017, Triadó-Margarit et al. unravelled

Table 5.1 Bioaerosol studies in various worldwide metro systems.

City	Reference	Sampling site	Target micro-organism	Bioaerosol characterisation method
Budapest	Szam et al. (1980)	Station platforms	Bacteria	Culture-based
	Szam et al. (1983)	Station platforms	Bacteria	Culture-based
Tashkent	Zhdanova et al. (1994)	Station platforms	Fungi	Culture-based
London	Gilleberg et al. (1998)	Station platforms	Bacteria & fungi	Culture-based
Milan	Picco & Rodolfi (2000)	Station platforms	Fungi	Culture-based
Cairo	Awad (2002)	Station platform	Bacteria & fungi	Culture-based
Washington D.C.	Birenzvige et al. (2003)	Station platform	Total bioaerosols & bacteria	UV fluorescence-based detection Culture-based, Flow cytometry, Epifluorescence microscopy, Cloning & sequencing of 16S rRNA gene
Tokyo	Seino et al. (2005)	Concourse conne-cting two stations	Bacteria	Culture-based
	Kawasaki et al. (2010)	Station platforms	Fungi	Culture-based
Seoul	Cho et al. (2006)	Station platforms	Fungi	Culture-based
	Hwang et al. (2010)	Station platforms	Bacteria	Culture-bas
	Kim et al. (2011)	Station platform & train	Bacteria & fungi	Culture-based
	Hwang & Park (2014)	Station platforms	Bacteria	Culture-based
	Hwang & Cho (2016)	Station platforms	Fungi	Culture-based
	Hwang et al.(2016a)	Station platforms	Fungi	Culture-based
	Hwang et al. (2016b)	Station platforms	Bacteria	Culture-based
	Heo & Lee (2016)	Station platforms	Bacteria & fungi	Culture-based
St. Petersburg	Bogomolova & Kirtsideli (2009)	Station platforms	Fungi & bacteria	Culture-based
Beijing	Dong & Yao (2010)	Station platform	Bacteria & fungi	Culture-based
	Fan et al. (2017)	Station platforms	Bacteria & fungi	UV fluorescence-based, Culture-based, 454 pyrosequencing of 16S rRNA gene & ITS region from isolates
Tehran	Naddafi et al. (2011)	Station platforms & trains	Bacteria	Culture-based
	Hoseini et al. (2013)	Station platforms & trains	Fungi	Culture-based
Shanghai	Zhou & Wang (2012)	Station platforms & trains	Bacteria & fungi	Culture-based
	Zhou & Wang (2013)	Station platforms	*Staphylococcus* bacteria	Culture-based & Sanger sequencing of 16S rRNA, mecA and qac genes
Oslo	Dybwad et al. (2012)	Station platform	Bacteria	Culture-based, MALDI-TOF MS & 16S rRNA gene sequencing from isolates
	Dybwad et al. (2014)	Station platform	Bacteria	Culture-based & MALDI-TOF MS
New York	Robertson et al. (2013)	Station platforms	Bacteria, archaea & Eukarya	DAPI counts, 454 pyrosequencing of 16S rRNA gene & Sanger universal
Mexico City	Hernández-Castillo et al. (2014)	Station platforms & trains	Bacteria & fungi	Culture-based
Hong-Kong	Leung et al. (2014)	Station platforms & trains	Bacteria	Illumina Miseq of 16S rRNA gene
Prague	Dybwad et al. (2017)	Station platforms	Bacteria	Culture-based, MALDI-TOF MS & 16S rRNA gene sequencing from isolates
Barcelona	Triadó-Margarit et al. (2017)	Station platforms & trains	Bacteria, *A. fumigatus* influenza A & B, and rhinoviruses	454 pyrosequencing of 16S rRNA gene & quantitative PCR

the bacterial diversity in the subway system of Barcelona based on 454 pyrosequencing of 16S rRNA gene. Such studies have expanded out view of the so far known -from the cultivation-dependent literature- microbial diversity and have shown that subway air microbial assemblages are mainly composed by human commensal bacteria (e.g. skin-associated) and outdoor-air originating micro-organisms (e.g. soil-dwelling bacteria). There is now increasing interest on characterising the microbiome in the underground systems as more surveys, part of a bigger international effort involving researchers from big cities around the world, are being published (MetaSUB International Consortium, 2016). Studies have already been conducted in Boston and New York (Hsu et al., 2016; Afshinnekoo et al., 2015), while investigations are still ongoing in Paris and Stockholm transit systems (www.metasub.org). However, the microbial profiling from the metro transportation systems in the particular surveys is based on samples collected from subway surfaces and air sampling is not included in their study design.

In this study we aimed to explore the microbial aerosols in the Athens underground railway system (Athens Metro). The Athens transit network (Fig. 5.1) consists of two underground lines (Lines 2 and 3) and one almost entirely overground railway line (Line 1). Although the overground section (Line 1, 25.6 km long with 24 stations) was electrified in 1904, the subway part of the network started operating in 2000 and it is among the most modern systems in Europe. The two underground lines (Lines 2 and 3) are currently 59.7 km long with 41 stations (including 20.7 km and 4 stations in common use with the Suburban Railway line) and it is estimated that they serve approximately 938,000 passengers on a daily basis (www.ametro.gr). The subway network is being continuously expanded as more stations and lines are under construction within the Athens Metro development plan.

To our knowledge, investigations on the air quality of the Athens Metro have been limited, mainly focusing on the physicochemical characterisation of the particulate matter and the microclimatic environmental conditions at platforms as well as in the interior of train cabins (Mendes et al., 2018; Assimakopoulos & Katavoutas, 2017; Mammi-Galani et al., 2017; Martins et al., 2017; 2016; Barmparesos et al., 2016; Katavoutas et al., 2016; Assimakopoulos et al., 2013). So far, PM measurements at several stations have shown that the particle mass concentrations in the Athens subway environment are relatively elevated (Mammi-Galani et al., 2017) and considerably higher compared to outdoors (Martins et al., 2017; 2016, Barmparesos et al., 2016), while the most enriched chemical element is iron (Fe)

originating from the abrasion and wear of train wheels, rails and brakes (Martins et al., 2017; 2016). The particular findings are in line with many other studies conducted in various metro systems worldwide (e.g. Wang et al., 2016; Carteni et al., 2015; Martins et al., 2015; Perrino et al., 2015; Mugica-Álvarez et al., 2012; Murruni et al., 2009; Aarnio et al., 2005; Johansson & Johansson, 2003, Adams et al., 2001).

Figure 5.1 Athens Metro network map.

Here, we provide the first biological characterisation of PM_{10} in the Athens underground system by determining the bacterial and fungal composition using high throughput sequencing techniques. Moreover, this is the first attempt to describe the fungal air microbiome in a metro environment based on internal transcriber region (ITS) amplicon sequencing.

5.2 Methods

5.2.1 Sampling

Air sampling for the purposes of bioaerosol analysis was performed during the Athens Metro aerosol measurement campaign (April-May 2014), in collaboration with N.C.S.R. “Demokritos”.

Sampling site

Sampling was conducted at the side platform (part of the paid zone) of the underground station “Nomismatokopio”. The station, which functions as a hub to allow passengers to board and disembark from trains in Athens Metro Line 3 (Blue Line), is situated at the northeastern part of Athens and commenced operating in September 2009. The dual-side platform station is located at 20 m below ground, underneath a highly trafficked road (see image of the surrounding area in Appendix), and it is mainly naturally ventilated (for illustration and a detailed description of the station see Mendes et al., 2018). The sampling site is marked on the Athens Metro Blue Line (3) map (Fig. 5.2). Trains run from 05:30 until 00:30 every day, except Friday and Saturday when trains run until 02:30, with a frequency between 4 and 15 min, depending on the day (weekday or weekend) and time of day (peak or off-peak hours).

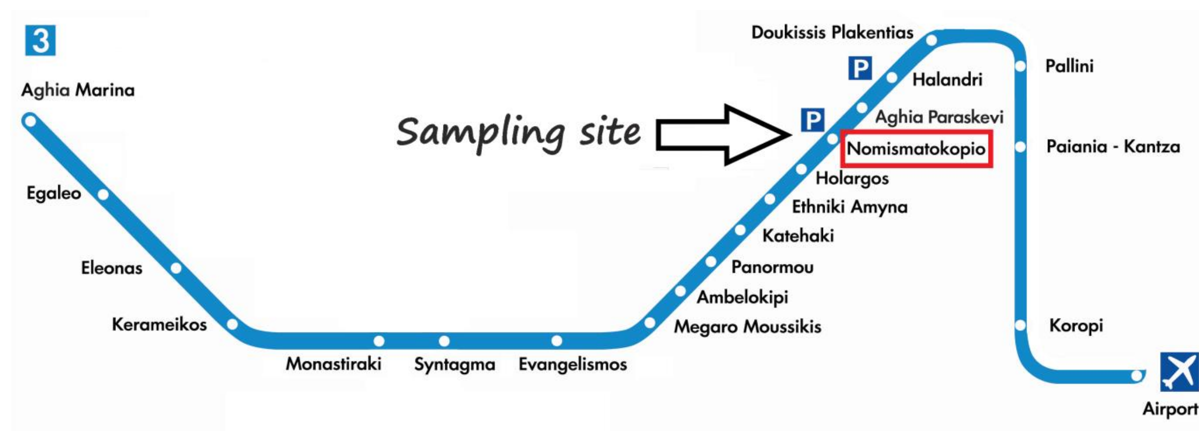


Figure 5.2 Athens Metro (Blue) Line 3 map. Sampling location (Nomismatokopio station) indicated by arrow.

Sample collection for bioaerosols analysis

Six samples of the PM_{10} size fraction were collected on 47-mm diameter PTFE filters (0.45-μm pore size, PALL Life Sciences, USA) using a low-volume gravimetric sampler (ENCO PM, TCR TECORA, Italy). The sampler was equipped with PM_{10} inlet, meeting the EN 12341 standard, and operated at 38 L/min. The instrument was placed in a central position on the platform, inside a metal cage with gridded walls. Sampling was carried out on weekdays and weekends during day-time (07:00 - 00:00) and night-time (00:00 - 05:30) on weekdays and weekends. Sampling dates and times are listed in Table 5.2. Prior to sampling, the sampler was sterilised with 70% (v/v) ethanol. After sampling, filters were placed in sterile petri-slides, sealed with parafilm and stored at -20°C. After the end of the campaign, samples were shipped in a cool box to the University of Essex (UK), where they were stored at -20°C until further analysis. Two unexposed filters (field blanks) were also analysed alongside those used for sampling to provide procedural controls. Environmental conditions (temperature and relative humidity) recorded during sampling are also shown in Table 5.2.

Table 5.2 Sampling details.

Sample identity	Sampling date (Start time)	Sampling duration	Mean Temperature	Mean Relative humidity
#1 - Weekday A	Tuesday 29/4/2014 (07:00)	16:57:40	23.6°C	38.0%
#2 - Weeknight	Thursday 1/5/2014 (00:00)	05:28:37	24.8°C	36.6%
#3 - Weekend day	Saturday 3/5/2014 (07:00)	16:57:41	23.6°C	45.5%
#4 - Weekday B	Wednesday 7/5/2014 (07:00)	16:57:40	24.2°C	30.7%
#5 - Weekend night	Saturday 10/5/2014 (00:00)	05:28:37	23.6°C	33.4%
#6 - Weekday C	Tuesday 13/5/2014 (07:00)	16:57:40	26.3°C	37.2%

The outdoor meteorological parameters during the sampling period (see Mendes et al., 2018) were typical for sping at this location, with daily average temperature varying between 16.2°C and 21.8 °C and daily average relative humidity ranging from 51.1% to 76.6%.

5.2.2 Sample processing

DNA extraction

Filters were aseptically inserted into sterile 5ml screw-cap tubes (Axygen Scientific Inc., USA) using autoclaved forceps by rolling the filter into a cylinder with the top side facing inward. Cells were lysed by bead beating with 0.75 g of sterile 0.1-mm zirconium/silica beads (Biospec, Bartlesville, OK, USA) in 1 ml extraction buffer [10mM Tris-HCl pH 8, 25mM Na_2EDTA pH 8, 1% (v/v) sodium dodecyl sulfate (SDS), 100mM NaCl and molecular

biology grade water] at Vortex Genie with a MOBIO Vortex Adapter at maximum speed for 10 minutes. For complete cell wall disruption, filter samples were incubated in a 70°C water bath for thirty minutes. The cell lysate was washed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) pH 8.0. The supernatant was isolated after centrifuging at 2,000×g for 5 minutes in a swing bucket centrifuge (Heraeus Megafuge 40, Thermo Fisher Scientific). This purification step was repeated twice to eliminate residual impurities. The nucleic acids were precipitated using equal volume of isopropanol and 2.5 µl of coprecipitant glycogen (20 mg/ml, Thermo Scientific) and incubated for 1 hour at room temperature. Samples were then centrifuged at 11,337×g for 25 mins in microcentrifuge (Minispin, Eppendorf), the supernatant was removed and the pelleted DNA was washed with 70% (v/v) ice-cold ethanol, air-dried at room temperature and resuspended in 35 µl sterile water. An extra ethanol precipitation step (2.5 volumes of absolute ethanol, 1/10 volume of 3M sodium acetate, pH 5.2) was included in order to further clean-up the DNA.

16S rRNA qPCR

Quantitative PCR was performed in triplicate using a CFX96 Real-Time System/C1000 Thermal cycler (BioRad, USA). For 16S rRNA gene quantification a SYBRgreen assay with universal bacterial primers Bakt_341F and Bakt_805R (Herlemann et al., 2011) was used. A standard curve was generated by serially diluting bacterial genomic DNA extracted from *Escherichia coli* K-12. Standards ranged from 1.9 x 10^7 to 1.9 x 10^2 16S rRNA gene copies per µl. The 16S rRNA gene copy number per µl was determined based on the measured DNA concentration and the average molecular weight of a base pair in double-stranded DNA (660 Da), along with taking into account the genome size of *E. coli* strain 4.6 Mbp (Blattner et al., 1997) and the number of 16S rRNA gene copies per genome (7) (Klappenbach, et al., 2001). Real-time PCR reaction mixture and cycling conditions are as described in Chapter 4. The reaction efficiency of the standard curve was 97.2% (slope = 3.39), with the correlation coefficient R^2 being 0.991, and the y-intercept value was 37.425. The Ct values obtained for the non-template controls were > 36. The resulting Ct values for the two field blanks (mean Ct = 31.9) was more than 3.3 cycles lower than the Ct of the NTC and therefore the maximum equivalent concentration from the two blanks (341 16S rRNA gene copies/µl) was subtracted from the sample concentrations.

Illumina MiSeq

Library preparation for Illumina MiSeq sequencing was performed as described previously, except the ITS1 region of the internal transcribed spacer region was targeted for amplicon sequencing for fungi using the fungal-specific primers ITS1F (Gardes & Bruns, 1993) and ITS2 (White et al., 1990) with Illumina overhang adapter sequences added (Appendix S5). Cycling conditions for "Amplicon PCR" (PCR1) of ITS1 region were: 3 min at 95°C, 30 times 30 s at 95°C followed by 30 s at 55°C, then 30 s for 16S rRNA primers and 1 min for ITS1 primers at 72°C, and finally 10 min at 72°C.

16S rRNA gene and ITS1 sequencing was performed on Illumina MiSeq platform with v3-chemistry and 20% PhiX (2×250 bp and 2×300 bp paired-end for bacteria and fungi, respectively), following methods described in previous chapters. Field blanks were only included in the 16S rRNA gene run, as they did not show any amplification of ITS1 region. No amplification was observed for the PCR negative controls during the library preparation but they were nevertheless included in the sequencing runs for both 16S rRNA gene and ITS1 for quality control.

MiSeq data processing for both bacterial and fungal sequencing reads was performed as described in Chapter 2, except for the ITS dataset chimeric sequences were identified and removed based on the UNITE UCHIME reference dataset (28.06.2017) for ITS1 (Nilsson et al., 2015) and taxonomy was assigned to OTUs using the QIIME-based wrapper of BLAST algorithm (Altschul et al., 1990) against the UNITE 7.2 database (28.06.2017) (Kõljalg et al., 2013).

5.2.3 Data analysis

Data analysis methods are as described in previous Chapters. For the purposes of analysis, in many cases, samples obtained during day-time ("Weekday A", "Weekday B", "Weekday C" and "Saturday-day") and night-time ("Week-night" and "Saturday-night") or during weekdays ("Weekday A", "Weekday B", "Weekday C" and "Week-night") and weekend ("Saturday-day" and "Saturday-night") were grouped in order to give an average representation of the microbial abundance and diversity.

5.3 Results

5.3.1 Abundance of airborne bacteria

Bacterial concentrations per each sampling period, as determined by qPCR quantification, varied between 9.89×10^4 (weekday B) and 6.71×10^5 (weekday A) 16S rRNA genes/m^3 of air, with an estimated mean abundance of 2.82×10^5 16S rRNA genes/m^3 of air (equivalent to 4.03×10^4 *E. coli* genome equivalents/m^3 of air) (Fig. 5.3). On average, the day-time concentration (estimated mean from "Weekday A", "B", "C" and "Saturday-day" samples) was found to be higher compared to night-time (estimated mean from "Week-night" and "Saturday-night" samples). However, there was no significant difference (independent samples t-test, $p > 0.5$) between day-time and night-time values. Likewise, even though bacterial concentrations on the weekdays were on average higher than those on weekends (with and without taking into account night-time samples), the difference was not substantial.

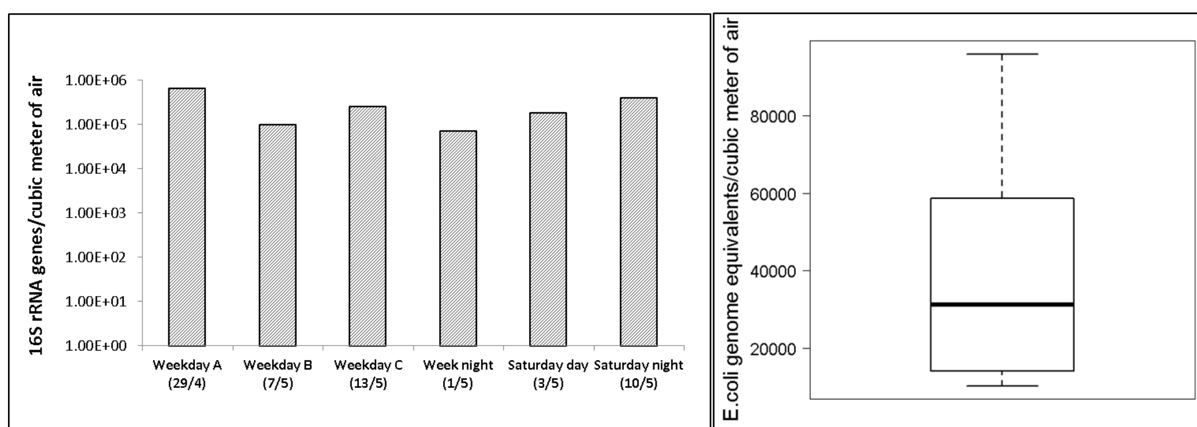


Figure 5.3 Bacterial abundance at the Athens subway station "Nomismatokopio", as determined by qPCR. Concentrations are presented in 16S rRNA gene copies/m^3 of air per each sampling period (panel on the left) and in *E.coli* genome equivalents/m^3 of air (panel on the right) in a boxplot. Box boundaries indicate the first (25th percentile) and third (75th percentile) quartiles. The median is given as the horizontal line within the box, while the whiskers (dashed lines) indicate the maximum and minimum values.

No significant association was found between bacterial abundance and any of the environmental parameters measured, as shown by Pearson correlation coefficient test (p-value > 0.05).

5.3.2 Bacterial and fungal diversity

A total of 218,488 bacterial 16S rRNA gene (6 samples, 2 field blanks and 1 PCR negative control) and 2,256,237 fungal ITS (6 samples and 1 PCR negative control) forward read

sequences were obtained. Following quality filtering and removal of chimeric sequences, 123,608 bacterial and 1,028,891 fungal sequences clustered into 6,265 and 12,084 OTUs, respectively. The resultant bacterial OTU table was filtered to remove unassigned reads, possible contaminant sequences/OTUs resulting from the blank/control samples and singletons. A total of 7,579 sequences (6.1% of total reads), representing 178 OTUs, were identified as chloroplast and were removed before further analysis. Following subtraction, a total of 5,923 bacterial OTUs (82,445 16S rRNA gene sequences, median/sample = 14,203 ± 4,211 read counts) were recovered. The resultant fungal OTU table was filtered to remove unassigned reads, sequences/OTUs detected in the negative controls and singletons and a total of 12,025 fungal OTUs (1,028,421 ITS reads, median/sample = 170,062 ± 40,771 read counts) were retained for further analysis. Rarefaction curves were constructed using the number of observed OTUs in the samples (see Appendix S5.2).

The OTU tables were normalised to the sample with the lowest number of total sequence reads (6,929 for 16S rRNA gene and 118,701 for ITS1 region) to account for differences in sequencing depth between samples, resulting in 41,574 bacterial 16S rRNA gene sequences (5,923 bacterial OTUs) and 712,206 fungal ITS sequences (12,025 OTUs). OTU tables were subsequently subjected to abundance-based filtering, removing low-abundance OTUs represented by 5 or fewer sequences in the datasets and a total of 19,785 bacterial and 670,014 fungal sequencing reads, corresponding to 216 bacterial and 1,206 fungal OTUs, respectively, were retained for downstream analysis.

Table 5.3 Alpha diversity measures for bacteria based on operational taxonomic units (OTUs) with 97% sequence similarity.

	Weekday (A)	**Weekday (B)**	**Weekday (C)**	**Week-night**	**Saturday-day**	**Saturday-night**
OTU richness	208	149	204	196	202	199
Shannon	4.77	4.34	4.75	4.60	4.83	4.72
Simpson	0.98	0.98	0.98	0.98	0.99	0.98
Chao1	210	176	206	199	204	201

The total number of bacterial OTUs detected varied from 149 (Weekday-B) to 208 (Weekday-A). Bacterial richness was strongly associated with bacterial abundance (5.3.1), as determined by Spearman's rank correlation coefficient (ρ=1, p-value =0.003). The number of bacterial taxa shared among all air samples, defined as core microbiome, was 118 (55% of total observed OTUs). 194 OTUs were common among Weekdays A, C and Saturday,

whereas 130 OTUs were shared among all day-time samples including Weekday B. 187 OTUs were present in both night-time samples. Within-sample (alpha) diversity was also characterised using Shannon index and Chao 1 measure. For the airborne bacteria (Table 5.3), Chao 1 estimator predicted 176 (Weekday-B) to 210 (Weekday-A) OTUs, whereas Shannon indices ranged from 4.34 (Weekday-B) to 4.83 (Saturday-day).

Table 5.4 Alpha diversity measures for fungi based on operational taxonomic units (OTUs) with 97% sequence similarity.

	Weekday (A)	Weekday (B)	Weekday (C)	Week-night	Saturday-day	Saturday-night
OTU richness	904	699	759	638	664	537
Shannon	4.73	4.37	4.42	4.40	3.96	3.93
Simpson	0.96	0.93	0.92	0.94	0.92	0.92
Chao1	922	718	788	657	680	560

.

Table 5.4 shows the alpha diversity metrics for the fungal communities. Fungal richness of individual air samples ranged between 904 (Weekday-A) and 537 (Saturday-night) OTUs. Across all days, 215 fungal OTUs (18% of total observed OTUs) were found to be present in all samples. A number of 351 OTUs were shared among day-time samples, while 328 OTUs were common between the night-time samples. The Shannon's Diversity Index and the Chao 1 estimated that the greatest level of fungal diversity was obtained for Weekday-A sample with values corresponding to 4.73 and 922 OTUs, respectively, while the lowest level of diversity was observed during Saturday night (3.93 and 560 OTUs for Shannon and Chao1 indices, respectively).

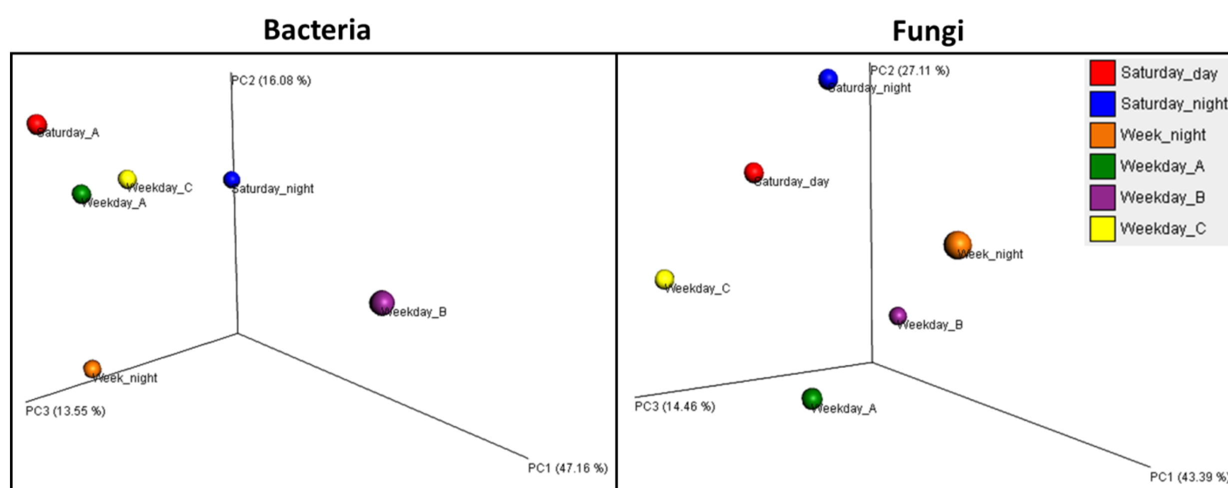


Figure 5.4 Principal coordinate analysis 3D-plots of bacterial and fungal beta diversity based on Bray-Curtis dissimilarity matrix.

Principal coordinate analysis (PCoA) was performed in order to assess clustering (and potential separation) of bioaerosol samples collected during different days, in terms of beta diversity. The microbial composition, both bacterial and fungal, was compared using the abundance-based Bray-Curtis index. For bacteria (Fig. 5.4, panel on the left), samples collected during night (Week-night and Saturday-night) were more dissimilar from those collected during the day (Weekday A, C and Saturday). However Weekday-B did not cluster with any of the other samples. When examining the similarity of the samples in terms of fungal OTUs (Fig. 5.5, panel on the right), all samples appeared to be scattered and there were no distinct groupings formed. PCoA analysis based on the incidence-based Jaccard index for both bacteria and fungi, as well as the phylogenetically-informed UniFrac distance metric (weighted and unweighted) for bacteria, also confirmed the same result for the bacterial and fungal OTUs (Appendix). The variation between weekdays (A, B, C and week-night) and weekend (Saturday-day and night) or between day-time and night-time was examined for both bacteria and fungi but there was no statistical significance found (PERMANOVA, p-value > 0.05).

5.3.3 Bacterial composition

The bacterial dataset contained 7 phyla and 14 classes (Fig. 5.5). The top four phyla, Actinobacteria - 40.9% (*Actinobacteria* class), Proteobacteria -37.3% (*Alphaproteobacteria* - 25.3%, *Betaproteobacteria* - 6.3% and *Gammaproteobacteria* - 5.7%), Firmicutes - 11.5% (*Bacilli* - 7.2%, *Clostridia* - 3.9% and *Erysipelotrichia* - 0.3%) and Bacteroidetes - 6.4% (*Cytophagia* - 4.5%, *Flavobacteriia* - 0.5% and *Sphingobacteriia* - 1.5%), made up over 96% of all reads. Deinococcus-Thermus (2.8%), Acidobacteria (0.8%) and Cyanobacteria (0.3%) comprised 3.9% of the sequences.

At the genus level, the bacteria that were detected at >1% mean relative abundance across samples comprised 24% of the total number of identified genera and accounted for 73% of the total reads. Figure 5.6 shows relative abundances of the 20 most abundant bacterial genera. *Paracoccus*, a member of the family *Rhodobacteraceae* within the class *Alphaproteobacteria*, which is associated with outdoor environmental sources (terrestrial and marine environment) (Liu et al., 2008; Kelly et al., 2006), was the most abundant genus in the air of the Athens station, with relative abundances ranging from 16% (Weekday-B) to 9.7% (Week-night) and a mean value of 12%.

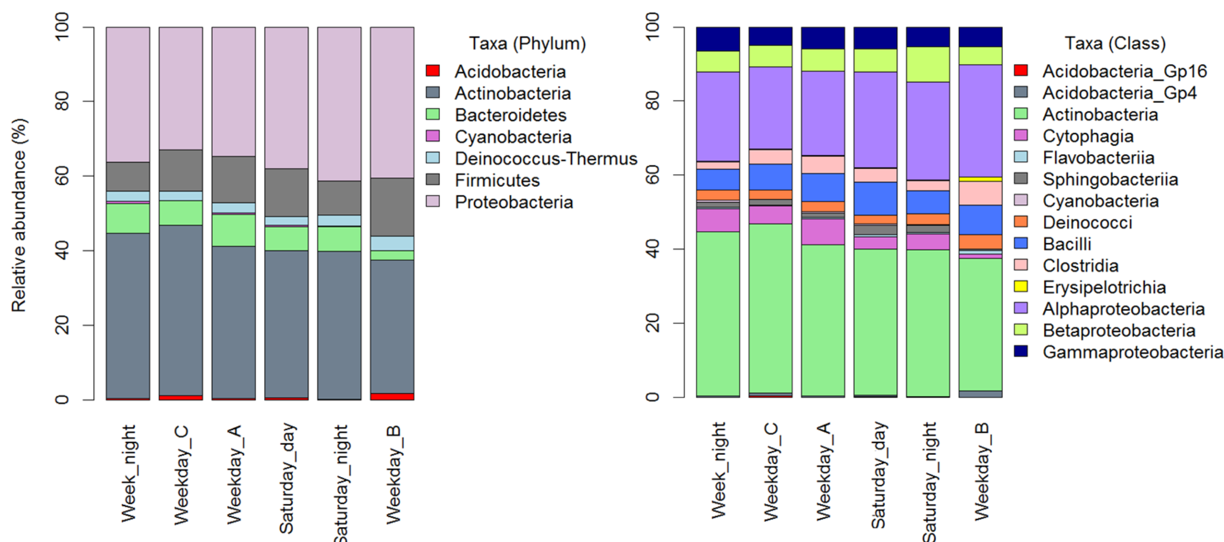


Figure 5.5 Relative abundance of bacterial OTUs at the phylum and class level per each sample.

Members of *Actinobacteria* class, *Kocuria (Micrococcaceae),* which is a common inhabitant of the human skin (Grice et al., 2008) and the oral cavity (Dewhirst et al., 2010), and *Modestobacter (Geodermatophilaceae)* that is commonly found in soil and sediments (Reddy et al., 2007; Mevs et al., 2000), were the second and third most abundant genera, with relative abundances ranging from 12% (Week-night) to 6.9% (Saturday day and night) and 8.4% (Week-night) to 3.2% (Weekday-B), respectively. Other taxa that were highly represented in the subway environment were the human-skin commensal bacteria *Corynebacterium* (4.1%) and *Staphylococcus* (1.4%) (Grice et al., 2009), the tick-associated *Diplorickettsia* (1.6%) (Mediannikov et al., 2010), while the abundantly detected *Actinobacteria*; *Blastococcus* (4.3%), *Nocardioides* (2.3%), *Arthrobacter* (2.1%), *Cellulomonas* (1.9%), *Ornithinimicrobium* (1.6%), *Dietzia* (1.6%) and *Geodermatophillus* (1.4%), *Alphaproteobacteria; Sphingomonas* (4.1%), *Skermanella* (2.2%) and *Rubellimicrobium* (2.0%), *Betaproteobacteria*; *Massilia* (2.9%) and members of *Comamonadaceae* (1.6%), *Cytophagia*; *Hymenobacter* (3.7%) and *Deinococci; Deinococcus (2.6%)* are taxa commonly found in soil, stones and aquatic habitats.

Whilst some bacteria were more abundant during day-time compared to night-time, such as the human commensals *Corynebacterium* (day-time mean 5.31% > night-time mean 1.82%) and *Staphylococcus* (day-time mean 1.86% > night-time mean 0.45%), the highest proportion of *Kocuria* (12.0%) was found during (week) night-time. Moreover, despite the average

proportions of several environmental taxa being more increased during night-time, the difference was not always substantial (e.g. *Blastococcus* day-time mean 4.15% < night-time mean 4.65%, *Sphingomonas* day-time mean 3.97% < night-time mean 4.42%). In addition, various genera of environmental origin were more enriched during day-time (e.g. *Paracoccus* day-time mean 13.0%. > night-time mean 10.4%, *Deinococcus* day-time mean 2.71% > night-time mean 2.40%).

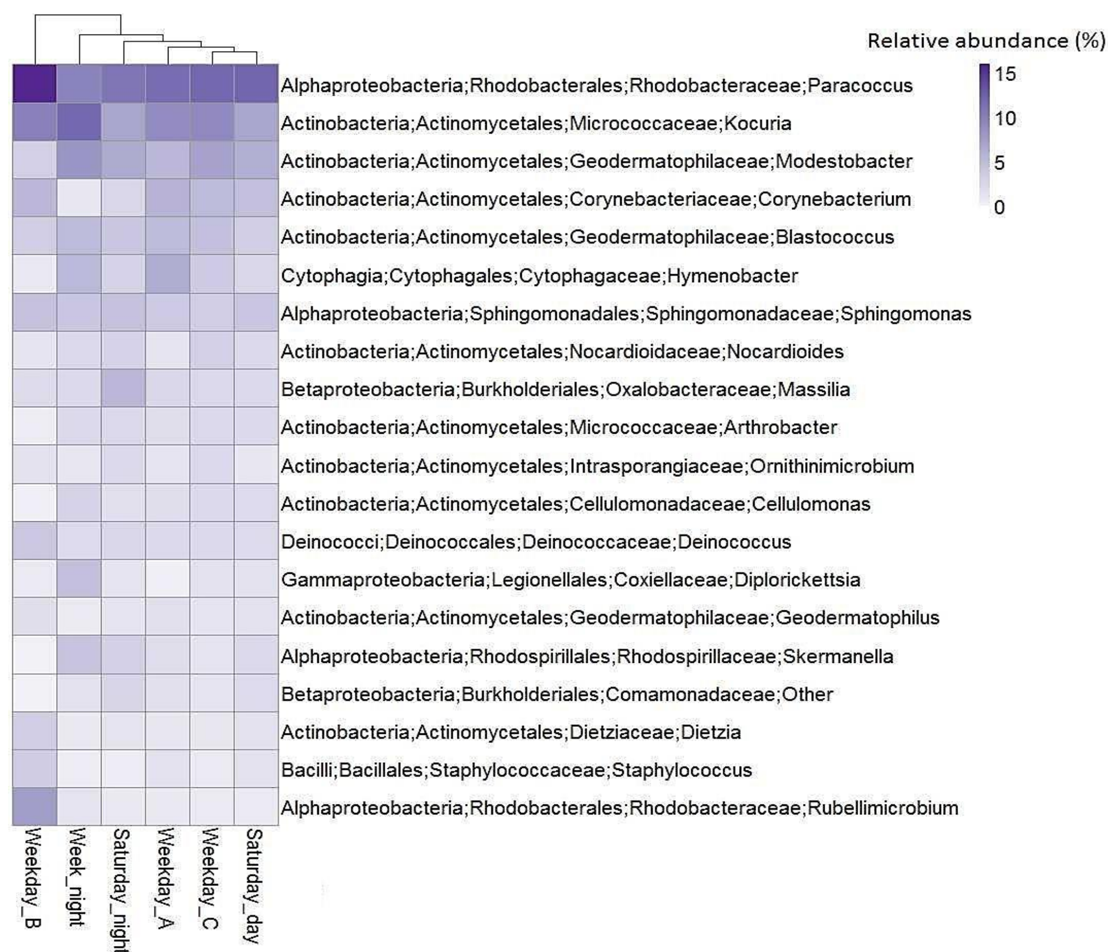


Figure 5.6 Heatmap displaying the relative abundance of the 20 most dominant bacterial genera (i.e. the 20 genera with the highest mean relative abundance) across the samples.

The rest of the bacterial genera that were encountered at mean relative abundance >1% across samples were the widely distributed *Roseomonas* (1.3%), *Bacillus* (1.1%) and *Exiguobacterium* (1.0%), which are associated with various environmental sources. In addition, the taxon *Aerococcus* that has been isolated from a wide range of ecological habitats and has also been associated with the human urinary tract (Lawson et al., 2001; Collins et al., 1999) was observed at 1.1% of total sequences. Even though the top (>1%) bacterial genera

associated with outdoor habitats collectively comprised 58% of the total reads identified at the genus level, compared to 16% for occupant-related (top) genera (including *Aerococcus*), several other taxa recognisable as bacteria associated with humans were detected at proportions <1%, including the skin-related *Acinetobacter* (Seifert et al., 1997), the oral cavity genus *Streptococcus* (Hardie & Whiley, 2006), the vaginal taxon *Lactobacillus* (Ma et al., 2012) and the human gastrointestinal microbiota *Enterobacter*, *Enterococcus*, *Anaerococcus*, *Blautia*, *Burkholderia* and members of *Ruminococcaceae* (Rajilić-Stojanović & de Vos, 2014; Rajilić-Stojanović et al., 2007).

5.3.4 Fungal composition

Figure 5.7 shows the phylum and class-rank compositions of fungi detected in the air samples. The majority of ITS reads belonged to Ascomycota (62.8%) and Basidiomycota (36.5%). A very low proportion of Mortierellomycota (*Mortierellomycotina* class - 0.01%) was also detected, while the rest of the sequences (0.6%) were unidentified.

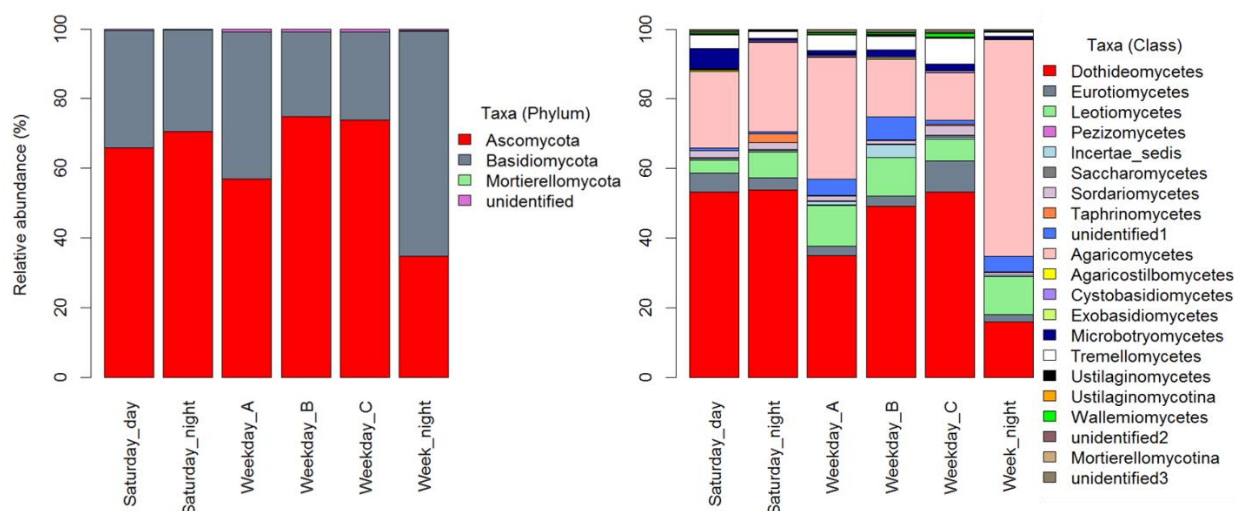


Figure 5.7 Relative abundance of fungal OTUs at the phylum and class level per each sample. "Unidentified 1" refers to Ascomycota unidentified class, "Unidentified 2" refers to Basidiomycota unidentified class and "Unidentified 3" refers to unidentified phylum and class. *Incertae sedis* belongs to Ascomycota phylum and refers to a detected class that has an uncertain taxonomic placement.

The *Ascomycota* encompassed seven major fungal classes, namely *Dothideomycetes* (43.4%), *Leotiomycetes* (8.5%), *Eurotiomycetes* (4.3%), *Sordariomycetes* (1.6%), *Taphrinomycetes* (0.6%), *Saccharomycetes* (0.3%), *Pezizomycetes* (0.1%), one with uncertain taxonomic

placement (*Incertae sedis* - 1%) as well as one unidentified ("1" - 3%). The majority of detected *Basidiomycota* species belonged to *Agaricomycetes class (29.2%),* whereas *Tremellomycetes* (3.8%), *Microbotryomycetes* (2%), *Cystobasidiomycetes* (0.4%), *Wallemiomycetes* (0.4%), *Ustilaginomycetes* (0.2%), *Agaricostilbomycetes* (0.2%), *Exobasidiomycetes* (0.1%), *Ustilaginomycotina* (0.1%) and unidentified ("2" - 0.2%) accounted for the rest of the total fungal sequences.

The fungal OTUs assigned at the genus level that were detected at a mean relative abundance >1% across samples comprised 5% of the total number of identified genera and accounted for 73% of the total reads. The two most dominant fungal genera (Fig. 5.8) were ascomycetes *Cladosporium* and *Mycosphaerella* (*Dothideomycetes)* with relative abundances ranging from 31 % (Weekday-C) to 11% (Week-night) and 18% (Saturday-night) to 2.2% (Week-night), respectively (mean values 25% and 11%). The third most abundant genus was the basidiomycetous *Antrodia* (mean = 7%), a member of *Agaricomycetes*, which was highly enriched in the air samples obtained during night-time (22%) and during weekend (Saturday-day 7.8% and night 7.2%). *Cladosporium* is one of the most widespread saprotophic molds found in the air. Species belonging to the fungal genus *Mycosphaerella* are some of the most common plant pathogens (Crous, 1998), while *Antrodia* is a common indoor wood-degrading fungal genus (Schmidt & Huckfeldt, 2011).

Alternaria (1.4%), *Aspergillus* (1.3%) and *Penicillium* (2.3%) were also among the predominant airborne fungal taxa found at the metro station. The rest of the highly abundant fungi mainly encompassed saprobic or plant-related taxa (epiphytes, endophytes or plant-pathogens) including representatives of *Agaricomycetes,* such as *Sistotrema* (2.4%) and other members of the order *Cantharellales* (0.90%), the genera *Bjerkandera* (2.4%), *Coriolopsis* (1.2%), *Steccherinum* (0.95%) and other members of *Polyporales* (1.8%), the genus *Stereum* (2.0%), which is a member of *Russulales*, as well as representatives of other classes, including *Tetrachaetum* (0.77%, *Incertae sedis*) and unidentified members of the orders *Helotiales* (5.0%, *Leotiomycetes* class), *Pleosporales* (2.2%, *Dothideomycetes class) and Filobasidiales* (1.4%, *Tremellomycetes* class). Basidiomycetous yeasts *Sporobolomyces* (*Microbotryomycetes* class) comprised 1.6% of total sequences. Various other genera commonly found in indoor environments were also detected at low proportions (mean relative abundance across samples <1%), such as *Aureobasidium*, *Botrytis*, *Wallemia*, *Acremonium* and *Filobasidium*. The human skin-associated yeasts of *Malassezia*, *Candida*,

Cryptococcus, Rhodotorula and *Trichosporon* (Findley et al., 2013) were also detected across all samples, but only at very low fractions (<0.5%).

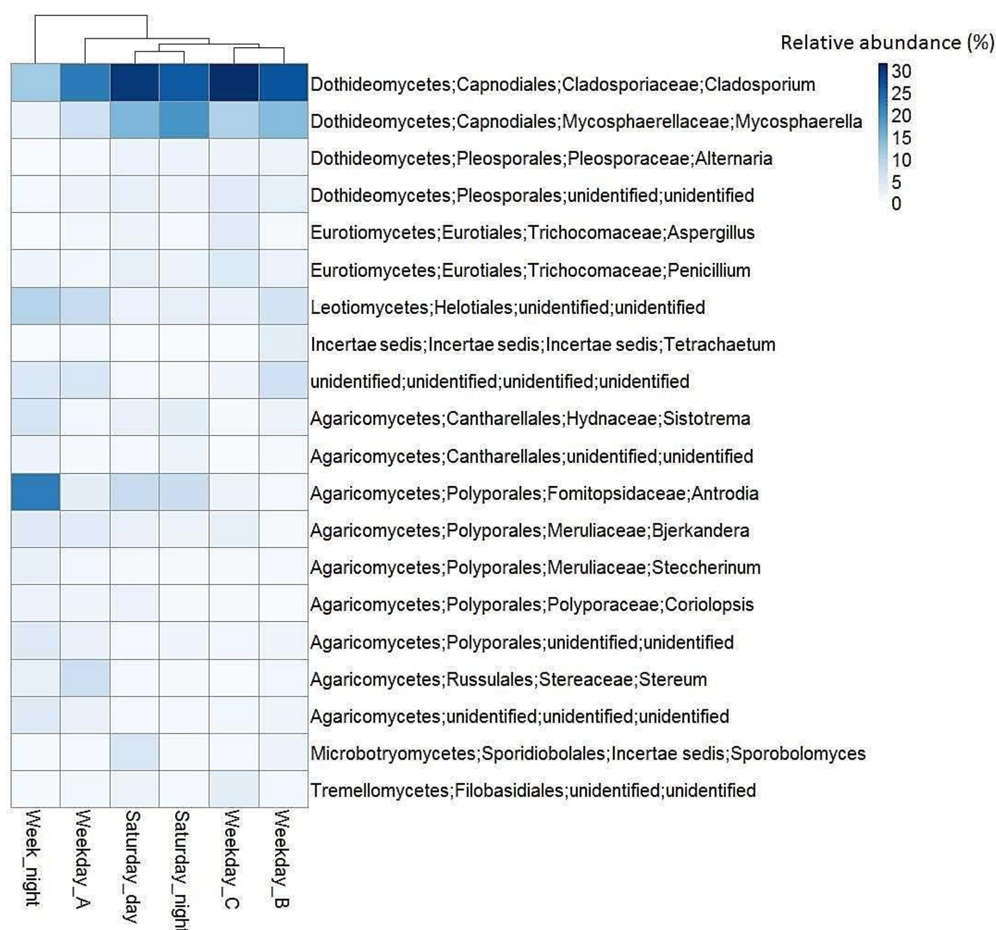


Figure 5.8 Heatmap displaying the relative abundance of the 20 most dominant fungal genera (i.e. the 20 genera with the highest mean relative abundance) across the samples.

The substantially reduced proportions of the predominant genera *Cladosporium* and *Mycosphaerella* during (week) night-time (11.44% and 2.02%, respectively) compared to day-time (mean values 27.6% and 11.2%) largely mirrored the big decrease of Ascomycota and *Dothideomycetes* observed in the particular air sample (Week-night). On the other hand, the (week) night-time enrichment of Basidiomycota and *Agaricomycetes* was highly represented by some of the dominant fungi such as *Antrodia* (night-time 21.88% > day-time mean 3.44%) and *Sistotrema* (night-time 5.37% > day-time mean 1.36%). However, the same trend was not observed during Saturday night, which mostly followed the day-time patterns.

5.4 Discussion

This study provides a first in-depth description of the airborne microbial abundance and diversity in the Athens Metro network, based on a sampling campaign conducted at the station platform "Nomismatokopio". In order to provide an overview of the aerosol microbial assemblages encountered in the Athens Metro, a long-term filter-based static sampling strategy was used, covering the whole-day metro operating hours (~17 h), as well as non-operating hours during night (~6 h), on weekdays and weekend. Our findings are discussed in the context of previous high throughput sequencing-based and culture-dependent work exploring the subway aerosol microbiome.

Bacterial abundance

Due to the limited number of culture-independent bioaerosol surveys in underground transport systems and the lack of qPCR-based estimates of total microbial concentrations, there is little information about the overall subway bioaerosols levels. Traditional quantification methods, reporting the counts of culturable micro-organisms in colony-forming units per volume of air sampled (CFU/m^3) that have been used by the majority of metro surveys (Table 5.1) are known to greatly underestimate the actual microbial densities in the air (Rappé & Giovannoni, 2003; Amann et al., 1995). In the present study, the mean bacterial concentration found in the Athens Metro station Nomismatokopio was estimated to be 2.82×10^5 16S rRNA genes/m^3 of air (equivalent to 4.03×10^4 *E. coli* genome equivalents/m^3 of air), as determined by qPCR. Similar levels have been reported by previous studies. Triadó-Margarit et al. (2017), that they also utilised qPCR, determined an average bacterial load of 4.46×10^4 equivalent *E. coli* genomes/m^3 of air, derived by samples collected inside trains and on subway platforms in Barcelona. Meanwhile, Robertson et al. (2013) measured the total microbial load using a non-culture, microscopy-based method (4',6-diamidino-2-phenylindole - DAPI cell counts), and, interestingly, found a concentration of the same order of magnitude (estimated average value: 2.2×10^4 cells/m^3) at the New York subway network. However, it has to be taken into account that quantitative metrics are incomparable when experimental protocol parameters vary, including sampling technique, sample collection and extraction efficiency as well as quantification assay design and efficiency, and caution must be taken when comparing results obtained from different studies. Moreover, the bacterial abundance estimated herein represents the inhalable fraction PM_{10} (particles with a nominal mean aerodynamic diameter ≤ 10 μm) and not the total airborne particles that are typically

analysed in bioaerosol molecular studies (Triadó-Margarit et al., 2017; Robertson et al., 2013) and can only provide a crude estimate of the actual exposure.

The day-time/night-time and weekday/weekend trends in bacterial levels observed in the Athens station are in agreement with the $PM_{2.5}$ concentration patterns described by Martins et al. (2016) who performed particle mass measurements at the same station and during the same period. Martins et al. (2016) found that the $PM_{2.5}$ levels were increased during the operating hours compared to the night-time service interruption hours as well as being on average higher on the weekdays compared to the weekends, even though the difference was not substantial, due to the higher frequency of trains and numbers of commuters. In addition, our findings are in agreement with the higher day-time levels of airborne cultivable bacteria, compared to the night-time concentrations that have been observed in other subway systems (Dybwad et al., 2014; 2012; Birenzvige et al., 2003). Most metro studies, though, have not included night-time bioaerosol monitoring. Nevertheless, a bigger sample size than the one that could be obtained in the current work would allow for a more robust quantification of the airborne microbial load and the evaluation of the inter-day variation.

Microbial diversity

Microbial diversity patterns did not demonstrate any statistically significant differences in terms of day-time/night-time as well as weekday/weekend variation. In the present study, 55% of the total observed bacterial OTUs were detected across all samples, with 3 out of the 4 daytime samples exhibiting the highest level of similarity. However, airborne fungi were more diverse across the days since only 18% of the total observed OTUs were found to be present across all samples. The detection of higher inter-day variability in bioaerosol fungal composition suggests that passengers commuting on a daily basis are likely to be exposed to a wider range of airborne fungi, compared to bacteria, indicating that different factors contribute to the shaping of the airborne bacterial and fungal assemblages found in the subway environment.

Bacterial composition

In terms of bacterial composition, the prevalence of the phyla Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes found in the present work has also been reported by previous HTS studies in the subway environments of New York (Robertson et al., 2013), Hong Kong (Leung et al., 2014) and Barcelona (*Triado-Margarit et al., 2017). Minor contributions from*

other phyla detected in the air of the Athens Metro system, such as Cyanobacteria and Deinococcus-Thermus, were also found in *New York and* Hong-Kong subway networks, respectively, along with the aforementioned phyla. The predominance of Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes has been consistently observed in several other sequencing-based surveys investigating indoor bioaerosols in various types of non-transportation environments (e.g. Gao et al., 2018; Prussin et al., 2016; Wilkins et al., 2016; Miletto & Lindow, 2015; Shin et al., 2015; Gaüzère et al., 2014; Park et al., 2014) and in the atmosphere (Behzad et al., 2015). Members of the particular phyla have also been found to dominate subway surfaces, as revealed by the metagenomic study in Boston's urban transit system (Hsu et al., 2016).

Results at the genus level showed that bacterial diversity at the Athens Metro station was dominated by a mixture of environmental taxa indicative of outdoor sources (soil, water etc.), with *Paracoccus* (*Alphaproteobacteria* class) being the most abundant genus, and human-related genera, including *Kocuria* and *Corynebacterium* (*Actinobacteria*) as well as *Staphylococcus* (*Bacilli*) among the top detected bacteria. The New York subway platforms were also found to be mainly comprised of bacterial genera associated with terrestrial and aquatic environments and human skin (Robertson et al., 2013). In particular, the human commensal *Staphyloccus* (*Staphylococcaceae*) was the most abundant genus, whereas soil-constituents and water-related bacteria, such as *Arthrobacter* spp. (*Micrococcaceae*), *Acinetobacter* spp. and *Psychrobacter* spp. (*Moraxellaceae*) -also found in our study- were highly abundant throughout the New York transit system. Likewise, the most common bacterial genera found within the urban subway network in Hong Kong were human skin-associated, including *Micrococcus*, *Enhydrobacter* -a genus associated mostly with the skin microbiome of Chinese individuals (Ling et al., 2013)-, *Propionibacterium*, *Staphylococcus* and *Corynebacterium*, while environmental bacteria, such as *Sphingobium*, *Blastomonas, Xanthomonas* and *Acinetobacter* -which apart from being ubiquitous in nature (Baumann, 1968) is also a human skin commensal (Berlau et al., 1999)- were also among the most commonly detected airborne micro-organisms (Leung et al., 2014). In contrast, Triado-Margarit et al. (2017) found that proportions of human-related bacteria (*Staphylococcus*, *Neisseria*, *Streptococcus*, *Corynebacterium*, *Enhydrobacter*) were below 1% and that aerosols in the Barcelona subway were more highly enriched for widespread environmental taxa, such as *Methylobacterium* and members of *Chitinophagaceae, Bradyrhizobium* and *Paracoccus* -also highly abundant in the present study-.

Moreover, overall results from culture-based studies from other metro systems worldwide (Table 5.1) have demonstrated a lot of commonalities in terms of detected airborne bacteria with the current and the aforementioned sequencing-based surveys. Despite the different bioaerosol collection methodologies and differences among the various underground systems in terms of architecture design, system age, ventilation type and geographic location of each network, the most prevalent identified cultivable taxa are human commensals (e.g. *Corynebacterium*, *Kocuria*, *Staphylococcus* etc.) and bacteria ubiquitously found in nature (e.g. *Bacillus*, *Pseudomonas* etc.). The detection of various common bacterial genera between culture-dependent and culture-independent studies implies that a big fraction of the observed subway bioaerosol diversity represents living micro-organisms and possibly metabolically active and not dead cells or microbial fragments suspended in the air.

Fungal composition

As expected, the aerosol fungal diversity encountered in the current investigation was mainly represented by the phyla Ascomycota and Basidiomycota, members of the subkingdom Dikarya, which are known to comprise almost 86% of the known taxa in the kingdom of Fungi (https://unite.ut.ee/, last updated: 2017-12-01). The predominance of *Dothideomycetes* and *Agaricomycetes* classes within their corresponding divisions is in line with other cultivation-independent indoor bioaerosol surveys (Shin et al., 2015; Hoisington et al., 2014; Adams et al., 2013). Likewise, in the New York network (Robertson et al., 2013), that the aerosol eukaryotic diversity was found to be mainly comprised by fungi, as determined by universal Sanger sequencing, the fungal composition was also represented by the aforementioned phyla. However, *Agaricomycetes*, instead of *Dothideomycetes*, was the most dominant class detected in the New York subway, followed by *Eurotiomycetes* (*Trichocomaceae*), which was the fourth most abundant class encompassing the Athens Metro air mycobiota.

At the genus level, *Cladosporium* was found to be the most abundant genus in the air of the Athens Metro system. *Cladosporium* is a ubiquitous phyloplane fungus commonly found to dominate the air mycobiome indoors and outdoors *(*Bensch et al., 2012; Fröhlich-Nowoisky et al., 2012; Kaarakainen et al., 2008). *Penicillium*, *Aspergillus* and *Alternaria* spp. were also among the prevalent airborne fungi sampled off the Athens station, even though at lower proportions compared to *Cladosporium*. The particular four saprotrophic genera are known for their wide distribution in the indoor environment due to their ability of producing

numerous conidia which can get easily aerosolised (Nevalainen et al., 2015). *Penicillium* and *Aspergillus* are members of the family *Trichocomaceae* (*Eurotiomycetes*), which was found to be dominant in the air of the New York transit system. *Davidiellaceae* (obsolete nomenclature of family *Cladosporiaceae*) that contains the genus *Cladosporium* (Hyde et al., 2013), was also one of the fungal groups detected in New York's subway aerosol samples (Robertson et al., 2013*).* The second most abundant airborne fungal genus encountered in the Athens Metro, *Mycosphaerella*, which mainly includes plant leaf infecting species, belongs to *Capnodiales* that was another one of the fungal orders identified in the study of Robertson et al (2013). The rest of the highly enriched taxa in the Athens subway air mycobiome were mainly saprobic or plant-related fungi (e.g. *Agaricomycetes*) associated with various natural sources in both terrestrial and aquatic environments, suggesting a potential outdoor origin. Several identified basidiomycetous genera, members of the mushroom-forming *Agaricomycetes*, detected in the Athens Metro, such as *Antrodia*, *Sistotrema*, *Bjerkandera*, *Coriolopsis*, *Steccherinum* and *Stereum*, are wood decomposers known to cause damage to structural timber (Hibbett et al., 2014). Robertson and colleagues (2013) also detected fungal wood decayers (genus not specified), which was hypothesised to be related to the wooden track structure of the particular subway network.

Apart from the common indoor molds *Cladosporium*, *Penicillium*, *Aspergillus* and *Alternaria* spp. and a limited number of ascomycetous genera such as *Botrytis* and *Acremonium*, which were also found at very low proportions in the Athens Metro, the presence of the majority of the aforementioned airborne taxa in a subway environment has not been reported previously (Table 5.1). The lack of any detected basidiomycetous fungi in the culture-dependent studies, apart from reflecting the known limitations of culture based-methods (Rappé & Giovannoni, 2003), is linked to the problematic identification of filamentous Basidiomycota by standard microbiology phenotypic methods, compared to the more readily culturable and identifiable Ascomycota, due to lack of morphologically distinguishing features (Chowdhary et al., 2014; Pashley et al., 2012).

Although HTS amplicon sequencing assay was employed for the investigation of the Athens Metro fungal bioaerosols, targeting the internal transcribed spacer region that enables great genus-level taxonomic assignment (Schoch et al., 2012), various detected fungal OTUs could not be classified at the sub-order level, with some of the most abundant taxa being identified as members of *Polyporales*, *Cantharellales*, *Helotiales*, *Pleosporales* and *Filobasidiales*, whereas a small proportion (0.2%) could not be defined beyond the fungal kingdom.

Limitations associated with the taxonomic identification of fungal sequences are attributable to the fact that the catalogue of annotated species in the kingdom of Fungi is far from complete (Blackwell, 2011) and the fungal databases lag way behind those for bacteria (Pautasso, 2013) with millions of species still awaiting description or having uncertain taxonomic placement ("*Incertae sedis"*) and new taxa being continually discovered (Hibbett et al., 2013; 2011; Kõljalg et al., 2013). Nevertheless, to our knowledge, this is the first study to apply high-throughput sequencing for the characterisation of the bioaerosol fungal diversity in a metro environment and therefore, results presented here provide novel information on a previously largely unexplored mycobiome.

Bioaerosol sources

It is well-established that human occupancy is one of the predominant sources of airborne microbial particles in the non-industrial indoor environment (Luongo et al., 2017; Bhangar et al., 2016; Adams et al., 2015; Gaüzère et al., 2014; Meadow et al., 2015; 2014; Hospodsky et al., 2012) and therefore, human microbiota originating from the commuters contribute significantly to the aerosol microbiome encountered in public transit systems. Humans can act as primary sources through direct emission of micro-organisms via talking, breathing, coughing, sneezing and skin shedding (Spendlove & Fannin, 1983), as well as secondary sources due to the movement-induced resuspension of particles deposited on the surfaces, floor, clothes and footwear (Tian et al., 2016; Chen & Hildemann, 2009; Täubel et al., 2009). The cumulative relative abundance of bacterial genera with presumptive human origin presented here, which was estimated to be at least 16% (taking into account only the dominant taxa observed at > 1% proportions), lies within the range of non-transportation studies in occupied settings, where human-associated bacterial taxa were found to comprise, on average, 12% (Gaüzère et al., 2014), 17% (Hospodsky et al., 2012; Qian et al., 2012) and 23.7% (Shin et al., 2015) of the total number of indoor air bacterial sequences. Minor enrichments (< 1%) of taxa associated with the human oral or gut microbiome (e.g. *Streptococcus*, *Enterobacter*, *Ruminococcaceae*) were also found.

Moreover, the association of the human commensal bacteria with the large numbers of commuters was further supported by the increased proportions of *Corynebacterium* and *Staphylococcus* observed during the Metro operating hours (day-time) compared to night-time. It is worth noting that *Kocuria* spp., which are part of the normal human skin and oral flora, did not exhibit elevated proportions during day-time. However, members of *Kocuria* have been also isolated from various environmental sources (Tang et al., 2009; Kim et al.,

2004; Reddy et al., 2003; Kovács et al., 1999). As the species identity of the OTUs belonging to genus *Kocuria* could not be specified, it is possible that some *Kocuria* OTUs could be originating from outdoors and therefore not being affected by the commuters influx during the metro operating hours. Moreover, it has been reported that human-associated taxa can also be observed in outdoor air (Adams et al., 2015; Qian et al., 2012), especially in urban locations. This could justify the increased presence of *Kocuria* spp. during non-occupied periods, suggesting that human commensals could also flow from outdoors into the subway platform, via the ventilation system. Nevertheless, further work is needed in order to accurately link the dispersed micro-organisms to their sources.

A metagenomic study profiling microbial communities across surfaces in Boston's metropolitan transit system found that the surface microbiome was dominated by various human skin and oral commensal bacteria, as commonly found in the present and previous subway air microbiome surveys, such as *Propionibacterium*, *Corynebacterium*, *Staphylococcus*, *Micrococcus*, *Rothia* and *Streptococcus*, while the non-bacterial composition was highly represented by the the human skin yeast *Malassezia* (Hsu et al., 2016). Moreover, another metagenomic investigation (Afshinnekoo et al., 2015) found that the human DNA recovered from New York subway surfaces mirrored the census demographic data of the populations living in the surrounding areas. Furthermore, in agreement with our results, bacterial taxa not associated with the commuters were also recovered at high proportions on the subway surfaces, including the soil-associated genera *Sphingomonas*, *Sphingobium* and *Methylobacterium* that is commonly found on plants (Hsu et al., 2016), as well as *Pseudomonas*, *Enterobacter*, *Stenotrophomonas* and *Acinetobacter* (Afshinnekoo et al., 2015). Hsu et al (2016) also extended previous findings indicating that bacterial communities on interior surfaces vary with the type of contact with the human body (Meadow et al., 2014b) by showing that the subway surface type is also a strong determinant of the microbial composition. Resuspension of settled material, generated by the air movement on the platform that is driven by the piston effect (i.e. forced air flow inside a railway tunnel caused by the trains passing through the tunnel) as well as the commuters, has been identified to be one of the major sources of airborne particles in the subway environment (in addition to the abrasion between rails, train wheels and brakes) (Moreno et al., 2018; Pan et al., 2013). The detection of various common human- and environmental- related taxa found between surface studies and aerosol investigations in metro systems suggests that biological particles

suspended in the subway air may originate from the surface microbiome and may, as well, contribute to it by depositing biological material on the various surfaces.

Since outdoor samples were not obtained and owing to the lack of any regional high throughput sequencing-based bioaerosol study in the urban area of Athens, tracking of outdoor air sources was mainly limited to identification of taxa indicative of outdoor sources such as soil, plants, water and sediments. Considering that 17 out of the 20 most enriched bacterial genera represent non-human associated taxa, constituents from various ecological habitats, and given that all the dominant fungal taxa are non-occupant related, strongly suggests that outdoor-derived microbes have a significant influence on the Athens Metro bioaerosol composition. In addition, the detection of a big percentage of phylotypes classified as chloroplast (6.1% of total reads prior exclusion from analysis), which denotes the presence of plant material and has been used as an indicator of outdoor vegetation-related sources (e.g. Barberán et al., 2015; Hospodsky et al., 2012), further supports the strong impact of the outdoor environment on indoor bioaerosols. Moreover, the presence of plant material in the subway air, tracked in chloroplast and plant-related microbial taxa, likely resulted from outdoors, is in accordance with the vegetated outdoor environment near the station as well as the warm spring period when the sampling took place.

As natural ventilation occurring through draught relief shafts and the passenger pathways is the main air exchange mechanism with the outdoor environment at the particular station (Mendes et al., 2018) where samples were collected, enhanced by the air motion produced by the train piston effect (Moreno et al., 2014), outdoor air appears to be an important contributor to the airborne microbial diversity. Ventilation strategy has been shown to play a major role in shaping the microbial composition in the built environment (Kembel et al., 2012; Kodama & McGee, 1986). Meadow and colleagues (2014) demonstrated that in well-ventilated occupied settings, the introduction of unfiltered air to the indoor environment has a significant impact on the indoor bioaerosol microbial diversity. Their findings showed that even though the presence of occupants provides a distinct signal of human-associated bacterial taxa, the use of natural ventilation tends to increase the composition similarity between indoor and outdoor air microbiota. In any case, both the outdoor air and the occupants have been identified as the main sources of micro-organisms found in indoor settings (Leung & Lee, 2016). However, the input of occupant-related direct emissions is greater for bacteria (skin-associated fungal yeasts were detected at fractions $< 0.5\%$), whereas fungal taxa found indoors mainly derive from the adjacent outdoor environment carried

inside via outdoor air (Barberán et al., 2015b; Adams et al., 2014). Previous literature has demonstrated that in the absense of dampness problems, fungal species in indoor environments mainly reflect the outdoor air fungal diversity of the surrounding environment and the local land sources (Adams et al., 2013; Fradkin et al., 1987) and that geographic location is a major factor driving fungal composition patterns (Grantham et al., 2015; Fröhlich-Nowoisky et al., 2012; Amend et al., 2010). The strong dependence of the mycobiome encountered indoors on the outdoor environment might explain the higher variation observed in the subway air fungal diversity across the days, compared to the bacterial composition, as outdoor atmospheric microbial aerosols have been shown to demonstrate high temporal variability between days within the same season (Pashley et al., 2012; Fierer et al., 2008).

Moreover, the input of resuspension of outdoor-derived environmental micro-organisms entering the metro areas through human vectors (e.g. on clothes or footwear) should not be disregarded (Adams et al., 2015), especially considering the big numbers of passengers that the system serves. It is worth noting that there was a big increase in the observed proportion of *Agaricomycetes* (Basidiomycota) during night-time and at the same time a reduction in the relative abundance of *Dothideomycetes* (Ascomycota). This observation could be related to the lack of occupancy during the night and might suggest that the specific group of fungi (*Agaricomycetes*) is likely to be transported from outdoors mainly via ventilation, while a big portion of taxa belonging to *Dothideomycetes* could be conveyed by commuters (e.g. *Cladosporium*, *Penicillium* and *Aspergillus* are widespread airborne taxa found in all types of environments) and subsequently resuspended. Even though the same pattern was not observed during Saturday night, this could be related to the prolonged operation of the Athens Metro on Saturday, as trains run until 02:30, and therefore, any compositional change is expected to be less pronounced. It is also possible that some of the airborne micro-organisms observed indoors could be deriving from indoor colonisers on the subway building structure materials, even though this would require specific conditions (e.g. indoor fungal growth requires water availability).

Martins et al. (2016) studied the $PM_{2.5}$ mass concentrations at the Athens station, as part of the same measurement campaign, and found a positive association between the indoor and ambient air levels recorded outdoors (squared Pearson's correlation coefficient R^2=0.60). Even though subway air particles ($PM_{2.5}$) were found to be much higher than those above ground (6.9 times on average), mainly due to the ferruginous character of the underground

aerosols produced by the contact between train wheels and rail track or the braking process (Moreno et al., 2015), and regardless of the different sources of biological and non-biological particles, the observed correlation still indicates that the outdoor-derived particles accumulating in the station affect the subway aerosol concentrations and that the outdoor air flowed into the platform has a substantial influence on the overall subway air quality. In addition, the chemical composition analysis performed on the Nomismatokopio station $PM_{2.5}$ samples (Martins et al., 2016) revealed that components of crustal origin (Al, Ca, K, Ti, Mg and P), which are mainly indicative of soil material and road dust typically present outdoors, were in higher levels indoors compared to outdoor air, providing further evidence about the outdoor origin of the subway particles. Moreover, the detection of crustal matter is in line with the identification of soil-related micro-organisms (e.g. *Paracoccus*, *Sphingomonas, Arthrobacter* etc.) in the air of the station, as suspended soil particles may have microbes attached to their surface and act as carriers of microbial material in indoor air. However, it has to be noted that crustal particles, apart from being transported into the metro environment through ventilation, they might be also transferred indoors through the underground rail commuters as well as deriving from erosion of building construction materials in the subway tunnel.

Moreover, findings from prior sequencing-based bioaerosol studies strongly recommend that outdoor air is a major driver of the aerosol microbial composition in underground rail transport systems. For instance, Robertson et al. (2013) suggested that the resemblance found between the New York subway and outdoor air microbiota was an indicator of good indoor and outdoor air mixing throughout the system. Similar to the Athens Metro, the New York network is mainly naturally ventilated and the effective air exchange is aided by the train piston effect-induced air movement. Likewise, Leung et al. (2014), found no significant differences between the subway and outdoor aerosol bacterial composition and showed that the outdoor air is a major source for the airborne bacteria in the Mass Transit Railway of Hong Kong. However, due to variations observed among different subway lines and differences in the contribution of outdoor air to the microbiome of different lines, Leung and colleagues concluded that complete indoor and outdoor air mixing does not occur, most likely due to the combined mechanical and natural ventilation system, as well as the presense of safety screen doors along various platforms.

Triado-Margarit et al. (2017) suggested that the high abundance of *Methylobacterium* observed in the air of Barcelona subway could be linked to the occurrence of vehicular

pollutants in the metro environment, such as PAHs (Martins et al., 2016b; Green, 2006). Members of the genus *Paracoccus*, which was highly abundant in the present study, are also known PAHs-degrading bacteria (Zhang et al., 2004). Various other microbial genera associated with known PAHs-degrading strains (Fernández-Luqueño et al., 2011) were also abundant in the air of the Athens Metro station, including *Sphingomonas*, *Cellulomonas*, *Arthrobacter*, *Dietzia*, *Cladosporium*, *Penicillium*, *Alternaria*, *Aspergillus*, *Bjerkandera* and *Coriolopsis*. Airborne PAHs are significant pollutants in urban areas and vehicle emissions are among the major sources of PAHs (Slezakova et al., 2013; Tang et al., 2005; Guo et al., 2003; Marr et al., 1999; Harrison et al., 1996). However, the trains in the Athens subway as well as in most underground systems are powered by electricity and the possible presence of PAHs, which has been reported in various other subway networks (Martins et al., 2016b; Yan et al., 2015; Velasco et al., 2004; Furuya et al., 2001), is mostly related to combustion sources from the outdoor urban environment or, in some cases, to diesel-powered trains used for maintenance works, usually taking place during nighttime (Moreno et al., 2018). Vehicular fuel combustion and exhaust emissions (followed by biomass burning used for household heating during wintertime) have been found to be the main contributors of PAHs in the atmosphere of Athens (Alves et al., 2017; Andreou & Rapsomanikis, 2009; Vasilakos et al., 2007; Valavanidis et al., 2006; Mantis et al., 2005). Although PAH concentrations were not determined inside and outside the station, elevated concentrations due to road traffic emissions are expected considering that Nomismatokopio station is located in a highly trafficked motorway site in the city of Athens. Accumulation of PAHs in urban roadside soil and street dust, either directly deposited from the air or via plant leaves (Essumang et al., 2011; Dong & Lee, 2009; Wang et al., 2009; Morillo et al., 2007; Ollivon et al., 2002; Yang et al., 1991; Takada et al., 1990), has been linked to occurrence and increased concentrations of microbial PAH degraders (Undugoda et al., 2016; Yutthammo et al., 2010; Johnsen & Karlson, 2007; Johnsen et al., 2006a; b) that they can be subsequently aerosolised, spread through the air and penetrate the station. Therefore, it might be speculated that particular environmental taxa might be related to the outdoor vehicular transport-associated PAHs concentrations. In addition, these observations provide further support for the hypothesis that the particular micro-organisms are more likely to originate from outdoor sources. Furthermore, apart from the ability of certain taxa to survive and grow in the presence of pollutants, such as vehicular fossil fuel products, it is worth noting that various microbes have adaptive mechanisms that allow them to tolerate harsh conditions (Pikuta et al., 2007). For instance, members of Deinococcus - Thermus (e.g. *Deinococcus* spp.) that were found in the

metro aerosol samples are resistant to extreme environmental stresses, such as dry conditions (e.g. asphalt) or high levels of UV radiation (Rainey et al., 2005; Hirsch et al., 2004).

Health-related taxa

The densely populated undergound transportation systems form an ideal basis for the rapid dissemination of infectious micro-organisms through the air and the uncontrolled spread of diseases by the airborne route as well as being considered to be ideal environments for the deliberate release of biohazardous agents. Therefore, monitoring of the bioaerosol background levels and surveillance of suspicious biocontaminants is essential for defining potential hazards and assessing related health risks.

Among the highly abundant bacterial taxa detected in the Athens metro, various genera are affiliated with health-related species. Apart from the well-known opportunistic pathogens belonging to genus *Staphylococcus*, such as *S. aureus* and *S. epidermidis* (Ziebuhr et al., 2006; Sheagren, 1984), the human-associated bacteria *Kocuria* and *Corynebacterium* also comprise some potentially pathogenic strains, such as *K. kristinae*, *C. amycolatum* and *C. jeikeium* (Purty et al., 2013; Bernard, 2012). Moreover, *Diplorickettsia*, a genus of intracellular parasites of arthropods, includes the human pathogen *D. massiliensis* that can be possibly transmitted by ticks (Subramanian et al., 2012). Also, some members of *Dietzia* (*D. cinnamea*, *D. maris* and *D. papillomatosis*), which is a widespread genus found in soil and marine sediments and it is also able to colonise the human skin, have been reported to cause infections in immunocompetent and immunocompromised patients (Koerner et al., 2009). Last, both *Roseomonas* and *Aerococcus* include clinically significant species that can cause human infections (Rasmussen, 2016; Rihs et al., 1993).

In terms of fungi, the abundantly detected *Cladosporium*, *Penicillium*, *Aspergillus*, *Alternaria*, which are some of the most common inhabitants in built environments, are well-known for their allergenic properties (Simon-Nobbe et al., 2008; Horner et al., 1995; Burge, 1985; Gravesen, 1979). *Aspergillus* is further important because several species (e.g. *A. fumigatus, A. flavus*) are the cause of severe fungal infections, mainly in immunocompetent and immunocompromised individuals (Denning, 1998). Sensitization to *B. adusta*, a member of the wood-rotting bacidiomycetous genus *Bjerkandera*, has been related to fungus-associated chronic cough (Ogawa et al., 2009), whereas *Sporobolomyces* spp. (*S. salmonicolor*, *S. holsaticus* and *S.roseus*), naturally occurring in the phyllosphere (Wang & Bai, 2004), have been associated with hypersensitivity pneumonitis (Cockcroft et al., 1983)

and have been implicated as the cause of infections in immunocompromised hosts (Plazas et al., 1994; Morris et al., 1991). However, it has to be noted that the current limitations associated with the amplicon sequencing analysis in providing sufficient resolution at the sub-genus level do not allow for the differentiation of the harmful species from their benign close relatives (Ranjan et al., 2016; Poretsky et al., 2014) and can only be used as a rough indicator for prediction of possible adverse health effects.

5.5 Conclusions

Despite the general conception that underground rail systems are unique micro-environments harboring uncommon microbial populations, our findings are in agreement with previous studies, showing that, the observed aerosol microbial diversity in the Athens Metro is similar to the one encountered typically in naturally ventilated occupied indoor settings. Overall, our results demonstrated that the metro air microbiome is mostly dominated by bacterial and fungal taxa of enviromental origin (soil, plants, water etc.) with a lower contribution of human commensal bacteria, underscoring the importance of both outdoor air and the commuters as sources in shaping the bioaerosol microbial composition. Additional efforts are necessary in further examining the bioaerosol load in more stations and during different seasons, with concurrent outdoor air sampling as well as surface sample collection, in order to provide a more comprehensive assessment of the airborne micro-organisms and the contribution of their sources.

The current study presented the first microbial characterisation of PM_{10} in the Athens Metro underground railway system, providing the foundation for further and more systematic investigation of the overall subway air quality beyond the typical physicochemical aerosol determination. Moreover, this work contributed to the growing body of the microbiome exploration in urban public transport systems by explanding the so far knowledge about the aerosol mycobiome in the subway environment. Results generated by this study can provide valuable background information that can be used for public health surveillance and prevention of transmission of infectious agents.

Chapter 6

Bioaerosol characterisation in the transportation environment; Bioaerosol exposure during commuting

6.1 Introduction

Scientific studies have shown that commuting results in significant contribution to the daily exposure to air pollutants (12-32% of daily exposure; Williams & Knibbs, 2016; Dons et al., 2012; 2011; Fondelli et al., 2008), which is highly affected by the mode of transport (Cepeda et al., 2017; Good et al., 2016; Karanasiou et al., 2014; Kingham et al., 2013). A growing literature on air quality and exposure assessment during commuting has mainly focused on the suspended particulate matter (e.g. Rivas et al., 2017; Moreno et al., 2015; Knibbs et al., 2011, Zuurbier et al., 2010; Gulliver & Briggs, 2004; Chan et al., 2002; Adams et al., 2001). Recently, the microbial fraction of particles has started receiving attention and a wide range of bioaerosol studies have been conducted covering various means of transport, from underground subways (e.g. Leung et al., 2014; Robertson et al., 2013; Dybwad et al., 2012 etc.) to even aircrafts (Gupta et al., 2012; Walkinshaw, 2010; Osman et al., 2008; Mangili & Gendreau, 2005).

Among the various different public transport modes, the metro system has perhaps been the most well-studied in terms of airborne micro-organisms. However, most studies have focused on subway station platforms rather than the interior of train cabins (see Chapter 5.1). At the same time, information on bioaerosol exposure in other in-transit environments is still limited in existing literature. Moreover, findings reported in literature are mainly based on culture-based methods. Wang et al. (2011; 2010) studied the size distributions of airborne viable fungi and bacteria in commuting trains and buses in Taiwan. According to their findings, the highest bacterial and fungal concentrations in buses occurred in the 2.1 - 3.3 μm size range (Wang, 2011), while the highest levels in trains were found in the 1.1 - 2.1 μm size range (Wang et al., 2010). In terms of composition, some of the most frequently identified micro-organisms found inside public buses are the common airborne allergenic fungi *Aspergillus* spp., *Alternaria* spp., *Penicillium* spp. and *Cladosporium* spp., as well as *Staphylococci* bacteria (Nowakowicz-Dębek et al, 2017; Prakash et al., 2014; Lee & Jo, 2005). Some types of potentially pathogenic bacteria, especially *Staphylococcus aureus*, can be resistant to

antibiotics and are likely to spread among crowds of people in shared areas, such as public transport vehicles (Lutz et al., 2014; Zhou & Wang, 2013; Yeh et al., 2011). Onat et al. (2016) investigated the bacterial concentrations inside public buses in Istanbul and found a significant correlation between airborne *S. aureus* levels and $PM_{2.5}$ levels. Among different means of transport, methicillin-resistant *Staphylococcus aureus* (MRSA) contamination, mainly identified on frequently touched surfaces, has been found in buses in Lisbon, as reported by three studies (Mendes et al., 2015; Conceição et al, 2013; Simoes et al., 2011), and in the Midwestern USA (Lutz et al., 2014). Low levels of MRSA contamination have also been found in Lyon's Metro (Gaymard et al, 2016) and in trains in Tokyo (Iwao et al., 2012). Even though the detection was based on hand-touched surfaces, MRSA can also be transmitted through the airborne route (Gehanno et al., 2009; Beggs, 2003; Shiomori et al., 2002). The spread of tuberculosis among commuters has also been a field of investigation and studies have shown that public transportation may play a critical role in sustaining tuberculosis transmission (Andrews et al., 2013; Feske et al., 2011).

Yet, commuting can also be performed by private vehicles. It has been estimated that a considerable amount of time, ranging from 79 mins to 100 mins is spent by the average person inside an automobile on a daily basis (Sattar et al., 2016; Klepeis et al., 2001). The amount of time inside motor vehicles can be substantially higher to certain individuals such as truck, van or taxi drivers. The literature has already shown that in-vehicle exposure is associated with elevated levels of several pollutants apart from PM, such as carbon monoxide, volatile organic compounds and polyaromatic hydrocarbons (Xu et al., 2016; Riediker et al., 2003; Jo & Park, 1999; Clifford et al., 1997; Chan et al., 1991). However, despite the fact that the inside of an automobile is considered to be an ideal environment for transmission of biological agents due to its confined space, information on bioaerosol exposure in car interiors is currently limited.

So far, various studies on in-cabin air quality have measured the bacterial and fungal CFU levels in passenger cars by employing culture-dependent methods and found that the most dominant fungal genera in automobiles (*Cladosporium, Penicillium, Aspergillus* and *Alternaria*) are similar to the ones found in public transport means (Wang et al., 2013; Jo & Lee, 2008; Lee & Jo, 2005; Simmons et al., 1997). According to the size distribution data presented by Wang et al. (2013), the highest concentrations of viable airborne fungi in automobiles were obtained for the size fractions 2.1 - 3.3 μm and 1.1 - 2.1 μm, same as found for public buses and trains (Wang et al. 2011; 2010). Stephenson et al. (2014) performed a

culture-independent survey in a car interior by using 16S rRNA pyrosequencing of samples from swabbed surfaces and their results showed that the most abundant bacterial genera were *Staphylococcus* and *Propionibacterium*. Moreover, findings reported in literature on air quality inside passenger vehicles' cabins have demonstrated that the automobile air conditioning (A/C) system can contribute significantly to the reduction of airborne microbial reagents to levels over 80% (Wang et al., 2013; Vonberg et al., 2010; Jo & Lee, 2008; Kumar et al., 1990). On the other hand, A/C systems in cars can also be a source of potentially pathogenic micro-organisms, such as *Legionella pneumophila*, via improperly maintained filters (Alexandropoulou et al., 2013; Sakamoto et al., 2009). Bacterial and mold spores collected in the car cabin air filter, which might thrive under high humidity conditions, can get aerosolised and released into the vehicle environment through the air stream (Li et al., 2013; Simmons et al., 1997; Kumar et al., 1984; 1981). In fact, it has been found that professional drivers may be at high risk of acquiring Legionnaires' disease from the automobile air-conditioning systems (Farnham et al., 2014; Polat et al., 2007). Windscreen wiper fluid reservoirs not containing screenwash (Palmer et al., 2012; Wallensten et al., 2010), as well as those with added washer fluid (Schwake et al., 2015), have also been identified as possible in-vehicle sources for *L. pneumophila*. Finally, the risk of airborne Influenza transmission in automobiles has been investigated by Knibbs and colleagues (2012). Their results showed that the infection risk inside the car cabins can be reduced by not recirculating air.

Lee & Jo (2005) studied the bioaerosol levels in both cars and public buses in Korea and found that the summertime in-vehicle viable bacteria concentrations were significantly higher for buses compared to passenger cars and that winter in-vehicle levels were similar for cars and buses. Nevertheless, there is not enough evidence to show whether the exposure to airborne micro-organisms in automobiles is more or less significant than the exposure in other in-transit environments. Hence, further research is required to understand the significance of bioaerosol exposure in the various means of transport.

The aim of this study was to investigate the abundance and diversity of the airborne micro-organisms in the transport environment. We aimed to explore whether the choice of commuting mode will have a significant impact on the bioaerosol microbiome that passengers are exposed to and whether commuters using public transport, rather than private automobiles, are going to be exposed to a different range of microbial taxa. In order to

address these questions, a comparative bioaerosol study was carried out in two different types of commuting micro-environments, a train and a private vehicle, covering the same route.

6.2 Methods

6.2.1 Study environments

Both travel scenarios were examined under a fixed route between Colchester, the second biggest town in the county of Essex (south-eastern UK), and London (UK). Colchester is located about 80 km northeast of London and is connected to the capital through a major road and a railway line (Fig. 6.1). Both routes are heavily used daily by people travelling to and from work, as well as by leisure commuters. The first part of study (train sampling) was conducted during September 2016, while the second part (car sampling) took place during February-March 2017. A total of six commutes were carried out for each transport mode.

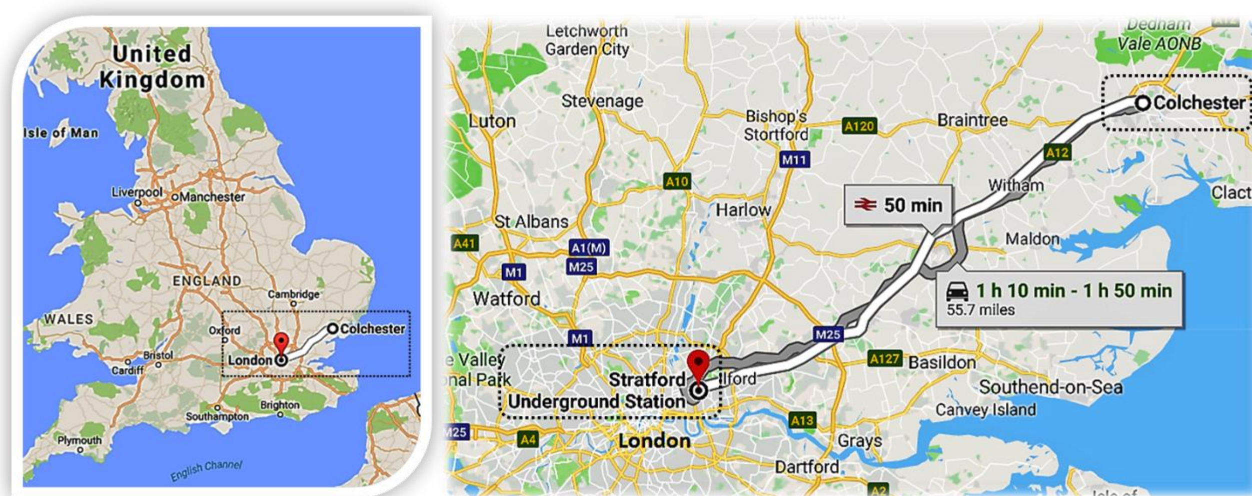


Figure 6.1 Location of origin and destination points of the study route (map on the left). The zoomed map on the right (https://maps.google.co.uk/) shows the railway line (white line) used for train sampling and the road transport route (grey line) used for car sampling.

Train

An overground UK national railway line operated by Greater Anglia, connecting Colchester town to metropolitan London zone 1, was selected for the first part of study. The chosen commute route for the purposes of sampling was a return trip from Colchester station to London Stratford station (East London), one stop before the line terminus (London Liverpool Street). Stratford is a major multilevel interchange station serving various overground and underground lines linking Stratford to other stations in central London, as well as other

counties in the UK. In 2015-16 Stratford was ranked as the 6th station in terms of passengers' number and station usage in Great Britain (https://www.gov.uk/government/statistics/rail-factsheets-2016).

Each sampling consisted of a two-way trip and included the time spent inside the train cabins, as well as the time spent waiting and walking on the (outdoor) station platform for the outward and the return journey. The average journey time between Colchester and Stratford London is 51 minutes (i.e. about 100 minutes for a return journey). However, the total duration of each sampling trip depended on the number of stops, as well as the waiting time between the arrival at the destination station and the departure of the return train, and ranged from 110 to 159 min. In order to get a better representation, sampling was carried out in different time periods, i.e. during morning, afternoon and evening hours. The duration of each journey, as well as the ventilation conditions, are shown on Table 6.1.

Table 6.1 Sampling duration per each two-way train journey, number of stops and ventilation conditions.

Sampling trip	Date	Time in train (min)	Time on platform (min)	Outward journey stops	Outward journey ventilation	Return journey stops	Return journey ventilation	Total duration of sampling at 8LPM (min)
Afternoon (A)	12/9/16	100	34	4	A/C ON	1	A/C ON	134
Evening (A)	12/9/16	111	48	6	windows open	4	A/C ON	159
Evening (B)	13/9/16	121	9	5	windows open	6	windows open	130
Morning (A)	15/9/16	106	23	4	-	6	A/C ON	129
Morning (B)	16/9/16	111	20	5	-	6	A/C on	131
Afternoon (B)	16/9/16	103	7	4	A/C ON	4	-	110

Car

Car sampling was carried out with two different petrol-fuelled vehicles, a 5-door hatchback (car A) and a 5-door wagon (car B). The number of regular passengers in car A varied between 1 and 2 adults, while car B was typically used by 1 - 4 people, including 2 toddlers. For the purposes of the study, the automobiles were occupied with two passengers during sampling. All round trips were carried out during afternoon hours with the duration of each one-way journey ranging from 83 to 106 min. During sampling trips, the vehicle windows were kept closed, while ventilation conditions were set to the personal comfort level of the occupants (Table 6.2).

Table 6.2 Sampling duration per each two-way car journey and ventilation conditions.

Sampling trip	Date	Time inside car/ total duration of sampling at 8LPM	Car ventilation
Car A (1)	11/2/17	193 min	A/C ON 22°C *
Car A (2)	19/2/17	192 min	A/C ON 21°C *
Car A (3)	11/3/17	190 min	A/C ON 20.5^{o}C *
Car B (1)	20/3/17	184 min	car ventilation ON
Car B (2)	22/3/17	173 min	car ventilation ON
Car B (3)	23/3/17	195 min	car ventilation ON

*A/C temperature set by the passengers.

6.2.2 Sampling

Air samples were collected using Advantec polypropylene filter holders (Cole-Parmer, UK) loaded with 47-mm nuclepore polycarbonate filters (0.4 µm-pore size, Whatman, UK). The filters used to collect air samples were sterilised by autoclaving. Filter holders were washed with 10% bleach, rinsed with ultrapure water (Milli-Q, Millipore) and autoclaved at 121°C for 15 mins. Each sampler was sealed with sterilised aluminum foil and was stored in a sealed sterile bag until ready for sampling. A portable battery-operated Leland Legacy Pump (SKC, UK) was used for sampling while commuting by train. The flow rate used was the maximum possible that could be achieved by the pump for the specific filter pore size (i.e. 0.4 µm), that is 8 LPM. Sampling in the cars was performed by drawing air through a Linear Diaphragm LD50 DE pump (Charles Austen, UK), operated at the same flow rate used for the train sampling. Flow rate was controlled with a throttling valve. Power for the pump was supplied by a car power inverter. Filter holders were connected to the pumps using Tygon tubing (R-3603, Sigma-Aldrich, UK). Real-time PM_{10} mass concentration monitoring was carried out using Aerocet 531 instrument (Met One Instruments Inc., USA) connected to a sampling tube (49 cm, 3-mm thick). Temperature, relative humidity and carbon dioxide concentration were recorded every minute using Rotronic CP11 indoor air quality meter. When sampling in train, all sampling equipment was carried out in a backpack that was specially reinforced with soundproofing foam and the inlets were positioned at the breathing height (~ 1.5 m). When sampling in the automobiles, sampling inlets were placed below the driver's seat headrest facing towards the back seats.

6.2.3 Sample processing for molecular analysis of 16S rRNA gene & ITS1 region

Filters were inserted into 5-ml screw-cap tubes containing 0.1-mm zirconium/silica beads and cells were disrupted by bead-beating in SDS-based extraction buffer and incubation at 75°C, followed by phenol-chloroform purification and isopropanol precipitation, with addition of co-precipitant glycogen, and final elution of pelleted DNA in 35 μl sterile water (see Chapter 2 for detailed protocol). The 341F/805R primer pair (Herlemann et al., 2011) amplifying the V3/V4 region of the 16S rRNA gene was used for qPCR quantification assay and Illumina Miseq sequencing using approaches described previously. The ITS1 region of the internal transcribed spacer region was also targeted using fungal-specific primers ITS1-F (Gardes & Bruns, 1993) and ITS2 (White et al., 1990) and used for amplicon sequencing. Liraries were prepared using Illumina Nextera XT Index kit, pooled with an internal control PhiX (20%) and loaded to a v3-chemistry 600 cycle-kit reagent cartridge (Illumina, Inc.). Negative controls (3 filter blanks and 3 no-template extraction controls) were included in all of the molecular analysis steps to check for contamination. Sequencing (2×300 bp) was performed on the Illumina MiSeq platform at the School of Biological Sciences, University of Essex. Sequence processing and data analysis was as described in Chapter 2.

Statistical analysis

Group means were compared using Student's t-tests and one-way analysis of variance (ANOVA) on normally distributed variables, whereas Mann–Whitney U and Kruskal-Wallis tests were used to compare group medians when non-parametric tests were necessary. Correlations between bacterial abundance, microbial richness and environmental variables were assessed using Spearman's rank coefficient analysis. Mantel tests were also used to examine the relationships between Bray-Curtis dissimilarity matrices and Euclidean distance matrices of bacterial abundance and environmental variables. Venn diagram analysis was performed using jvenn (Bardou et al., 2014). For the purposes of analysis, in many cases, samples obtained during commuting by train in the morning (n=2), in the afternoon (n=2) and in the evening (n=2) as well as samples obtained in car A (n=3) and car B (n=3) were grouped in order to give an average representation of the microbial abundance and diversity.

6.3 Results

6.3.1 PM_{10} levels and environmental parameters

Results showed that median PM_{10} concentrations differed significantly among journeys (Kruskall Wallis, p-value < 0.05), ranging from 4 μg/m^3 (afternoon) to 10 μg/m^3 (morning) and 23 μg/m^3 (evening) for trains, while medians in automobiles were found to be in the range from 4 μg/m^3 (car B) to 7 μg/m^3 (car A). The real-time profiles of PM_{10} mass and CO_2 concentrations per each train and car journey are presented in Figures 6.2 and 6.3. Summary boxplots are also provided (Fig. 6.4 and 6.5).

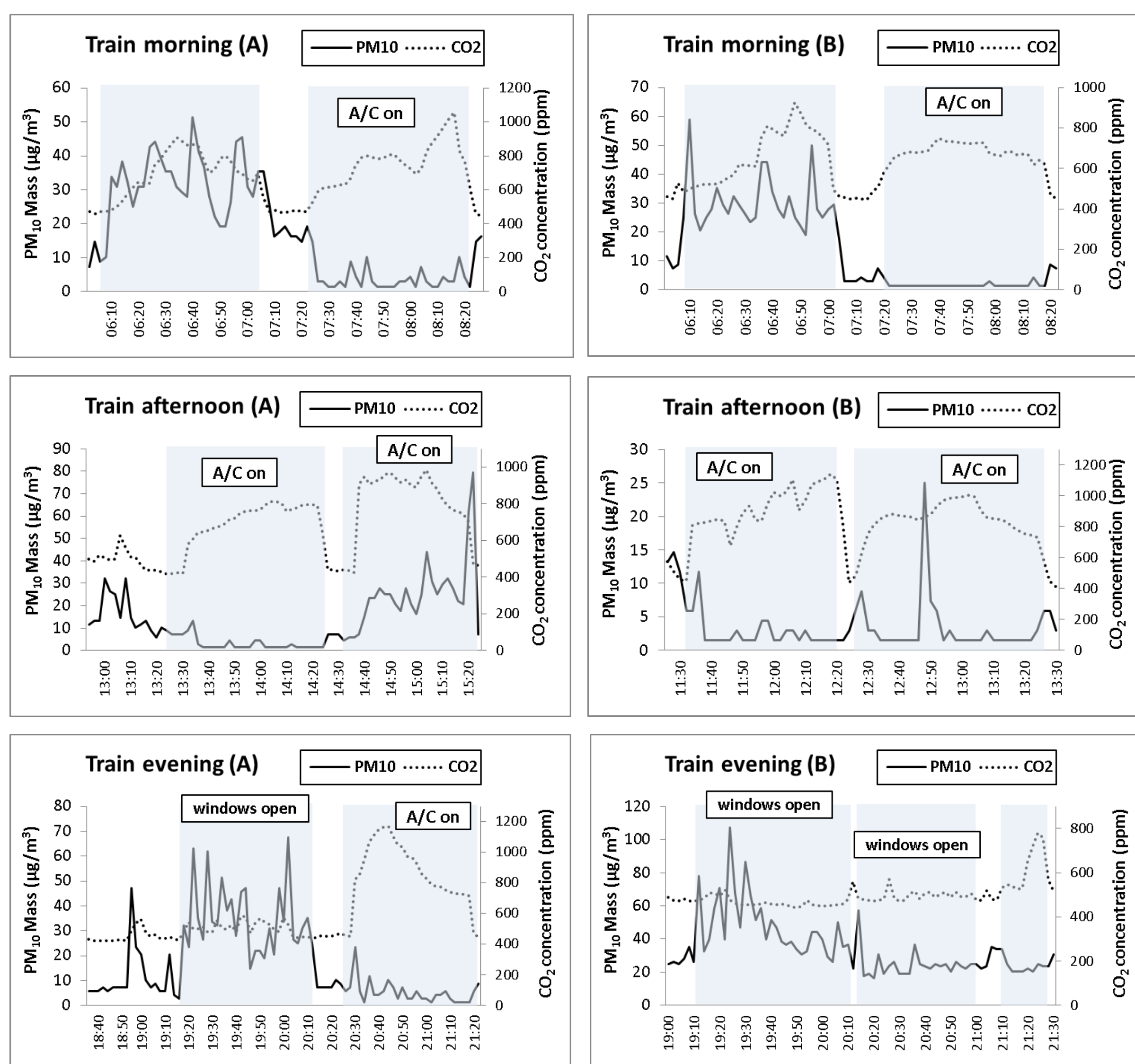


Figure 6.2 Time series of PM_{10} mass and CO_2 concentrations during each train sampling trip. The shaded regions denote the periods of time spent inside the train cabins (outward and return journey).

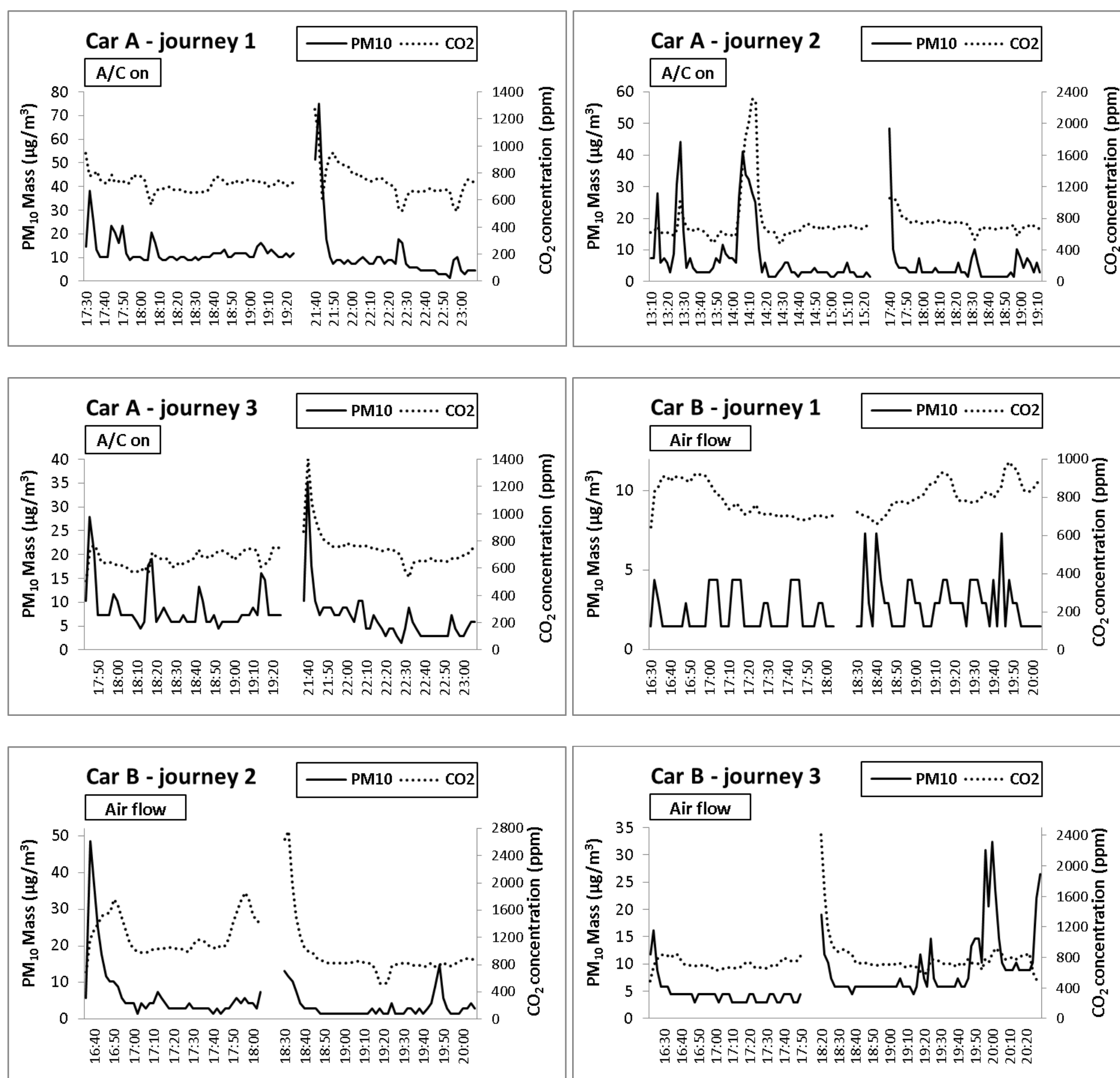


Figure 6.3 Time series of PM_{10} mass and CO_2 concentrations during each round trip by car.

Similarly, the CO_2 levels also varied significantly among the journeys (Kruskal Wallis, p-value < 0.05). The lowest median concentration (478 ppm) was observed during the evening trips that windows in the trains were kept open. Median values for the morning and afternoon trips were 669 ppm and 792 ppm, respectively. Between the two automobiles, car A that had the A/C system switched on during all three sampling trips exhibited lower median concentration (697 ppm) compared to car B (median = 781 ppm).

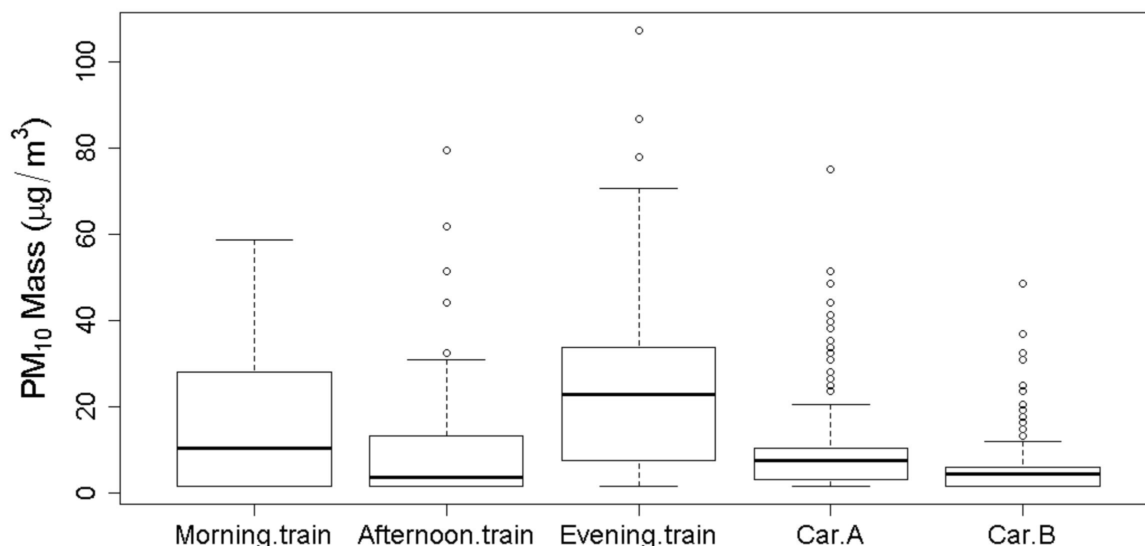


Figure 6.4 Box and whisker plots of PM_{10} mass concentration in µg per cubic meters of air per time-zone of train journey (morning, afternoon and evening, n=2) and type of car (A and B, n=3). Outliers are indicated with circles beyond the whiskers.

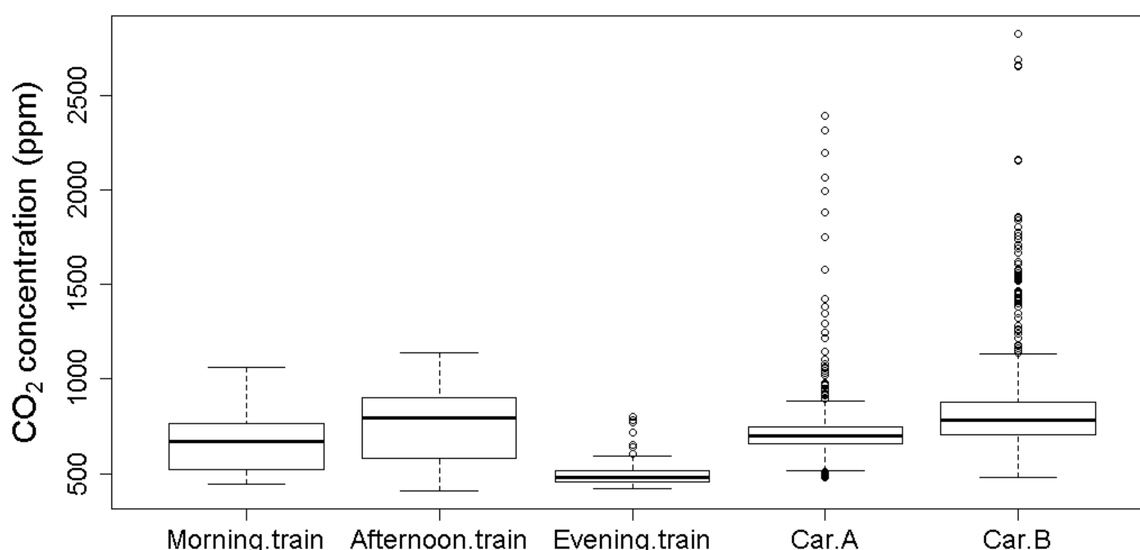


Figure 6.5 Box and whisker plots of carbon dioxide concentration in ppm per time-zone of train journey (morning, afternoon and evening, n=2) and type of car (A and B, n=3). Outliers are indicated with circles beyond the whiskers.

Table 6.3 shows the range of the recorded microclimate parameters. For the train trips, the median temperature ranged from 23.3°C (morning A and B) to 27.1°C (evening B) and the median relative humidity varied between 46% (afternoon A) to 71% (morning B). For the car journeys, the median temperature was higher in car A (18.7 - 20.8°C), due to the use of the air conditioning system, compared to car B (16.4 - 18.6°C). Finally, the relative humidity median levels were lower in car A (31- 39%) than in car B (43 - 59%).

Table 6.3 Temperature and relative humidity levels (median ± S.D) measured during each return journey by train and by car.

TRAIN			CAR		
Sampling trip	**Temperature (°C)**	**Rel. Humidity (%)**	**Sampling trip**	**Temperature (°C)**	**Rel. Humidity (%)**
Morning (A)	23.3 ± 1.6	63 ± 5	Car A – journey 1	20.8 ± 2.2	31 ± 5
Morning (B)	23.3 ± 1.6	71 ± 6	Car A – journey 2	20.2 ± 1.0	36 ± 4
Afternoon (A)	25.8 ± 2.8	46 ± 7	Car A – journey 3	18.7 ± 0.8	39 ± 4
Afternoon (B)	23.8 ± 1.6	62 ± 6	Car B – journey 1	16.8 ± 1.0	59 ± 3
Evening (A)	25.4 ± 1.1	56 ± 5	Car B – journey 2	16.4 ± 1.9	56 ± 8
Evening (B)	27.1 ± 1.1	57 ± 4	Car B – journey 3	18.6 ±1.9	43 ± 5

6.3.2 Abundance of airborne bacteria

The bacterial abundance was determined by qPCR of 16S rRNA gene based on a standard curve constructed using *E.coli* genomic DNA (qPCR efficiency = 97.2%, slope = 3.39, R^2=0.99, y-intercept=37.43, Ct values of the non-template controls > 36) and concentrations were expressed as the number of gene copies per cubic meter of air. Bacterial levels during commuting by train ranged from 4.03×10^5 to 6.57×10^5 16S rRNA gene copies/m^3 of air during morning, from 3.07×10^5 to 5.52×10^5 gene copies/m^3 during afternoon and from 6.61×10^5 to 8.97×10^5 copies/m^3 during evening journeys. Bacterial concentrations during automobile journeys varied from 3.53×10^5 to 4.78×10^5 16S rRNA genes/m^3 in car A and from 4.21×10^4 to 1.54×10^5 gene copies/m^3 in car B.

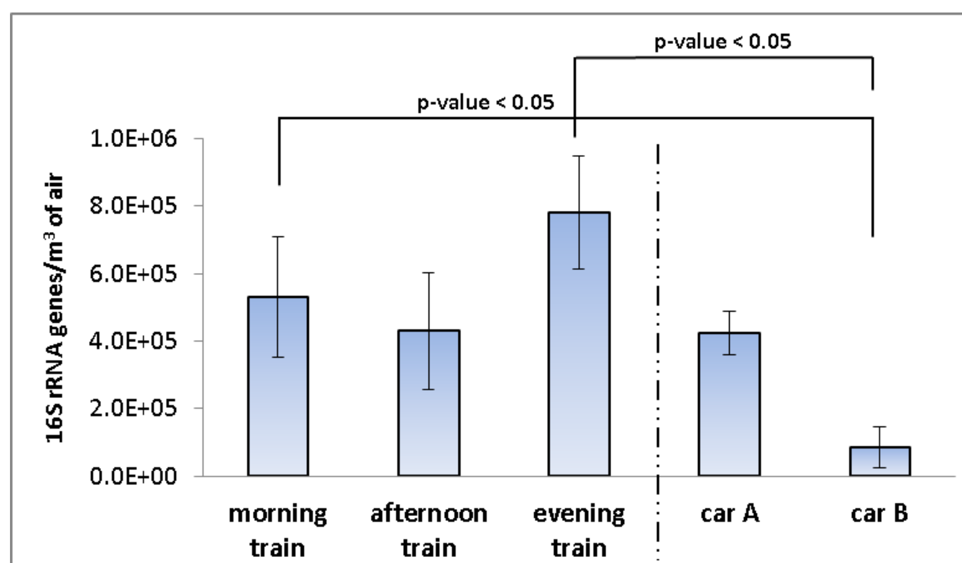


Figure 6.6 Number of 16S rRNA gene copies per cubic meter of air per time-zone of train journey (morning, afternoon and evening, n=2) and type of car (A and B, n=3), as determined by qPCR. Error bars represent standard deviation of the mean. Tukey's HSD pair-wise comparisons were used to denote statistical significance (p-value < 0.05) between types of journeys.

Overall, bacterial levels were found to vary significantly among commuting trips (one-way ANOVA, p-value = 0.005). Subsequent post hoc Tukey's honest significant difference (HSD) tests indicated that there were no significant differences found between train journeys during different time periods or between journeys by train and car A (p-value>0.05). However, bacterial concentrations in trains were found to be significantly higher compared to car B, during morning (p-value = 0.03) and during evening (p-value = 0.003) trips. Results are summarised in Fig. 6.6.

When comparing the two types of transport mode (Fig. 6.7), independent samples t-test confirmed that bacterial levels were overall significantly greater in train journeys compared to car journeys (p-value = 0.019 < 0.05). Likewise, PM_{10} concentration was also significantly higher during commuting by train (Mann-Whitney U test, p-value < 0.001).

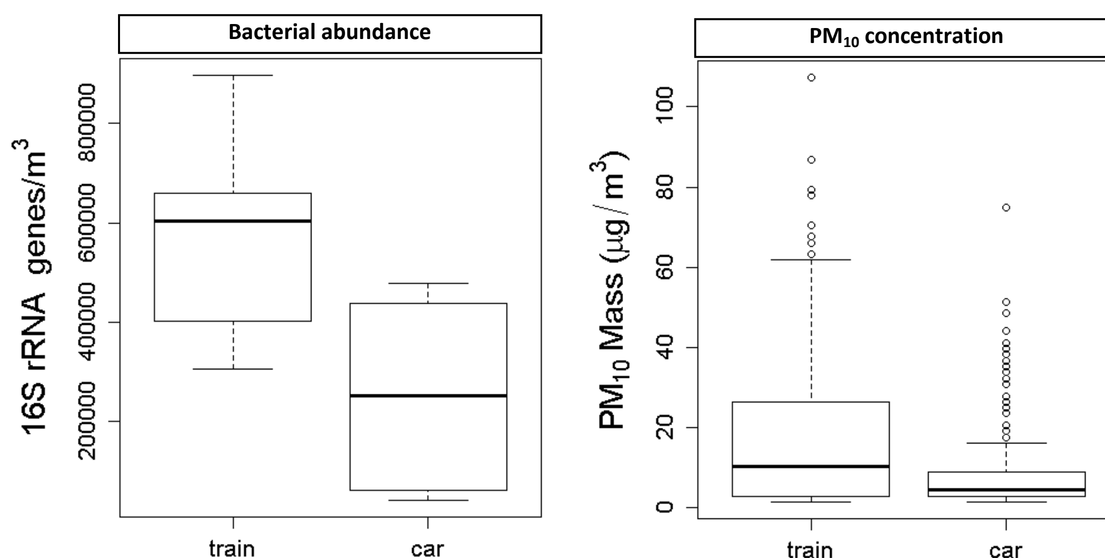


Figure 6.7 **(a)** On the left panel: Bacterial abundance (16S rRNA gene copies/m^3) and **(b)** On the right panel: PM_{10} mass concentration (µg/m^3) for the total of air samples collected during commuting by train and by car.

Spearman's rank coefficient analysis (Table 6.4) demonstrated that there is a significantly strong positive association between bacterial abundance and PM_{10} concentration (ρ = 0.85) and also with temperature (ρ = 0.85). A negative correlation was found between bacterial abundance and carbon dioxide levels (ρ = 0.64).

Table 6.4 Spearman's rank correlations between bacterial abundance, as measured via qPCR, and recorded environmental parameters. Bolded values indicate statistical significance (p-value < 0.05).

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	PM_{10}	CO_2	T	RH
Rho (ρ)	**0.85**	**-0.64**	**0.80**	0.02
p-value	< .001	0.030	0.002	0.948

6.3.3 Diversity of airborne bacteria and fungi

A total of 625,162 bacterial and 629,002 fungal sequencing reads were generated by the MiSeq run for all samples (n=12) and negative controls (n=6). After quality trimming and chimera filtering, a total of 312,155 16 rRNA gene and 335,359 ITS region sequences clustered into 10,060 bacterial and 2,970 fungal OTUs, respectively. After removal of unassigned reads, non-bacterial/non-fungal sequences and subtraction of the number of sequencing reads in the controls from the samples, 203,668 16S rRNA gene reads (median/sample = 11,040 ± 10,251 read counts), representing 8,394 bacterial OTUs, and 332,307 ITS reads (median/sample = 27,374 ± 4,462 read counts), corresponding to 2,413 fungal OTUs were retained. The numbers of sequences per samples were normalised based on the number of sequences obtained from the smallest library (3,837 sequences for bacteria and 20,066 for fungi). The total numbers of remaining sequences were 46,044 for bacteria and 240,792 for fungi. Abundance-based filtering carried out to remove OTUs with 5 or fewer counts further reduced the numbers to 19,618 bacterial and 224,321 fungal sequences represented by 139 bacterial and 347 fungal OTUs.

Table 6.5 OTU richness for bacteria and fungi detected in the air of trains and automobiles, based on operational taxonomic units (OTUs) with 97% sequence similarity.

TRAIN			CAR		
Sampling trip	**Bacteria**	**Fungi**	**Sampling trip**	**Bacteria**	**Fungi**
Morning (A)	115	186	Car A – journey 1	114	158
Morning (B)	109	193	Car A – journey 2	98	161
Afternoon (A)	115	116	Car A – journey 3	101	120
Afternoon (B)	104	148	Car B – journey 1	52	99
Evening (A)	118	178	Car B – journey 2	63	118
Evening (B)	108	169	Car B – journey 3	74	88

The number of different bacterial OTUs (Table 6.5) detected in the air samples was nearly the same for the train (104 - 118) and car A (98 - 114), whereas a lower level of (alpha) diversity was observed for car B (52 - 74). The OTU richness was higher for fungi compared to the

one obtained for bacteria, in all the samples, ranging from 116 to 193 in the train, 120 to 161 in car A and 88 to 111 in car B.

Significant positive correlations were found between bacterial OTU richness and bacterial abundance (ρ = 0.84), PM_{10} concentration (ρ = 0.66) and also with temperature (ρ = 0.79), as shown by Spearman's rank test (Table 6.6). Fungal OTU richness was only found to be significantly associated with carbon dioxide concentration (ρ = -0.66).

Table 6.6 Spearman's rank correlations between OTU richness, bacterial abundance, as determined by qPCR, and recorded environmental parameters. Bolded values indicate statistical significance (p < 0.05).

	Bacterial diversity					**Fungal diversity**			
	Abundance	**PM_{10}**	**CO_2**	**T**	**RH**	**PM_{10}**	**CO_2**	**T**	**RH**
Rho (ρ)	**0.84**	**0.66**	-0.42	**0.79**	0.12	0.36	**-0.66**	0.51	0.39
p-value	< .001	0.019	0.170	0.002	0.712	0.247	0.022	0.087	0.207

Beta diversity analyses using the abundance-based Bray-Curtis dissimilarity metric revealed different patterns across bacterial and fungal datasets. For bacteria, principal coordinate analysis (PCoA) (Fig. 6.8a) showed that samples obtained during commuting by train were grouped together. In terms of commuting time zone, more clear clustering was observed between the two afternoon, as well as between the two evening, samples. Air samples collected in car A clustered together, whereas samples obtained during commuting with car B appeared to be less similar. However, for fungi, samples corresponding to car A were scattered, in contrast to car B that two of the three samples were closer together. Similarly to results obtained for bacteria, all points representing fungal composition in trains were grouped together, with more clear clustering observed between the two samples collected during evening trips.

Permutation-based multivariate analysis of variance (PERMANOVA) performed on the Bray-Curtis matrices demonstrated that the variation between the train and car samples was statistically significant for both bacteria (pseudo-$F_{1,10}$ = 2.90, R^2= 0.22, p-value = 0.002) and fungi (pseudo-$F_{1, 10}$ = 8.58, R^2= 0.46, p-value = 0.002), with the variation between car A and car B being insignificant (bacteria: pseudo-$F_{1,4}$ = 4.07, R^2= 0.50, p-value = 0.1, fungi: pseudo-$F_{1,4}$ = 2.56, R^2= 0.39, p-value = 0.1).

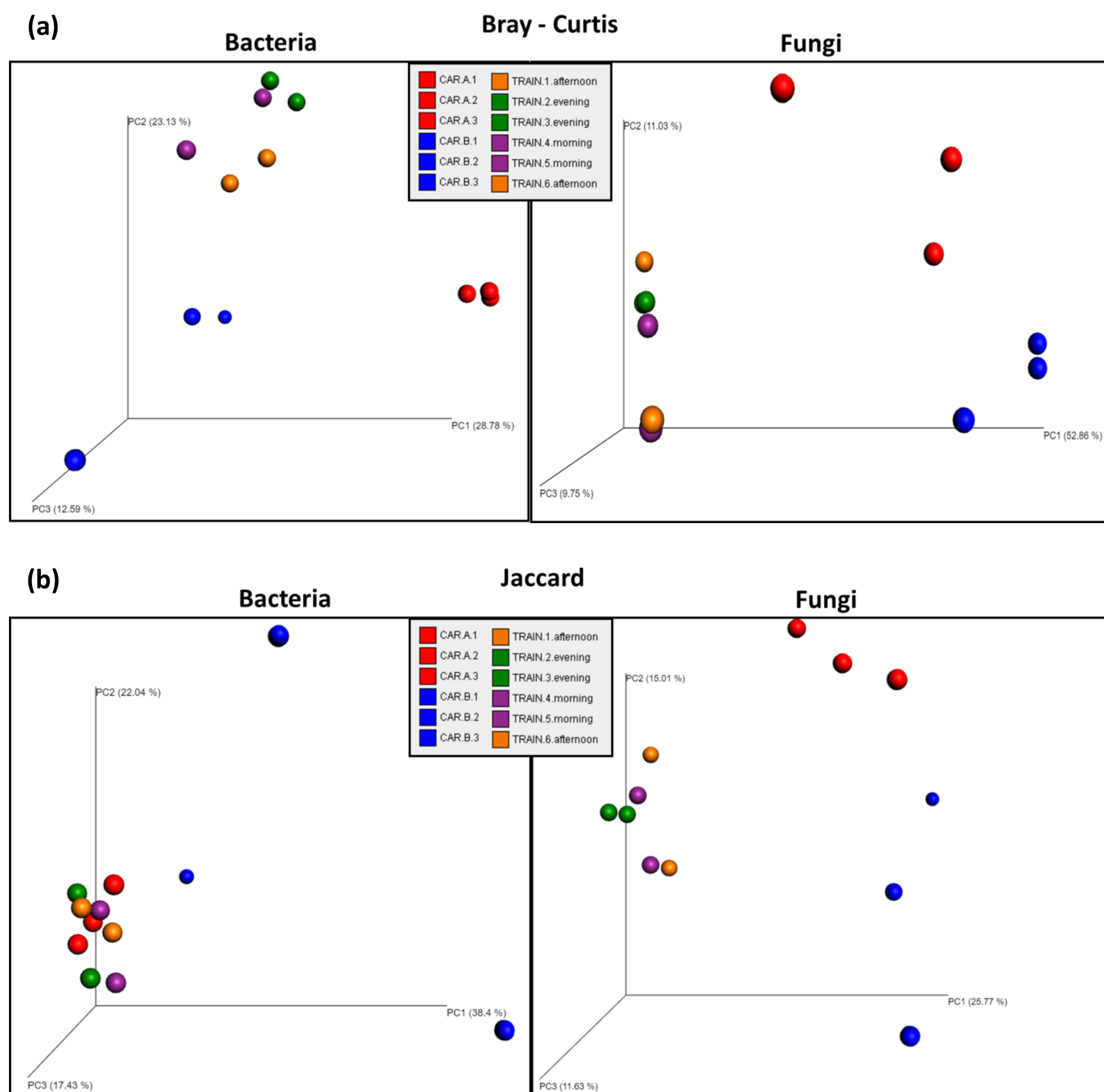

Figure 6.8 Principal coordinate analysis 3D-plots of bacterial and fungal beta diversity based on Bray-Curtis dissimilarity **(a)** and Jaccard distance matrix **(b)**. Samples TRAIN morning 4 & 5 correspond to "Morning (A)" & "(B)", TRAIN afternoon 1 & 6 correspond to "Afternoon (A)" & "(B)" and TRAIN 2 & 3 evening correspond to "Evening (A)" & "(B)". Points in greater proximity correspond to more compositionally similar samples.

However, comparison via Jaccard's distance (Fig. 6.8b), a qualitative diversity metric which is based on presence or absence of taxa, demonstrated clearly that air samples from car A appeared to be more similar to samples collected during train commutes and more dissimilar to the ones from car B, in terms of bacterial composition (see also distance boxplots in Appendix S6.2). Therefore, the transport mode had a weaker overall effect on bacterial

composition, as revealed by PERMANOVA (pseudo-$F_{1,10}$ = 1.82, R^2= 0.15, p-value = 0.007). The type of transport environment was also found to be a less strong factor for the fungal composition (pseudo-$F_{1,10}$ = 3.18, R^2= 0.24, p-value = 0.002), compared to the Bray-Curtis-based estimation. Nevertheless, the effect size was still larger compared to the one on bacteria, in agreement with results obtained based on the Bray-Curtis metric.

The bacterial composition between the two transport modes was also compared using the phylogenetically-informed UniFrac metric and principal coordinate analysis of bacterial beta diversity based on the weighted and unweighted distance matrices exhibited similar patterns with the aforementioned results obtained using Bray-Curtis and Jaccard indices, respectively (see Appendix S6.2).

Finally, Mantel tests used to investigate the relationship between beta diversity (Bray-Curtis) and the variation in environmental parameters between samples (Euclidean distance), showed significant and positive correlations only for qPCR-estimated bacterial abundance for bacteria ($r = 0.52$, p-value<0.001) and temperature for both bacteria ($r = 0.48$, p-value<0.001) and fungi ($r = 0.78$, p-value<0.001).

6.3.4 Overview of microbial composition

Airborne bacteria

As illustrated in Fig. 6.9, both air sample types were dominated by bacterial OTUs affiliated with the phyla Proteobacteria (mean relative abundance in trains 37% and in cars 30%), Firmicutes (train 20%; car 36%), Actinobacteria (train 34%; car 16%) and Bacteroidetes (train 6.1%; car 13%). Smaller proportions of Cyanobacteria (train 3.1%; car 4.4%) and Deinococcus-Thermus (train 0.32%; car 0.76%) were also detected. At the class level, *Actinobacteria* showed the highest relative abundance across all train samples (train 37%, car 16%), followed by *Gammaproteobacteria* (train 22%; car 14%). For the car samples, the most abundant bacterial classes on average were *Bacilli* (car 33%; train 18%) and *Actinobacteria* (Fig. 6.9).

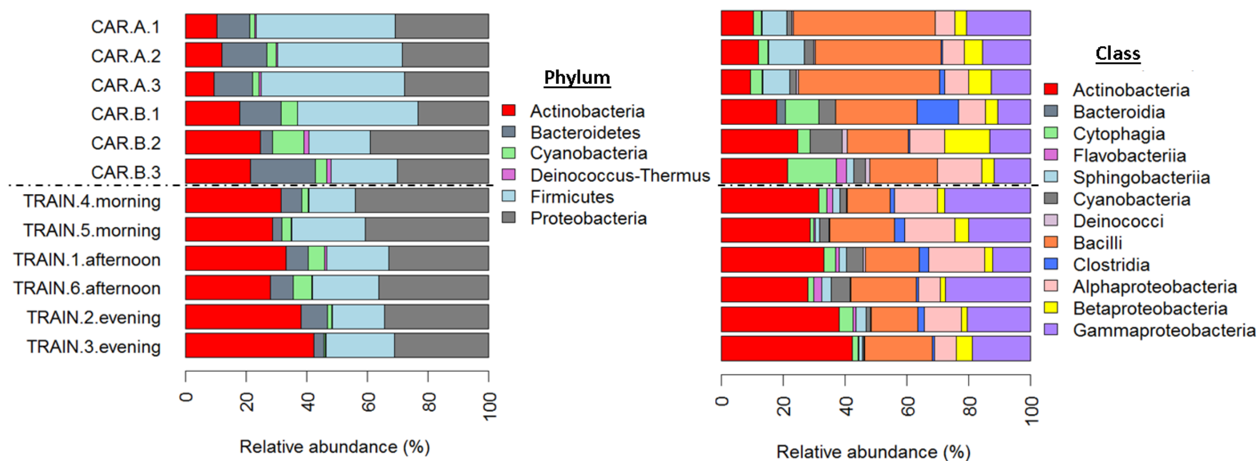

Figure 6.9 Relative abundance of bacterial OTUs at the phylum and class level per each sample. Samples TRAIN morning 4 & 5 correspond to “Morning (A)” & “(B)”, TRAIN afternoon 1 & 6 correspond to “Afternoon (A)” & “(B)” and TRAIN 2 & 3 evening correspond to “Evening (A)” & “(B)”.

At the genus level (Fig. 6.10), on average, the most abundant detected bacteria across the train samples were human commensal genera residing on skin or in the oral cavity (Grice & Segre, 2011; Dewhirst et al., 2010), including *Corynebacterium* (11.2%), *Staphylococcus* (10.5%), *Acinetobacter* (7.3%) and *Kocuria* (4.5%). *Corynebacterium* (10.9%) was also the most dominant genus encountered in car B. *Pseudomonas spp.*, that are widely distributed in the environment, were also highly enriched in the train samples (8.1%) and in car A (11.2%). Interestingly, samples obtained from car A exhibited high relative abundance of *Carnobacterium* (24.5%) and some genera originating from soil, water and plants, such as *Planomicrobium* (8.0%), *Pedobacter* (9.3%) and *Massilia* (4.9%). These taxa were present at much lower relative abundances in the other samples (car B 0.7%; train 0.4%). *Hymenobacter* (5.8%) and *Cyanobacteria GpI* (6.6%), which are both genera associated with outdoor environmental sources, were more abundant in the air samples collected during commuting with car B. *Streptococcus and Gemella*, that are part of the oral microbiome (Dewhirst et al., 2010), were also present at higher relative abundance (8.5% and 4.3% respectively) in car B compared to car A (1.37% and 0.34%) and the trains (2.45% and 0.64%).

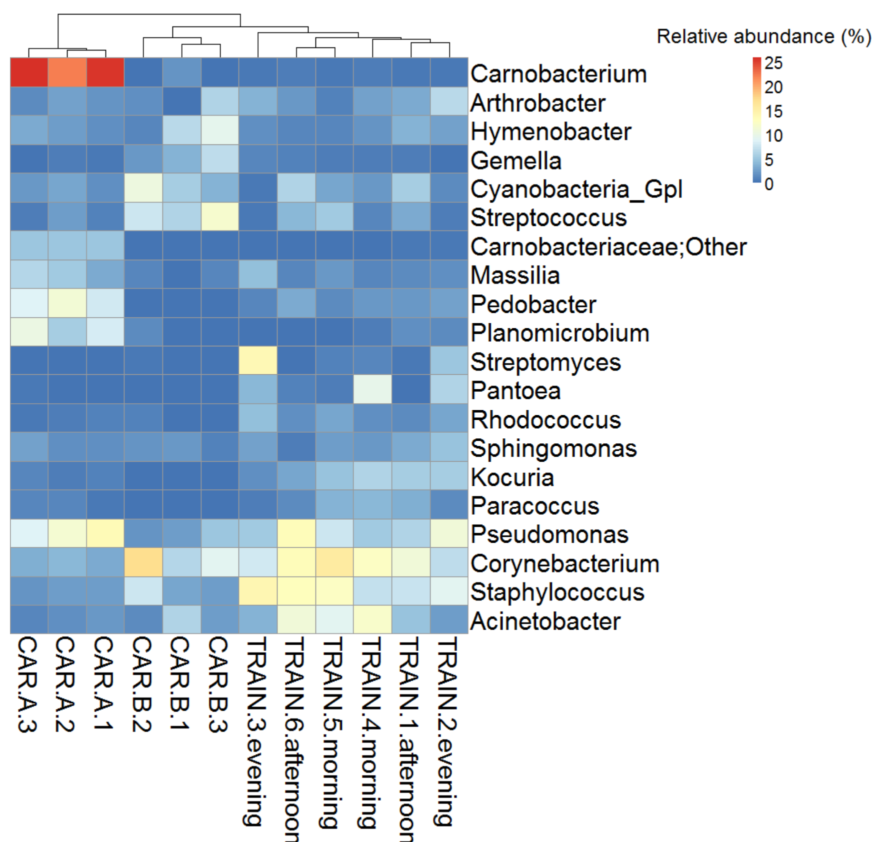

Figure 6.10 Heatmap displaying the relative abundance of the 20 most dominant bacterial genera across the samples. Samples TRAIN morning 4 & 5 correspond to "Morning (A)" & "(B)", TRAIN afternoon 1 & 6 correspond to "Afternoon (A)" & "(B)" and TRAIN 2 & 3 evening correspond to "Evening (A)" & "(B)".

Among the most frequently encountered bacteria, genera that comprise opportunistic pathogenic species are *Staphylococcus* (Arcer, 1998), *Streptococcus* (Stevens et al., 1989), *Corynebacterium* (Barka et al., 2016), *Acinetobacter* (Weber et al., 2016), *Kocuria* (Purty et al., 2013), *Pseudomonas* (Stover et al., 2000) and *Gemella* (Anil et al., 2007). *Pantoea* genus that was detected at high abundance in Morning train A (TRAIN 4 - 9.7%) and Evening train B (TRAIN 2 - 6%) has also been implicated as the cause of significant infections (Cruz et al., 2007). Last, *Streptomyces* bacteria that were mainly detected during the evening commutes by train (4.9% and 13.8% for TRAIN 2 & 3 respectively) have also been reported as infrequent human pathogens (Kirby et al., 2012).

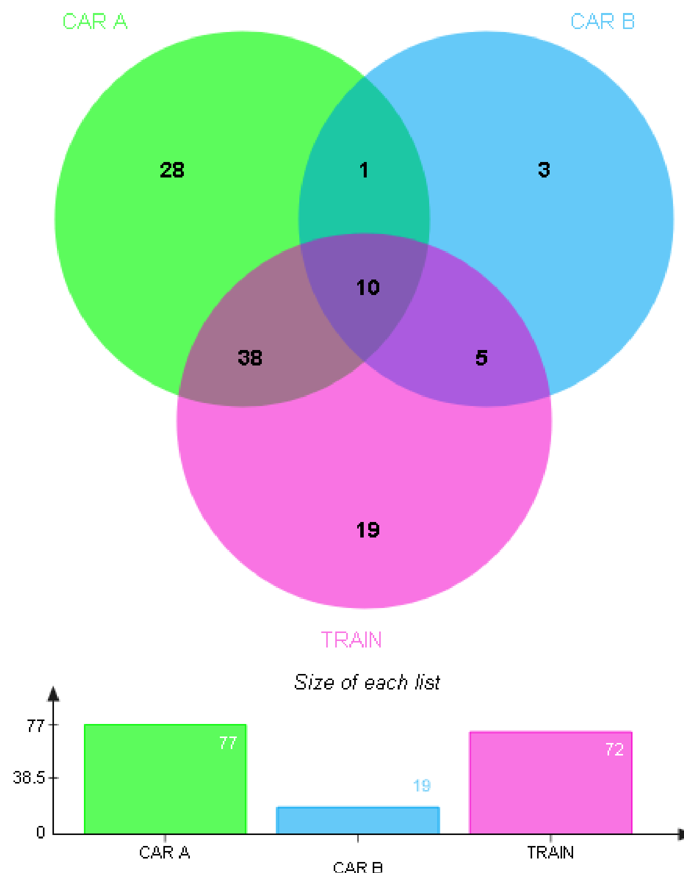

Figure 6.11 Venn diagram displaying the number of shared (overlapping regions) and unique bacterial core OTUs among the two automobiles (car A and car B) and the train. The number of common OTUs present across all samples (i.e. 'core OTUs') per each transport environment is shown in the bar graph below.

The common bacteria found across all air samples ('core OTUs') collected during commuting by each transport mean (i.e. train, car A and car B) were also determined. Fig. 6.11 shows the numbers of shared and unique OTUs among the 3 core datasets. In total, 53 OTUs were found to overlap between the 85 identified core OTUs from both automobiles and the 72 core OTUs from the train. From those, 48 OTUs were shared between the train and car A, while 15 OTUs were present in both the train and car B. However, only a set of 11 OTUs was found to be common between the two vehicles, from which 10 OTUs were also present across all the train samples. The bacteria that were found in varying abundances in the air of all three transport microenvironments belonged to the human-associated *Corynebacterium* spp. (2 OTUs), *Streptococcus* spp., *Staphylococcus* spp. (2 OTUs) and outdoor environment-related taxa *Blastococcus*, *Brachybacterium*, *Hymenobacter*, *Arthrobacter* and

Carnobacterium. The genera representing the unique OTUs present in each core dataset are shown in Table 6.7.

Table 6.7 Genus of unique core OTUs encountered only in the train or the car environment. The number in brackets denotes the occurrence of the OTU.

Train	Automobile	
Pedobacter	**Car A**	*Microbacteriaceae* (unidentified)
Pseudomonas	*Skermanella*	*Sphingomonas* (2)
Acinetobacter (3)	*Acetobacteraceae* (unidentified) (2)	*Neisseria*
Corynebacterium (4)	*Algoriphagus*	*Massilia* (2)
Finegoldia	*Carnobacteriaceae* (unidentified)	*Acinetobacter*
Empedobacter	*Virgibacillus*	*Polaromonas*
Romboutsia	*GpI* (unidentified) (2)	*Thermoactinomyces*
Lactobacillus	*Streptococcus*	*Arthrobacter*
Brevibacterium	*Psychrobacter* (2)	**Car B**
Streptomyces	*Mycobacterium*	*Gemella*
Brachybacterium	*Planomicrobium* (2)	*Haemophilus*
Blastococcus	*Sanguibacter*	*Neisseria*
Pantoea	*Carnobacterium*	**Car A & Car B**
Moraxellaceae (unidentified)	*Pseudomonas* (2)	*Nocardioides*

Airborne fungi

Fungal diversity was represented by Ascomycota and Basidiomycota, with distinct different proportions observed for the two types of transport means (Fig. 6.12). 70% of the OTUs obtained for the car samples (mean relative abundance) were assigned to Basidiomycota**,** with the remaining reads assigned to Ascomycota. In contrast, the predominant detected phylum in all the train samples was Ascomycota (80% of total reads, on average). The majority of Basidiomycota in cars was represented by *Agaricomycetes* (53% of the overall population), *Tremellomycetes* (8%) and *Wallemiomycetes* (4.4%), while the second most abundant class detected in the automobile environment belonged to Ascomycota (*Dothideomycetes* - 19%). The top three most abundant classes in the trains and their averaged relative abundances were Ascomycetous *Dothideomycetes* (58% of the overall population), and *Agaricomycetes* (8%) and *Sordariomycetes* (7.1%) within the division of Basidiomycota.

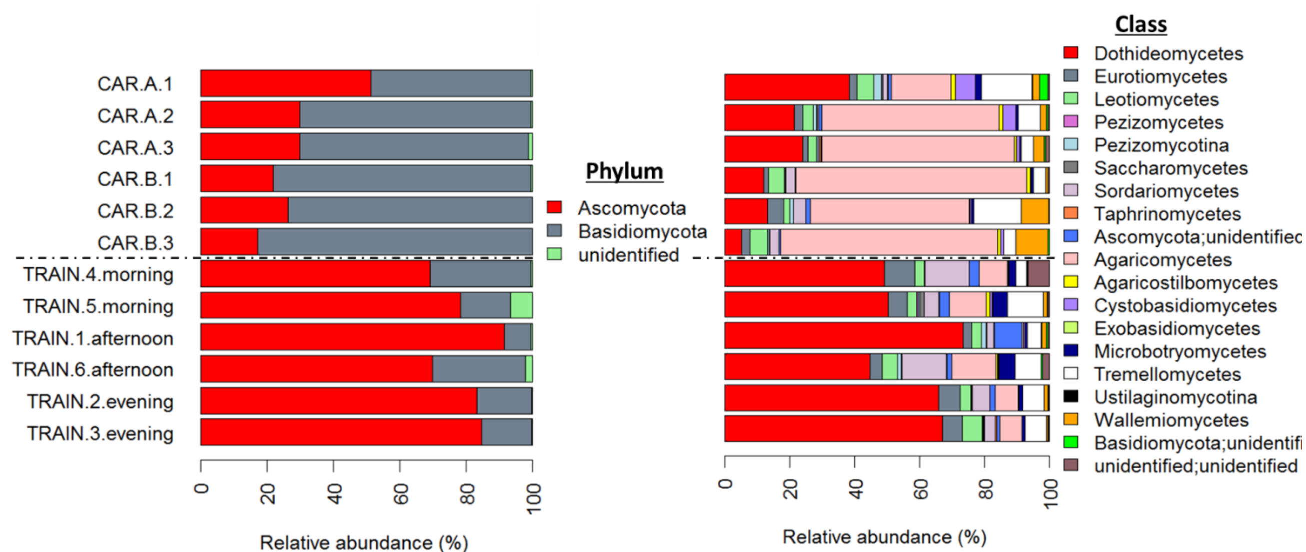

Figure 6.12 Relative abundance of fungal OTUs at the phylum and class level per each sample. Samples TRAIN morning 4 & 5 correspond to “Morning (A)” & “(B)”, TRAIN afternoon 1 & 6 correspond to “Afternoon (A)” & “(B)” and TRAIN 2 & 3 evening correspond to “Evening (A)” & “(B)”.

Fig. 6.13 shows the 20 most dominant fungal taxa at the genus level. On average, the most abundantly detected fungal genera (Fig. 5B.12) in both trains (26%) and car A (19%) was *Cladosporium* spp. (Ascomycota, *Dothideomycetes*), while the wood-degrading basidiomycete *Trametes* (*Agaricomycetes*) was the most abundant genus in car B (19%). Other abundant genera that were more highly represented in the train environment, compared to the car interiors that they were present in lower proportions (0.04 - 3%), were ascomycetes *Mycosphaerella* (13%), *Alternaria* (6%), *Aureobasidium* (5.1%) and *Penicillium* (4.7%). From the other hand, apart from *Trametes* (car A 13%; car B 19%), *Agaricomycetes Bjerkandera* (car A 14%; car B 15%) and *Daedaleopsis* (car A 4.3%; car B 4%) were highly enriched mainly in the two study cars. Other highly abundant fungi that could not be attributed to a genus (unidentified) belonged to Ascomycota and saprotrophic or plant-related taxa, such as the class of *Agaricomycetes*, the orders of *Cantharellales* (class *Agaricomycetes*) and *Helotiales* (class *Leotiomycetes*, Ascomycota) and the family of *Diatrypaceae* (order *Xylariales*, class *Sordariomycetes*, Ascomycota). Air samples from car A were also found to be more enriched (mean relative abundance 3.61%) in the skin-associated yeast *Cryptococcus* (Findley et al., 2013), compared to samples from trains (0.89%) and car B (0.09%). The human skin-associated yeasts of *Malassezia*, *Candida*,

Rhodotorula and *Trichosporon* were also detected in the majority of the samples, but only at very low fractions (<0.5%).

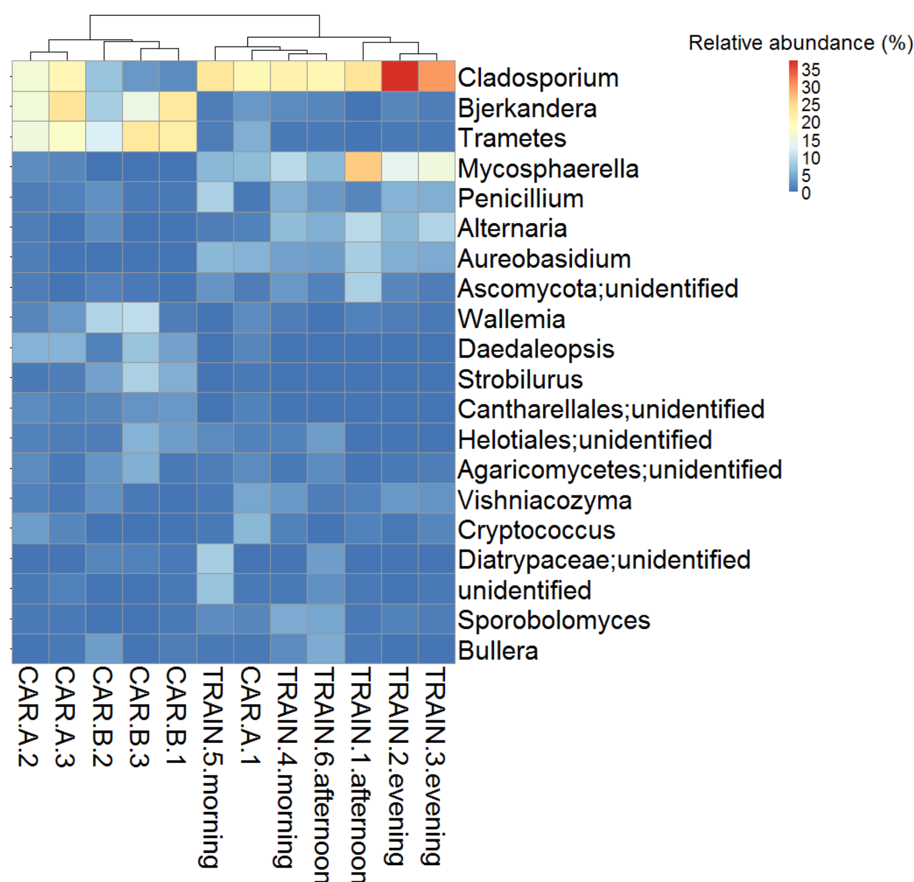

Figure 6.13 Heatmap displaying the relative abundance of the 20 most dominant fungal genera across the samples. Samples TRAIN morning 4 & 5 correspond to "Morning (A)" & "(B)", TRAIN afternoon 1 & 6 correspond to "Afternoon (A)" & "(B)" and TRAIN 2 & 3 evening correspond to "Evening (A)" & "(B)".

Among the highly abundant genera detected in the samples, *Cladosporium*, *Alternaria* and *Penicillium* are well-known genera associated with important allergenic species (Burge, 1985; Gravesen, 1979; Hyde et al., 1956). Sensitization to the ubiquitous Ascomycetous *Aureobasidium* and the wood-rotting Basidiomycetous *Bjerkandera* has been linked to the development of allergic rhinitis and asthma (Niedoszytko et al., 2007; Taylor et al., 2006) and allergic fungal cough (Ogawa et al., 2009), respectively. *Wallemia* genus (basidiomycete, trains 0.67%; car A 2.35%; car B 6.38%), one of the common molds found indoors (Adan & Samson, 2011), has been identified as a potential fungal allergen (Sakamoto et al., 1989), too. Last, detected basidiomycetous yeasts *Cryptococcus* (train 0.89%; car A 3.61%; car B 0.09%)

and *Sporobolomyces* (train 2.26%; car A 0.89%; car B 0.32%), comprise medically important infectious species (Khawcharoenporn et al., 2007; Loftus et al., 2005; Morris et al., 1991; Cockcroft et al., 1983). The common widespread genus *Aspergillus*, one of the best-known causes of fungal diseases (Marr et al., 2002), was only detected at low proportions in the air of the study trains (0.45%) and automobiles (car A 0.12%; car B 1.27%).

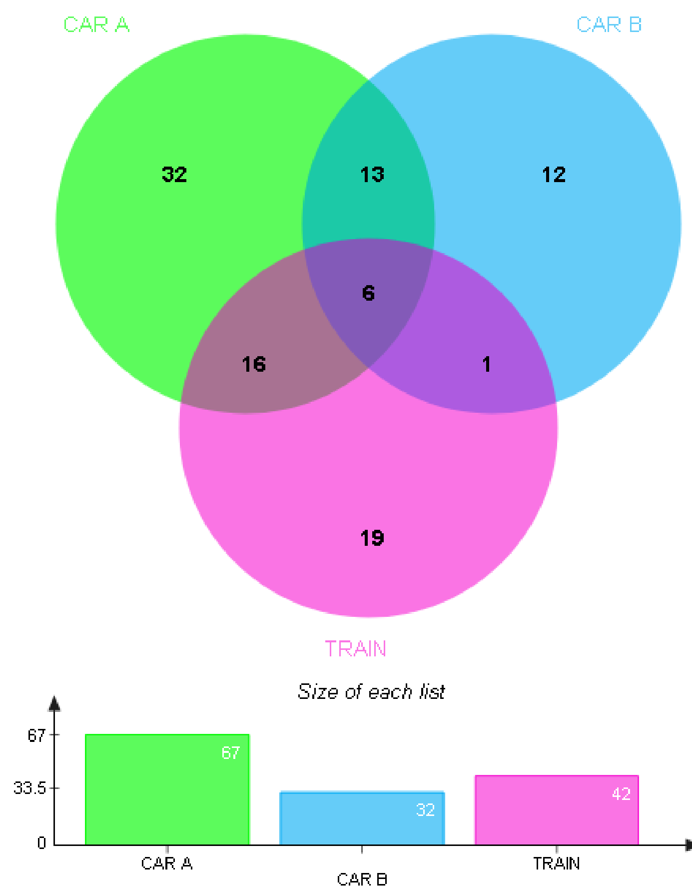

Figure 6.14 Venn diagram displaying the number of shared (overlapping) and unique fungal core OTUs among the two automobiles (car A and car B) and the train. The number of common OTUs present across all samples (i.e. 'core OTUs') per each transport environment is shown in the bar graph below.

The numbers of common and distinct fungal phylotypes among the core OTUs identified for each study transport site is shown in Fig. 6.14. Overlapping the OTUs showed that the total number of common OTUs between the automobiles and the train core datasets was 23, from which 22 were shared between car A and the train, while only 7 taxa were present in both car B and the train. The OTUs shared between the two study cars were 19, whereas only 6 OTUs

were found in all three fungal core datasets. The shared taxa present in both cars and train air samples were affiliated with the ubiquitous airborne fungi *Cladosporium* spp. (2 OTUs) and *Alternaria* spp., as well as the saprotrophic fungi *Trametes* spp., *Mycosphaerella* spp. and *Sporobolomyces* spp. The unique OTUs found in each transport environment are listed in Table 6.8.

Table 6.8 Genus of unique core OTUs encountered only in the train or the car environment. The number in brackets denotes the occurrence of the OTU.

Train	**Automobile**	
Articulospora	**Car A**	**Car B**
Hypocreaceae (unidentified)	*Buckleyzyma*	*Hyphodontia*
Neodevriesia	*Saccharomyces*	*Vestigium*
Phlebia	*Ascomycota* (unidentified) (2)	*Aspergillus*
Plectosphaerella	*Ramularia*	*Helotiales* (unidentified) (2)
Dissoconium	*Extremus*	*Steccherinum*
Debaryomyces	*Articulospora*	*Agaricomycetes* (unidentified)
Phaeosphaeria	*Flammulina*	*Skeletocutis*
Basidiomycota (unidentified)	*Cladosporium*	*Diatrypaceae* (unidentified)
Pyrenochaetopsis	*Agaricales* (unidentified)	*Dioszegia*
Cordycipitaceae (unidentified)	*Itersonilia* (2)	*Zymoseptoria*
Cladosporium	*Chalastospora*	*Entylomatales* (unidentified)
Cordyceps	*Symmetrospora* (2)	**Car A & B**
Sarocladium	*Nectriaceae* (unidentified)	*Resinicium*
Ascomycota (unidentified)	*Cantharellales* (unidentified) (3)	*Aspergillus*
Penicillium	*Postia* (2)	*Itersonilia*
Monographella	*Sporobolomyces*	*Phlebiella*
Bullera	*Chaetothyriales* (unidentified)	*Cantharellales* (unidentified)
Unidentified	*Kondoa* (2)	*Daedaleopsis*
	Tetrachaetum	*Strobilurus*
	Unidentified (3)	*Bjerkandera*
	Cryptococcus	*Piptoporus*
	Polyporales (unidentified)	*Wallemia* (2)
	Guehomyces	*Agaricomycetes* (unidentified)
		Stereum

6.4 Discussion

Determining the microbial content of the air in the interior of means of transport used daily for commuting is essential for assessing the exposure to potential health risks associated with poor indoor air quality. Overground rail has been one of the public transport modes that has been under-represented in bioaerosol studies, mainly due to its association with long distance journeys. However, in large urban areas that a lot of people prefer living on the outskirts or nearby towns, railway train serves as the main mean of public transport used daily by passengers to commute to work. According to the Official Statistics from the UK Department from Transport (DfT), Great Britain's (surface) railway trips accounted for 20% of passenger journeys in 2015-16, among all public transport trips. Moreover, the number of rail journeys recorded in the UK was the second highest in the European Union in 2014, after Germany (https://www.gov.uk/government/statistics/rail-factsheets-2016). However, a lot of people prefer commuting through road transport by using their own cars. In 2015 it was estimated that 68% of all trips to work in Great Britain was by private car or vans (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/576095/tsgb-2016-report-summaries.pdf). Even though the in-vehicle airborne microbiome has attracted some attention in the recent years, there is currently a lack of molecular data on the airborne micro-organisms found in the confined and often shared space of automobiles.

In the current study, the bioaerosol exposure of commuters was evaluated by elucidating the microbial diversity of the air in a comparative survey between public railway trips and private vehicle journeys, covering the route between London and the town of Colchester (UK). Even though the small sample size might be considered a limitation in the present study, this is the first investigation to provide important insights into the microbial composition of aerosols in the indoor environments of railway trains and automobiles, based on high throughput sequencing data. Overall our results showed that the aerosols in the interior of the two transport microenvironments harboured distinct bacterial and fungal populations. At the same time our findings also revealed the presence of a shared microbiome between the two transport means, as well as the presence of microbial taxa of particular concern to human health.

All individual examined rail trips revealed a high degree of overlapping in terms of bacterial aerosol composition as more than 65% of all bacterial OTUs (average across samples) appeared in all trips. Air samples were more dissimilar in terms of fungal diversity as only

25% of all fungal OTUs (average of all samples) were found to be present across all train journeys. Even though compositional variation is expected due to the use of different train carriages per each sampling trip, as well as various other factors contributing to the shaping of the indoor air microbiome in the trains, such as number of passengers, outdoor air composition and ventilation conditions (Meadow et al., 2014), our results indicated the presence of a common assemblage of airborne micro-organisms in all examined trains. Moreover, the examination of a variety of trips throughout the day (morning, afternoon, evening) allowed us to evaluate the overall exposure of commuters as it is unlikely that a passenger will commute by the same exact train vehicle each time.

From the other hand, our intentional differentiation of the two automobiles by our analysis scheme was consistent with the findings of the study since samples taken from the two cars did not cluster, as revealed by principal coordinate analysis. In fact, in terms of presence or absence of observed micro-organisms, both bacterial and fungal OTUs appeared in the air from car A were more similar to the ones appeared in the train carriages, rather than in car B. In particular, the fraction of core OTUs shared between the two automobiles was less than 20% for both bacteria and fungi. Even though the same route with the same number of passengers was examined for both automobiles, each transport vehicle cabin is a unique microenvironment with its own distinct characteristics (design, materials, ventilation settings etc.) and microbial background, mainly affected by its usage and passengers. Meadow et al. (2015) showed that occupants emit a unique identifiable microbial cloud in the surrounding environment and therefore private vehicles carry a distinct microbial signature linked to the passengers (i.e. owner, family etc.) using them. Nevertheless, despite the fact that commuting by an owned car means that passengers are exposed repeatedly to the airborne environment of the same enclosed space, variation is also expected among different journeys with the same vehicle (74% of all bacterial OTUs and 46% of all fungal OTUs were common among all trips with car A; 30% of all bacterial OTUs and 31% of all fungal OTUs were shared across all journeys by car B), mainly driven by the aforementioned factors.

Consistent with these interpretations, the above observations were also supported by the different levels of microbial richness encountered in the two cars. Interestingly, the air samples from car A yielded more similar (average) species richness with the one observed inside trains, rather than with car B. Moreover, bacterial abundance, as determined by qPCR, largely mirrored results of richness (Spearman's rank, $\rho = 0.84$). Examination of the quantitative data showed that the highest bacterial densities among all examined commuting

trips, which were found in the air of evening and morning trains, were significantly greater than the ones in car B, suggesting that train passengers are likely to be exposed to higher average levels of bacterial contamination in the air in comparison with car passengers. However, the airborne bacterial abundance in car A did not differ significantly from the one encountered during commuting by train. Moreover, the strong correlation found between bacterial levels and PM_{10} mass concentration, indicates that the airborne bacteria are associated with the thoracic fraction of particles (i.e. the mass fraction of inhaled particles that can penetrate the thoracic region of the human respiratory tract, U.S. EPA, 1987) and thus, they are of particular importance for the evaluation of commuters' exposure. On the other hand, fungal richness was not found to be significantly associated with the PM_{10} concentration, indicating that the detected airborne fungal species diversity covers a broad size range distribution, covering fungal material that is also > 10 μm in size (e.g. multicellular spores, fungi attached to large particles etc.). Yamamoto and colleagues studied the size distribution of fungal communities in indoor (2015) and outdoor air (2012) and detected a variety of taxa associated with large (> 9 μm) and smaller (< 9 μm) aerodynamic diameters.

Apart from PM_{10} mass, carbon dioxide (CO_2) levels were also measured throughout the commuting trips. CO_2 concentration is used as an indicator of other occupant-generated pollutants and ventilation adequacy. CO_2 medians in both trains and automobiles met the recommended ventilation standard of maximum 1000 ppm (based on 300 ppm outdoor level, ASHRAE, 1989). In contrast, in the study of Wang et al. (2010) it was found that the average CO_2 concentrations inside train cabins exceeded the guidance level of 1000 ppm, indicating an insufficient supply of outdoor air. In the present work this threshold was only exceeded for few minutes in the interior of the cars, as revealed by the real-time measurement profiles. Even though the trains can accommodate a bigger number of passengers and therefore, they are more likely to demonstrate more elevated CO_2, compared to a small vehicle, it has to be taken into account that occupant densities in the confined space of an automobile might be similar to a crowded train and thus accumulation of occupant-generated CO_2, as well as other pollutants, might result in similar or even higher concentrations indicating insufficient fresh air and consequent poor air quality. The negative correlation found in the current study between the CO_2 and airborne bacteria, as well as PM_{10}, meaning that the highest bacterial concentrations were found in the absence of elevated CO_2, might suggest that indoor levels are strongly affected by sources other than the occupants. Therefore it is reasonable to hypothesise that outdoor particles introduced indoors with ventilation air play a major role in

the increase of non-biological and biological particles and hence, in the shaping of the microbial assemblages in indoor air. Likewise, Wang et al. (2010) also found a negative correlation between CO_2 and total (viable) bacterial concentrations inside trains. In terms of microbial (alpha) diversity, even though the bacterial richness, same as abundance, was found to be significantly associated with the temperature, there was no strong correlation found with CO_2. On the other hand, the fungal richness exhibited a significant negative association with carbon dioxide concentration ($\rho = -0.66$). Consistent with the above hypothesis, this indicates that ventilation-based supply from outdoor air is an important contributor to the airborne fungal diversity in transport vehicles. This is line with prior work that has demonstrated that fungal aerosols in indoor environments mainly reflect the outdoor fungal composition (Adams et al., 2013).

Interestingly, among the measured environmental variables, only the differences observed in temperature between journeys were found to be significantly associated with the variation in both bacterial and fungal aerosol composition. Moreover, the differences in bacterial abundance between samples were found to correlate with the variability in bacterial composition, meaning that commutes with more similar levels of bacterial concentrations in the air had also more similar bacterial communities. Nevertheless, a more extended collection of samples would be required in order to evaluate possible links between the environmental parameters and the in-transit bioaerosol microbiome variability.

In terms of bacterial composition, a high abundance of Proteobacteria was observed in both rail and road transport modes, as it has been consistently observed in several other sequencing-based studies investigating indoor bioaerosols in various types of different environments (e.g. Wilkins et al., 2016; Gaüzère et al., 2014; Kembel et al., 2012). However, the predominant class inside the trains was *Actinobacteria* (*Corynebacterium*, *Kocuria*, *Streptomyces*, *Arthrobacter* and *Rhodococcus* spp. accounted for 25% of total bacterial OTUs), followed by *Gammaproteobacteria* (*Pseudomonas*, *Acinetobacter* and *Pantoea* spp. represented 19% of total reads), whereas a dominant abundance of *Bacilli* was observed in the interior of both automobiles (41% of total reads for car A comprised *Carnobacterium* and other *Carnobacteriaceae*, *Planomicrobium* and *Staphylococcus* spp., while 17% of total reads for car B belonged to *Streptococcus*, *Staphylococcus* and *Gemella* spp.). The prevalence of Proteobacteria and Actinobacteria phyla has also been detected in bioaerosols inside subway trains (Leung et al., 2014). To our knowledge, this is the first culture-independent study to present microbial composition data of bioaerosols in commuting overground trains. Wang et

al. (2010) studied the bioaerosol levels in railway trains in Taiwan using cultivation methods and found that Gram-positive and Gram-negative bacteria accounted for 70.5% and 20.5% of the total bacteria, respectively. Nevertheless, culture-dependent bioaerosol studies have been biased towards the over-representation of airborne Gram-positive bacteria compared to Gram-negative taxa (e.g. Rendon et al., 2017; Fang et al., 2014; Tsai & Macher, 2005; Seino et al., 2005; Shaffer & Lighthart, 1997; Simard et al., 1983; Mancinelli & Shulls, 1978). In our study, along with the highly enriched Gram-positive Actinobacteria (34%), also Gram-positive Firmicutes *Bacilli* (e.g. *Staphylococcus*, *Streptococcus*) represented a high majority (18%) of the observed taxa in trains. However, Gram-negative Proteobacteria, Bacteroidetes and Cyanobacteria accounted for about 46% of the identified OTUs. The in-vehicle predominance of *Bacilli* was in line with the findings of two other culture-independent studies (Stephenson et al., 2014; Li et al., 2013), that even though they collected dust samples, and not air samples, inside vehicle cabins, they also reported that the highest proportions within their samples belonged to the same class. In particular, Li et al. (2013) collected samples from the automobile air conditioning system filters of various vehicles in China, while Stephenson et al. (2014) swabbed frequently touched car interior surfaces. Both techniques, i.e. settled dust sampling from surfaces or from HVAC filters have been widely adopted and applied as alternative approaches of passive collection of airborne particulates replacing the active (pump-driven) bioaerosol sampling methods (Nygaard & Charnock, 2017; Hoisington et al., 2014; Rintala et al., 2012; Noris et al., 2011).

Furthermore, results showed that the air in both transport modes shared a common bacterial signature, mainly comprised from human-related (*Corynebacterium*, *Streptococcus, Staphylococcus)* and outdoor environment-associated species (*Blastococcus*, *Brachybacterium*, *Hymenobacter*, *Arthrobacter* and *Carnobacterium*). It has to be specified that the taxa mentioned represent bacterial OTUs that they cannot be identified at the species level. However, many commonalities were also found at the genus level for both occupancy-associated (e.g. *Acinetobacter*) and environmental (e.g. *Pseudomonas*) bacteria in the air of trains and cars. The great contribution of bacteria originating from the occupants to the aerosol microbiome encountered in the indoor environment has been well-established (Luongo et al., 2017; Adams et al., 2015; Gaüzère et al., 2014; Hospodsky et al., 2012). Public transport means, such as trains, are enclosed spaces associated with the passage of a big number of people that apart from being the source of the direct emission of human-related bacteria (skin, oral-associated microbiota), they can also act as secondary sources due to the

movement-induced resuspension of particles deposited on the floor and surfaces (e.g. seat fabrics), as well as on their clothes and shoes (Liljegren et al., 2016; Tian et al., 2016; McDonagh et al., 2014; Qian et al., 2014; Täubel et al., 2009). Similarly, even though, automobiles are not expected to serve big numbers of passengers, the contribution of humans as sources of bacteria is expected to be substantial due to high occupant density occurring in the small volume of vehicle cabins. On the other hand, similarities found between the two transport modes in terms of bacterial taxa commonly detected in soil and aquatic environments are most likely related to the geographic location where the sampling trips took place, as the same route was examined for both train and cars.

It is worth noting that there was a big difference in the proportions of human microbiota between the two cars (e.g. mean relative abundance of *Corynebacterium* was 11% in car B and 3.7% in car A, *Streptococccus* accounted for 8.5% in car B and 1.4% in car A, while *Gemella* represented 4.3% and 0.34% of total reads for car B and A respectively), which might be related to the regular usage of car B by more passengers (3-4, including toddlers) compared to car A (1-2 people). Moreover, differences were also exhibited among train journeys. Even though on average, more that 11% of all sequences was represented by *Corynebacterium* spp. in trains, this proportion varied, with the highest fractions occurred during morning (14%) and afternoon (12%) commutes and the lowest fractions (7.3%) encountered in the air of the evening trains. Likewise, *Acinetobacter* was also present at lower relative abundance in the air of the evening trains. At the same time, evening journeys exhibited higher fractions of outdoor-originating taxa, such as *Arthrobacter* or *Rhodococcus*, which might be linked to the natural ventilation occurring through open windows at the specific trains. It is worth noting that evening journeys by train exhibited the lowest CO_2 levels and the highest PM_{10} and bacterial concentrations. Even though increased ventilation rate can contribute to dilution of indoor pollutants and microbial agents (Luongo et al., 2016; Sundell et al., 2011; Li et al., 2007), provided that the outdoor air is uncontaminated, it can also introduce bioaerosols in the indoor environments, as already discussed. Yet, it has to be taken into account that, as mentioned previously, passengers can also be potential passive transport vectors of microbes from outdoors and therefore, introduction of outdoor bacteria is not only affected by the ventilation system.

Apart from the oral-associated genera *Streptococcus* and *Gemella*, which were more abundant in car B, overall, the presence of human-skin associated *Staphylococcus*, *Acinetobacter* and *Kocuria*, including the aforementioned *Corynebacterium* spp., was in total

a lot more enriched in trains (34% of total sequences), compared to cars (car A 8.5%; car B 19%). Among these bacteria, *Staphylococcus* spp., including *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* (MRSA) -a major causative agent of invasive infections that has been associated with healthcare facilities (Naimi et al., 2003)- are bacteria commonly detected in public transport buses, either on surfaces that come into contact with exposed skin (e.g. Conceição et al., 2013; Simoes et al., 2011), or in the air (e.g. Nowakowicz-Dębek et al., 2017; Onat et al., 2016), as revealed by previous investigations. Similarly, a microbial investigation in the automobile environment has also shown that *Staphylococcus* is one of the predominant bacterial genera on the car interior hand-touched surfaces, including the steering wheel, the gear shifter and the center console (Stephenson et al., 2014), indicating that surfaces represent an important reservoir for deposition and dissemination of *Staphylococci* bacteria.

Moreover, *Staphylococcus* spp. was found to be among the predominant bacteria in dust samples collected from automobile air conditioning filters (Li et al., 2013), revealing another important source and dispersal mechanism for the specific genus. Apart from *Staphylococcus*, various other genera that were found on the automobile A/C filters, such as *Kocuria*, *Arthrobacter*, *Massilia*, *Pseudomonas*, *Pantoea* and *Acinetobacter*, were also detected in the air samples of the investigated cars in the present work (*Pantoea* was found only in car A). Ventilation and air-conditioning (HVAC) systems when improperly maintained are known indoor sources of airborne microorganisms in the built environment (Prussin & Marr, 2015; Bluyssen et al., 2003). Early studies investigated the potential of microbial colonisation in automobile air conditioning systems (Simmons et al., 1999; 1997; Kumar et al., 1990) and its association with health implications (Kumar et al., 1984; 1981), while various articles have pointed out the survival and growth capability of bacteria and fungi on air filters, which are enhanced under high humidity conditions, especially for fungal spores (e.g. Forthomme et al., 2014; Maus et al., 2001; Simmons & Crow, 1995).

Furthermore, evidence in the literature suggests that the use of A/C in automobiles increases the prevalence of bioaerosols smaller than 2.5 μm in size (Wang et al., 2013; Li et al., 2013) and that overall concentrations tend to be elevated between the first 5 and 15 min, immediately after switching on the A/C (Li et al., 2013; Jo & Lee, 2008). Even though our study did not include real-time bioaerosol monitoring, taking into account the strong positive correlation between the levels of airborne bacteria and the median PM_{10} concentrations, PM_{10} peaks shown in real-time particle profiles of car A during the first minutes of the sampling

(the A/C was turned on the minute the car was on) might be an indication that a big part of airborne particles might be of biological origin. It should be noted that the A/C filter in car A had not been changed or cleaned. In another study, Vonberg et al. (2010) demonstrated that increase in microbial counts when the A/C is turned on is mainly associated with the state of the filter and when old and not properly disinfected filters were replaced with new ones, the in-vehicle microbial air quality was significantly improved.

Nevertheless, despite the possibility of reaerosolisation of biological material deposited in the filter dusts, the contribution of properly maintained A/C to the overall reduction of indoor airborne micro-organisms through filtration of outdoor particles and dilution of indoor particles by the filtered air coming indoors, should not be disregarded (Wang et al., 2013; Vonberg et al., 2010; Jo & Lee, 2008; Hamada & Fujita, 2002). Examination of the quantitative data obtained in the present work shows clearly the influence of the air conditioning system on the air quality of the transport micro-environments. The significant contribution of the A/C to the decrease of the PM_{10} -significantly correlated with bacteria- in the air is evident from the real-time concentration profiles of the train routes that had mixed conditions, i.e. either the outbound or the inbound train had the A/C turned on, such as morning - A, B and evening - A. However variation in the concentration levels is expected due to various other factors including the background levels, passengers movements, resuspension from vehicle floors and fabric seats, the opening/closing of the doors during the stops as well as the outdoor conditions, especially when the windows are open (evening - A and B).

Differences were also observed in the enrichment of non-human related taxa among the transport sites, such as the higher percentage of specific genera associated with outdoor environmental sources in the automobile in-vehicle environment (higher abundance of *Pedobacter*, *Planomicrobium*, *Massilia* and *Pseudomonas* in car A, or *Hymenobacter* and *Cyanobacteria GpI* in car B), compared to the train cabins. Although the factors that may be responsible for the specific pattern cannot be specified, having excluded the effect of seasonality due to the proportional differences observed even between the two cars (both cars were investigated during the same season), possible reasons driving these differences might be related with the ventilation system efficiency.

The most pronounced difference in terms of non-occupancy associated bacteria was the high abundance of *Carnobacterium* (24.5% while < 1% in trains and car B) and other members of

the family *Carnobacteriaceae* (5% while < 0.2% in trains and car B) exhibited in car A. *Carnobacterium* are psychrophilic bacteria, belonging to order *Lactobacillales*, within the phylum Firmicutes, that have mainly been associated with food products (e.g. Jöborn et al., 1999) and cold environments (e.g. Franzmann et al., 1991). Interestingly, *Carnobacterium* spp. has also been detected on filters of automobile air conditioning systems in China (Li et al., 2013). Moreover, the unique presence of other psychrohiles in the air of car A, such as *Polaromonas* and *Psychrobacter* (see Table 5B.7), that normally live in extremely cold habitats like the Antarctic ice (Bozal et al., 2003; Irgens et al., 1996), indicates that part of the microbiome can survive under low temperature conditions, e.g. when the car is not in usage during winter. Moreover, *Psychrobacter* has also been detected on automobile A/C filters (Li et al., 2013) and therefore, in agreement with results previously discussed, it might be speculated that the specific bacteria colonise the air conditioning components, from which they can get dispersed into the vehicle cabin. Even though this might require further investigation with source-tracking analysis, detection of thermophilic *Thermoactinomyces*, which are known contaminants of HVAC systems (Banaszak et al., 1970), in the air of car A (Table 5B.7), might provide further evidence that the air conditioning system consists a major source of bioaerosols in the automobile cabin environment. Inhalational exposure to *Thermoactinomyces* can cause hypersensitivity pneumonitis, also known as extrinsic allergic alveolitis or farmer’s lung (Selman et al., 2017).

In terms of fungal composition, the air samples corresponding to the two examined commuting scenarios yielded significantly different proportions of the two major fungal phyla, Ascomycota and Basidiomycota (Fröhlich-Nowoisky et al., 2012; 2009; James et al., 2006; O'Brien et al., 2005). A high abundance (80%) of Ascomycota and in particular of the class *Dothideomycetes* (*Cladosporium*, *Mycospaerella*, *Alternaria* and *Aerobasidium* spp. comprised almost half of the total reads) was found to dominated the airborne fungi in the train cabins, whereas Basidiomycota, mainly represented by *Agaricomycetes* (*Trametes*, *Bjerkandera* and *Daedaleopsis* spp. accounted for about 35% of the overall population), encompassed the majority (70%) of fungal taxa detected in the car interiors. *Dothideomycetes* are saprotrophic molds that frequently find suitable growth condition indoors, while *Agaricomycetes* are mainly outdoor mushroom-forming and plant-decaying fungi. The prevalence of the two classes within their divisions has been also reported in previous indoor (Shin et al., 2015; Hoisington et al., 2014; Adams et al., 2013) and outdoor (Xu et al., 2017; Yamamoto et al., 2012) bioaerosol surveys. The presence of the well-known and commonly

detected indoors ascomycetes *Cladosporium*, *Alternaria* and *Aerobasidium,* along with *Eurotiomycetes* fungi *Penicillium* and *Aspergillus* (Nevalainen et al., 2015), has been found in a variety of culture-based surveys investigating public transport vehicles, such as buses (Nowakowicz-Dębek et al, 2017; Prakash et al., 2014; Lee & Jo, 2005) and underground trains (Hernández-Castillo et al., 2014; Hoseini et al., 2013; Kim et al., 2011), as well as private automobiles (Wang et al., 2013; Jo & Lee, 2008; Lee & Jo, 2005; Simmons et al., 1997). Moreover, the frequent detection of the specific genera in bioaerosol investigations has been facilitated by the much less complicated cultivation of Ascomycota compared to Basidiomycota species that have been largely missed by culture-dependent indoor air studies (Simon-Nobbe et al., 2008; Pitkäranta et al., 2008). Due to lack of sequence-based studies on the fungal bioaerosol microbiome in the transportation environment, the current survey provides novel information on a previously undetected airborne fungal diversity harbouring the interior of train and automobile cabins.

Similarly to many other indoor and outdoor air studies in various environments, *Cladosporium* spp. was found to be the most prevalent fungi sampled off the interior of trains and car A (Kaarakainen et al., 2008). *Cladosporium* is a worldwide widespread allergenic fungal genus (e.g. *Cladosporium cladosporioides*) that grows naturally on plant leafs (Bensch et al., 2012). The occurrence of *Alternaria* and *Aureobasidium* genera, that are ubiquitous phyloplane fungi (e.g. *Alternaria alternata*, *Aureobasidium pullulans*), along with Penicillium (e.g. *Penicillium chrysogenum*) that originates from soil substrates and can be also found on food, *is* also common indoors and well-known from cultivation-based studies (Nevalainen et al., 2015). Even though the higher prevalence of the specific molds inside train carriages, compared to the interior of cars, might be associated with the higher number of passengers using rail services, as many fungi enter indoor environments via humans (e.g. on footwear), it has to be taken into account that fungi exhibit strong seasonal patterns. As vegetation is one of the main natural sources of fungi, it has been reported that the highest concentrations in the air are typically found during summer and autumn (Frankel et al., 2012; Yamamoto et al., 2012; Kaarakainen et al., 2008; Shelton et al., 2002). Despite the fact that absolute concentrations for fungi were not determined in the present work, it is possible that some of the relative abundance differences observed between train and automobile journeys, in terms of fungal composition, are attributed to the seasonal factor as the two types of transport were examined during two different periods of the year.

On the other hand, the fact that the particular genera were still present and quite abundant in the indoor air mycobiome encountered in the automobile cabins during winter, even though at lower percentages compared to trains, indicates that the in-vehicle microenvironments provides suitable growth reservoirs. Culture-based literature has reported that *Cladosporium*, *Penicillium*, *Altermaria* and *Aerobasidium*, as well as *Aspergillus*, have been found to colonise A/C filters and vents in automobiles (Simmons et al., 1997; Li et al., 2013, Jo & Lee, 2008), indicating that the automobile air conditioning system can be a significant source of micro-organisms in indoor air of vehicles, as discussed previously. Moreover, the higher occurence of xerophilic genus *Wallemia*, which is another typical mold found in indoor settled dust (Adan & Samson, 2011) and foods with high concentrations of salt or sugar (Kunčič et al., 2013), in the air of both cars, as well as *Cryptococcus*, which is considered to be part of the human skin microbiome (Findley et al., 2013), mainly in car A, provides additional evidence that indoor sources contribute to the in-vehicle airborne fungal assemblages. Yamamoto et al. (2015) demonstrated that the fungal diversity in indoor air of occupied classrooms was largely dominated by occupant-related emissions.

However, as it has been well-documented, the origin of fungi found indoors is primarily located outdoors in various natural sources, mainly associated with plant material and rotting wood. Apart from the aforementioned taxa, the vast majority of airborne fungi that commuters using both rail services and automobiles are exposed to, were related to vegetation, either as plant-pathogens, epiphytes, endophytes, or just wood-decomposers (genera of *Bjerkandera*, *Trametes*, *Mycospaerella*, *Daedaleopsis*, *Strobilurus*, *Vishniacozyma*, *Bullera* and *Sporobolomyces* yeasts, family of *Diatrypaceae*, orders of *Cantharellales* and *Helotiales*, class of *Agaricomycetes*). From the detected taxa, OTUs belonging to *Cladosporium* spp. (2 OTUs), *Alternaria* spp., *Trametes* spp., *Mycosphaerella* spp. and *Sporobolomyces* spp. were found to be shared among all three examined transport means, which might suggest that the particular fungi are the main indicator taxa of the outdoor sources that represent the local surrounding environment, as location has been identified to be a strong determinant of the fungal composition in the indoor environment (Adams et al., 2013; Amend et al., 2010). Moreover, results were in agreement with the negative correlation found between indoor fungal richness and carbon dioxide concentration.

Interestingly, among the taxa identified there were few basidiomycetous yeasts that are known to exhibit superior adaptation to low temperatures and which were mainly detected in car A. The higher relative abundance of psychrophilic *Vishniacozyma* and *Cryptococcus*

genera encountered in car A, along with the occurrence of unique OTUs of *Cryptococcus* and *Sporobolomyces* spp. only in car A, as well as other cold-adapted yeasts, such as *Buckleyzyma*, *Kondoa* (2 OTUs) and *Guehomyces* (Sannino et al., 2017; Tasselli et al., 2017; Zalar & Gunde-Cimerman, 2014) is in agreement with results previously discussed about detection of psychrophilic bacteria in the air of car A. In general, fungi can remain viable within a wide range of temperatures and apart from psychrophiles, even some of the mesophiles detected, such as *Cladosporium* and *Penicillium*, are able to grow at freezing cold temperatures (Adan & Samson, 2011). The detection of the particular cold-adapted taxa, supports the argument that the A/C unit in car A, which was switched on during all sampling journeys, in contrast to car B that was only using mechanical ventilation, might provide suitable growth substrates and the use of it could be the cause of their dispersal in the air.

In terms of particle size, consistent with the lack of association found between fungal richness and PM_{10} concentration, the prevalence of Ascomycota in trains and Basidiomycota in cars, suggests that fungal material detected covered a wide range of sizes. In general, it has been reported that fungal spores of Ascomycota are larger than spores of Basidiomycota (Yamamoto et al., 2012), many of which exceed 10 μm, such as *Alternaria* spp. (McCartney et al., 1993). Nevertheless, a bigger sample size than the one that could be obtained in the current work, would allow us to examine correlations for each transport mean separately. In addition, it has to be taken into account that many of the fungal bioaerosols detected using sequencing-based methods represent fungal fragments, rather than intact spores, and therefore occur in smaller sizes (Górny et al., 2002).

Last but not least, despite the commonly held belief that using public transportation might be considered as a higher risk factor for acquisition of infectious diseases, several genera affiliated with opportunistic pathogenic or allergenic species were found in varying relative abundances in both types of transport environments. Many of the opportunistic pathogens detected, are bacteria living on the human skin (Roth & James, 1988), including *Corynebacterium*, *Staphylococcus*, *Acinetobacter* and *Kocuria*, or they are members of the oral microbiome (Kumar et al., 2005), such as *Streptococcus* and *Gemella*, and they are considered to be part of the normal human microflora. *Cryptococcus*, which is also a common skin coloniser, is a basidiomycetous yeast genus that comprises human- pathogenic species (e.g. *Cryptococcus neoformans*) that can cause medically important infections mainly to immunocompromised and immunocompetent individuals (Kwon-Chung et al., 2011). Widely distributed taxa such as *Pseudomonas*, as well as *Pantoea* and *Streptomyces* that were mainly

detected in the trains, are also associated with potential pathogenic strains. Moreover, there were also bacterial OTUs detected uniquely in each transport environment that are responsible for various infectious human diseases, such as *Finegoldia* (Goto et al., 2008) in trains, *Thermoactinomycetes* (Selman et al., 2017) in car A, *Haemophilus* and *Neisseria* (Stephens & Farley, 1990) in car B. In terms of the well-known fungal allergens, apart from *Wallemia* genus that was found to be more abundant in the interior of automobiles and *Aspergillus* spp. that was more enriched in car B, *Cladosporium*, *Alternaria*, *Penicillium* and *Aureobasidium* were found to be more predominant in the trains, possibly due to seasonal variation, as discussed previously. Even though the knowledge of the basidiomycetous health-associated fungi lags behind that of Ascomycota (Simon-Nobbe et al., 2008), apart from Cryptococcus, two other medically important members of Basidiomycota, mainly associated with respiratory conditions, were detected; *Sprorobolomyces* yeasts (Cockcroft et al., 1983), which are also associated with infections (Morris et al., 1991) and were mostly present in trains, and *Bjerkandera*, (Ogawa et al., 2009), which was more enriched in the two study cars.

Transport vehicles, public or private, are considered to be ideal environments for the dissemination of microbial agents through the air. However, the inability to specify the OTU identity at the sub-genus level does not allow us to elucidate the species of the specific micro-organisms of concern. Existing databases and tools used for taxonomy assignment, such as the Ribosomal Database Project (RDP) (Cole et al., 2008), cannot classify sequences below the genus level and therefore the taxonomic description is not sufficient for assessing the potential health risk and further identification to species level is essential. Moreover, the fact that DNA-based molecular methods cannot distinguish live from dead or active from inactive microbial cells adds another factor of uncertainty to the prediction and evaluation of possible health effects. Yet, detection of high abundance of pathogenic/allergenic microbial agents is still considered to be a good indicator to signal potential health problems. Previous literature has shown that many of the airborne pathogens and allergens, that were identified in our study, such as *Staphylococcus*, *Cladosporium, Penicillium* and *Alternaria* have also been collected using viable bioaerosol sampling methods (i.e. culture-based) either in means of public transport (e.g. Nowakowicz-Dębek et al., 2017; Onat et al., 2016; Prakash et al., 2014; Lee & Jo, 2005) or in private vehicles (e.g. Wang et al., 2013; Jo & Lee, 2008; Lee & Jo, 2005; Simmons et al., 1997), indicating that the specific OTUs represent micro-organisms have developed survival mechanisms and they were possibly viable when detected in the air

of the transport environments. Li et al. (2013) demonstrated that many of the micro-organisms detected on the automobile air-conditioning filters are metabolically active, suggesting that many of those agents can still be viable when aerosolised. Moreover, in consistence with this hypothesis, they showed that viable bioaerosols associated with aerodynamic diameters of 2.5 μm, were found to be at peak concentrations after use of the A/C for 5 min, as determined by on-line fluorescence measurements.

6.5 Conclusions

This study examined the aerosol microbiome encountered in public and private transport micro-environments, covering the same commuting route, using culture-independent high throughput sequencing approaches. In terms of quantitative metrics, our findings showed that airborne bacteria were, on average, more abundant in the interior of rail trains, compared to the automobiles, and were found to be significantly associated with the PM_{10} concentrations. Moreover, both bacterial and PM_{10} levels appeared to be affected by the ventilation conditions, as indicated by the negative correlation found with the CO_2 concentration. In terms of microbial composition, overall results revealed the presence of various common bacterial and fungal core OTUs shared between the two transport modes, with more common taxa observed between the trains and one of the two examined cars. However, the most abundant taxa driving the composition contributed to the shaping of distinct patterns in the aerosol microbiome for the two types of commuting environments, with a larger effect exhibited for the fungal composition, suggesting that the choice of transport mode has a significant impact on the bioaerosol microbiome that passengers are exposed to. More studies utilising the latest molecular techniques are required to further explore and characterise microbial aerosols in the transportation environment in order to evaluate their significance in exposure during commuting.

Chapter 7

Concluding remarks and Future considerations

The aim of this thesis was to explore the microbial diversity and abundance of bioaerosols in the indoor environment. Molecular-based microbial characterisation of bioaerosols from various types of indoor micro-environments, that people spend considerable amount of their time on a daily basis, was performed.

Before it is possible to understand and assess the effect of the airborne micro-organisms that share the environments where we live and work with us, the first step is to know who they are. The advent of molecular biology techniques and the rapid development of high throughput sequencing (HTS) technologies have enabled the exploration of the microbial content of the airborne particulate matter overcoming the issues associated with culture-dependent methods (Chapter #1). However our knowledge of the bioaerosol microbiome is still limited and certainly lags behind the knowledge we have of the terrestrial and aquatic microbial inhabitants.

The current work presented HTS-based microbial data of the airborne bacterial and fungal communities in a variety of environments, including residential (Chapter #4) and educational environments (Chapters #2 & #3), public transport (Chapters #5 & #6) as well as private vehicles (Chapter #6). Although several molecular indoor air studies have investigated bacterial and fungal bioaerosols in houses and classrooms, the number of HTS-based surveys is still limited. Moreover, important insights into the microbial composition of bioaerosols in private vehicles (Chapter #6), trains (Chapter #6) and a metro station (Chapter #5) were provided for the first time. Due to lack of sequencing-based studies on the aerosol microbiome in the transportation environment, the current work provided novel information on the bacterial and fungal composition of aerosols in automobiles and commuting railway trains (Chapter #6). The microbial characterisation of a subway station in the Athens metro system was attempted for the first time, whereas the mycobiome in a metro environment based on ITS amplicon sequencing has not been previously described (Chapter #5). Nonetheless, indoor air microbiome data from various other types of indoor settings are largely missing and require exploration.

Due to lack of standardised methods for bioaerosol collection, various different sampling approaches were employed for our investigations, including active sampling (i.e. filtration, impaction) and passive collection methods (i.e. settled dust swabbing, suspended petri dishes) using different sampling durations (1 month for the passive collectors, 48 and 12 hours for the impactors and 48, 16, 8, 4, 2 & 1 hours, 30, 20 & 10 minutes for the filter-based samplers). Filter-based sampling was performed using different types of collections substrates (polycarbonate, mixed-cellulose ester, gelatin, polytetrafluoroethylene, cellulose acetate, cellulose nitrate). Moreover, non size-specific and size-selective sampling approaches (i.e. PM_{10} fraction, Andersen impactor cut sizes, May impactor cut sizes) were also utilised. In addition, a modified version of the widely used viable six-stage Andersen impactor for the purposes of non-viable sampling was presented (Chapters #2 and #4), whereas the novel use of the seven-stage May impactor for sampling aimed for molecular analysis was demonstrated (Chapter #4).

Overall results from the several investigations described here, including comparative sampling experiments (i.e. comparison between different filter types, sampling times, size-resolved and non size-resolved, active and passive collection), pointed out the significance of the microbial biomass and the impact it can have on the generated data. Our findings showed that high biomass capture is crucial for generating robust and reproducible data. Therefore, collection of bigger sampling volumes, which could be achieved either by extending the sampling time or by increasing the flow rate/using high-volume samplers, are strongly recommended for air sampling. Moreover, prolonged active sampling or use of passive sampling approaches can overcome issues associated with the bioaerosol temporal variability if the goal of the study is to provide a representative description of the airborne microbial load to which individuals are exposed in a particular environment and not just a “snapshot”. Protocols produced and described in the current thesis could be useful for researchers working on the microbial characterisation of aerosols. However, additional efforts are necessary in further examining possible ways of optimising methods of bioaerosol collection to achieve molecular analysis under optimum conditions as it is crucial that reliable and efficient monitoring methods for detecting and identifying airborne biological particles be developed.

However, it is important to mention that the implementation of a sampling approach aimed for optimum microbial DNA recovery is not always feasible due to the intrusive nature of air sampling in occupied private or public environments. One of the main drawbacks of the

particular type of sampling is the high level of noise generated by the pumps required for the purposes of active sampling in combination with the need for extended periods for sampling which could cause inconvenience to the occupants of an indoor setting. For instance, for the study performed in the residential flats (Chapter #4) the recruited home occupants had to allow the use of several pumps for a whole daytime duration (12 hours). Even though the pumps were placed in boxes reinforced with soundproofing foam, the noise disturbance could not be reduced sufficiently. Therefore, difficulties might be encountered when trying to find participants willing to participate in a sampling study in their living environments. Moreover, carrying out a discrete sample collection, especially in public and typically crowded places might not be always achievable. For example, portable battery-operated pumps, which are convenient for sampling inside trains during commutes, tend to have low flow rates compared to the typically noisy, larger and heavier static sampling devices and therefore, collection of sufficient sampling volumes for downstream molecular applications might not be, in some cases, possible. Another possible limitation that could be encountered in bioaerosol investigations is the inability to perform parallel sampling of indoor and outdoor air, due to technical limitations or due to outdoor conditions. For instance, outdoor air sampling could not be performed during commuting with public transport (Chapter #6). In addition, even though the unpredictable weather conditions are not an issue for the indoor sampling, in some cases they might not allow the parallel sampling of outdoor bioaerosols for extended periods (e.g. when using passive collectors - Chapter #4). However, in order to avoid an incomplete assessment of the indoor air quality in the built environment, it is critical to consider both indoor and outdoor bioaerosols. All the aforementioned issues should be considered when developing strategies for bioaerosol sampling in indoor environments.

Another important consideration, which is especially important for low biomass samples, is the risk of contamination which can occur throughout any step from sample collection to sample processing. An additional factor of complexity in the case of indoor air microbiome studies is that the contaminant taxa found in the sampling and laboratory consumables as well as working reagents can often be similar to the micro-organisms detected genuinely present in the air samples, as they are commonly introduced from the wider environment. Apart from careful laboratory hygiene when processing samples (e.g. using a laminar flow hood for setting up PCR reactions, working with filter tips, cleaning pipettes regularly), a proper decontamination of the sampling equipment and collection substrates is also highly important to avoid possible contamination, which can have a significant impact on the interpretation of

data generated by molecular analysis of bioaerosol samples. The latter was particularly illustrated during the sampling study conducted at various university campus locations (Chapter #3). Our data demonstrated the presence of PCR-amplifiable bacterial DNA in gamma-irradiated gelatin blank filters and its impact on the microbial profiling of bioaerosol samples throughout molecular sample processing. The suitability of the gelatin filter as a collection substrate for sampling of airborne bacteria, aimed for culture-independent DNA-based analysis, has not been previously addressed. This particular finding highlights the importance of including blank negative controls during any investigation as the contamination background from the collection substrate could compete with the low concentration of collected sample. Moreover the traditional sterilisation techniques should be re-considered when it comes to sampling aimed for molecular analysis. Ideally, samplers and collection substrates should be DNA-free and not just sterile. In the work presented here, when possible, sodium hypochlorite solutions were used for the decontamination of sampling equipment in order to eliminate DNA present. However, as most items are subjected to material damaging limitations, including filters, alternative less effective methods, such as autoclaving or dry heat sterilisation, had to be used in most cases.

Overall, data produced from sampling in different indoor settings revealed the large input of human associated taxa that leave a strong bacterial signature in the built environment and which is one of the predominant factors that differentiate the indoor air microbiome from the aerosol microbial composition outdoors. On the other hand, the indoor air mycobiome mostly reflects the outdoor air fungal composition. Although the particular results have been previously reported, our data further supported those earlier findings in the context of different case studies. The HTS-based bioaerosol characterisation allowed us to address several research questions in relation to the microbial exposure in particular environments. The examination of the aerosol microbiota encountered in public and transport micro-environments showed that that the choice of transport mode has a significant impact on the bioaerosol microbiome that passengers are exposed to (Chapter #6). The investigation of bioaerosols in a subway environment revealed that the observed aerosol microbial diversity in a metro station could be in fact similar to the one encountered typically in naturally ventilated occupied indoor settings (Chapter #5). The longitudinal study conducted in two naturally ventilated houses (Chapter #4) demonstrated that seasonality is a strong predictor of the indoor aerosol bacterial composition variability. At the same time the aerosol composition was found to vary significantly between the two study houses only indoors, but not outdoors,

despite being located in two different types of environments (i.e. urban and semi-urban), indicating that the differences were occupancy-driven. The two latter surveys (Chapters #5 & #4) demonstrated the importance of the type of ventilation in built microbiome studies and, particularly, the significant effect of the natural ventilation on the indoor bioaerosol composition due to mixing of outdoor-originating environmental micro-organisms with human commensals.

One of the main limitations in the current work was the lack of source-tracking analysis which could evaluate possible links between potential sources and detected airborne taxa. Apart from some DGGE data (Chapter #3) that were presented for bacterial bioaerosols and settled dust samples collected from universal settings (which revealed some similarities between them indicating that some of the airborne bacteria could be originating from the interior surfaces), most of the potential sources of the identified micro-organisms were speculated based on the general knowledge of the microbial ecology. Bioaerosols are a complex mixture of microbes originating from many different sources including the outdoor air. Except for the human commensals, most of the microbes originate from natural habitats such as soil, plants and water bodies and can be carried directly from outdoors through ventilation. However some of those taxa could be also indoor colonisers. Potential source samples have been collected from most of the study sites and follow-up investigations are underway in order to complement and confirm already reported results. In particular, apart from the various settled dust samples collected from the university campus (Chapter #3), surface samples from potential sources which may account for attracting biological material as well as deposition and resuspension of aerosols (e.g. kitchen bench, bathtub, floor) have been obtained from the residential flats (Chapter #4). In order to investigate whether the riverside location affected the microbial composition of the indoor air in the study apartments, water samples from the adjacent rivers (River Colne in Colchester and Bow Back Rivers in East London) were also collected (Chapter #4). Unfortunately, it was not possible to obtain source samples from the transportation environments (Chapters #5 & #6).

Furthermore, apart from identifying the airborne micro-organisms and their potential sources, in order to be able to evaluate the degree of microbial exposure in a given environment it is crucial to specify the related particle size. Size-segregated data are of fundamental value for developing insights regarding health effects of exposure to bioaerosols and currently HTS-based data are largely missing. The present work provided the first in-depth size-resolved characterisation of bacterial aerosols in the domestic environment and at the same time

presented novel data on the seasonal dynamics of size-resolved bacteria-laden particles revealing that seasonality was mostly driven by the coarse particles (Chapter #4). In agreement with earlier findings, there was no size-related composition pattern observed, with bacterial taxa being dispersed across particles of various different sizes and peaked concentrations in the size ranges 2 - 4 μm and 4 - 8 μm. Further investigation is ongoing in order to evaluate the size-resolved fungal diversity of the same samples. Moreover, the present work presented novel information for bacterial aerosols over 10 μm. Results suggested that the large-sized particles were mostly of outdoor origin, whereas the smaller particles were mostly associated with in-house sources. As fungal aerosols are expected to be associated with the larger sizes, the particle size distribution over a wider size range will be of particular interest. Apart from the aforementioned study, a microbial characterisation of bioaerosols based on size-specific data was also provided for the subway investigation (Chapter #5). Results presented were of major importance as they were representative of the inhalable fraction PM_{10} and not the total suspended particulate matter that are typically analysed in bioaerosol molecular studies. However the limited number of samples obtained might be considered a limitation of the study.

Last, the current work also included parallel monitoring of the airborne particulate matter in two of the reported case studies (Chapters #4 & #6). Although microbial aerosols constitute part of the airborne particulate matter, PM levels are rarely included in bioaerosol studies. Despite the different origin of biological and non-biological components of the airborne particulates, possible correlations between the microbial data and the $PM_{2.5}$ or PM_{10} mass concentrations could provide useful information. For instance, bacterial and PM_{10} levels were found to be strongly associated in the transportation environment (Chapter #6), indicating that a big part of the airborne particles could be of biological origin. At the same time, based on the negative correlation found between CO_2 and airborne bacteria, as well as PM_{10}, it was speculated that the increase of bioaerosols was most likely due to outdoor particles introduced indoors with ventilation air rather than being associated with the passengers. Also, the lack of strong correlation between the aerosol fungal richness and PM_{10} mass that was found in the same study could be an indication of the broad size range of fungal aerosols which could exceed 10 μm. Positive associations between levels of airborne bacteria and $PM_{2.5}$ were also observed at the residential flats investigated (Chapter #4). Although the presence of occupants mainly increased the bacterial concentrations in the form of direct emissions (e.g. skin shedding) or through physical movements causing resuspension, the

elevated $PM_{2.5}$ concentrations were mainly due to combustion-related ordinary household activities taking place during occupancy.

In conclusion, the present thesis provided insights into the microbial composition and abundance of bioaerosols in indoor air from various environments based on molecular techniques. Results presented here provide novel information on a previously largely unexplored microbiome. The information generated in this thesis is expected to assist in developing strategies for bioaerosol exposure monitoring.

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Chapter 4

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Chapter 5

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Chapter 6

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Appendix S2

Table S2.1 Primers used in the current study (Herlemann et al., 2011).

Primer name	Primer sequence (5' – 3')
Bakt_341F	CCTACGGGNGGCWGCAG
Bakt_805R	GACTACHVGGGTATCTAATCC
Bakt_341F with Illumina overhang forward adapter sequence added	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
Bakt_805R with Illumina overhang reverse adapter sequence added	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Table S2.2 Top 10 OTUs identified across blank filter controls (n = 3).

#OTU ID	Taxonomy	Mean Relative abundance
311898	*Enterobacteriaceae*	16.13%
161245	*Methylobacterium*	10.11%
783719	*Ralstonia*	8.06%
New.CleanUp.ReferenceOTU465015	*Pelomonas*	5.66%
1084865	Unassigned	3.01%
888466	*Staphylococcus*	3.01%
209511	*Actinomyces*	2.71%
68458	*Acinetobacter*	2.41%
1084950	*Methylobacterium*	1.62%
177991	*Methylobacterium*	1.02%

Table S2.3 Top 10 OTUs identified across no-filter controls (n = 3).

#OTU ID	Taxonomy	Mean Relative abundance
911129	Unassigned	10.49%
New.ReferenceOTU819	*Clostridium sensu stricto*	5.26%
New.ReferenceOTU911	*Hymenobacter*	4.21%
16144	*Clostridium sensu stricto*	4.20%
885496	*Intrasporangiaceae*	4.05%
583102	*Sphingomonas*	3.21%
1060565	*Methylobacterium*	2.97%
821608	*Sphingomonas*	2.32%
New.CleanUp.ReferenceOTU758329	Unassigned	1.94%
1000876	*Nocardioides*	1.87%

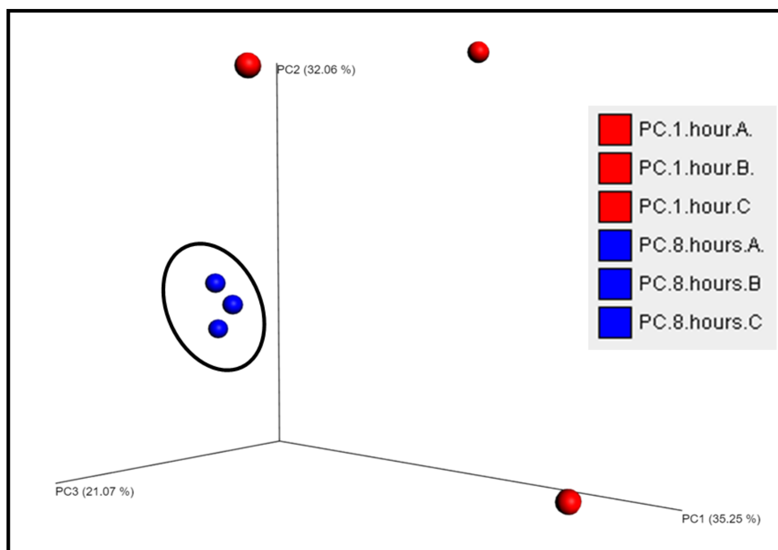

Figure S2.1 Principal coordinates analysis 3D-plot of bacterial beta diversity based on unweighted UniFrac metric.

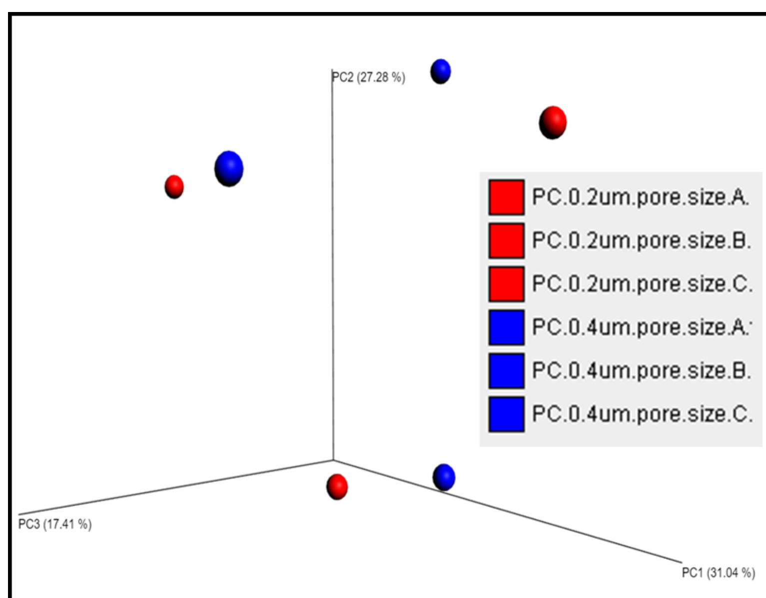

Figure S2.2 Principal coordinates analysis 3D-plot of bacterial beta diversity based on unweighted UniFrac metric.

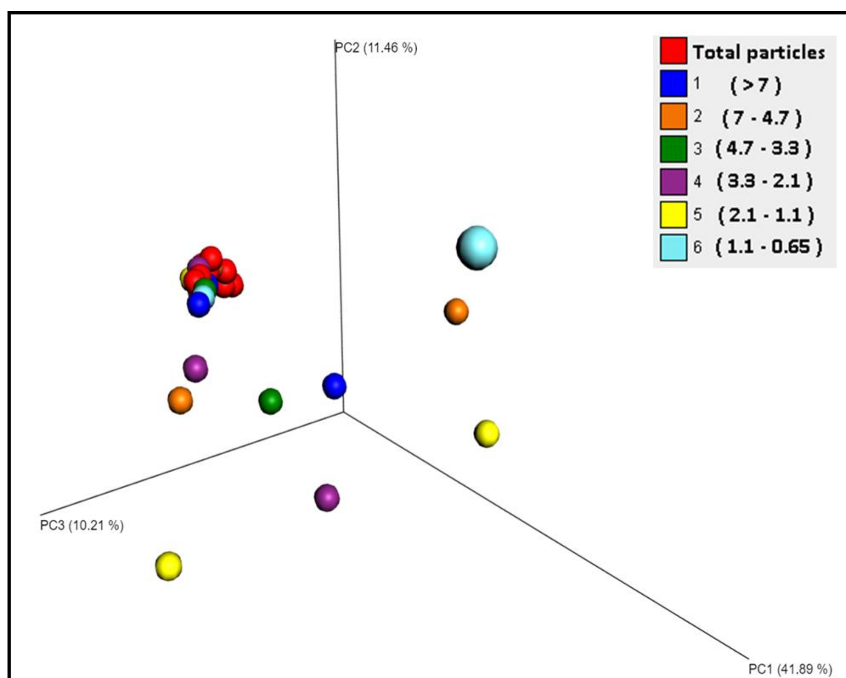

Figure S2.3 Principal coordinates analysis 3D-plot of bacterial beta diversity based on unweighted UniFrac metric.

Appendix S3

Table S3.1 Sequence-based identification and taxonomic affiliation of bands excised from DGGE gels derived from 16S rRNA gene amplicons from gelatin filter blanks. The closest match was obtained from searches in the GenBank with BLASTn and bands are numbered according to the order in Fig. 3.4 (starting from the top).

Band*	Sequence length	Closest affiliation (GenBank Accession no.)	Similarity (%)
#1	124 bp	*Bacillus* sp. R-32528 (AM691585.1)	98%
#2	141 bp	*Bacillus* sp. GR1-10 (KM057801.1)	99%
#3	145 bp	*Bacillus* sp. R-31856 (AM691561.1)	98%
#4	79 bp	*Bacillus* sp. NA275 (AB927772.1)	92%
#5	139 bp	*Bacillus* sp. R-32528 (AM691585.1)	99%
#6	138 bp	*Bacillus* sp. R-32851 (AM691580.1)	100%
#8	124 bp	*Bacillus* sp. GR1-10 (KM057801.1)	98%

*Bands #7 and #9 did not yield successful sequencing results.

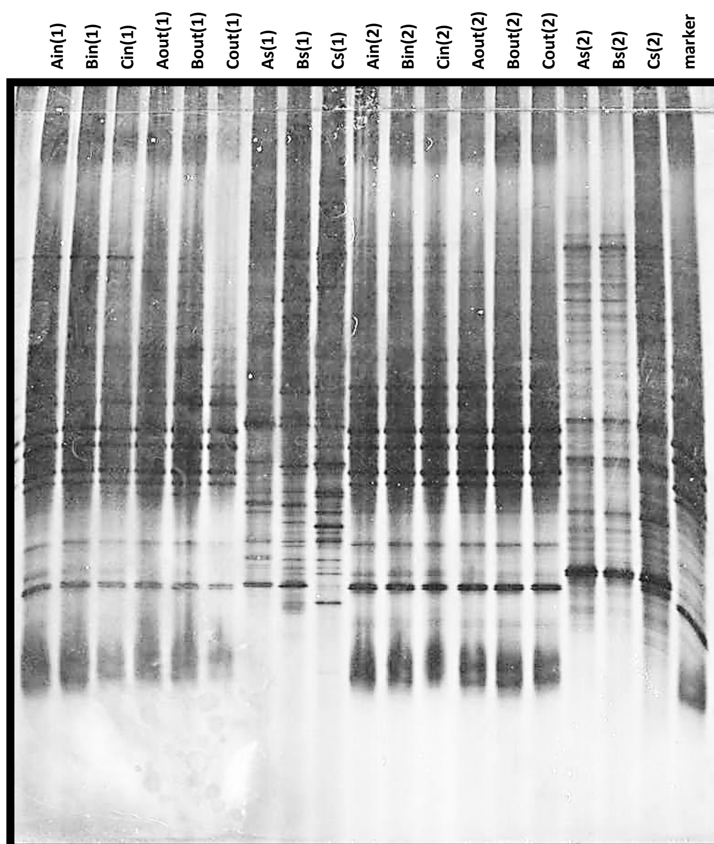

Figure S3.1 DGGE fingerprints of 16S rRNA amplified from various indoor and outdoor air samples obtained with gelatin filters, as well surface samples. Number "1" indicates samples collected at the student's accommodation site, while index "2" corresponds to the university gym.

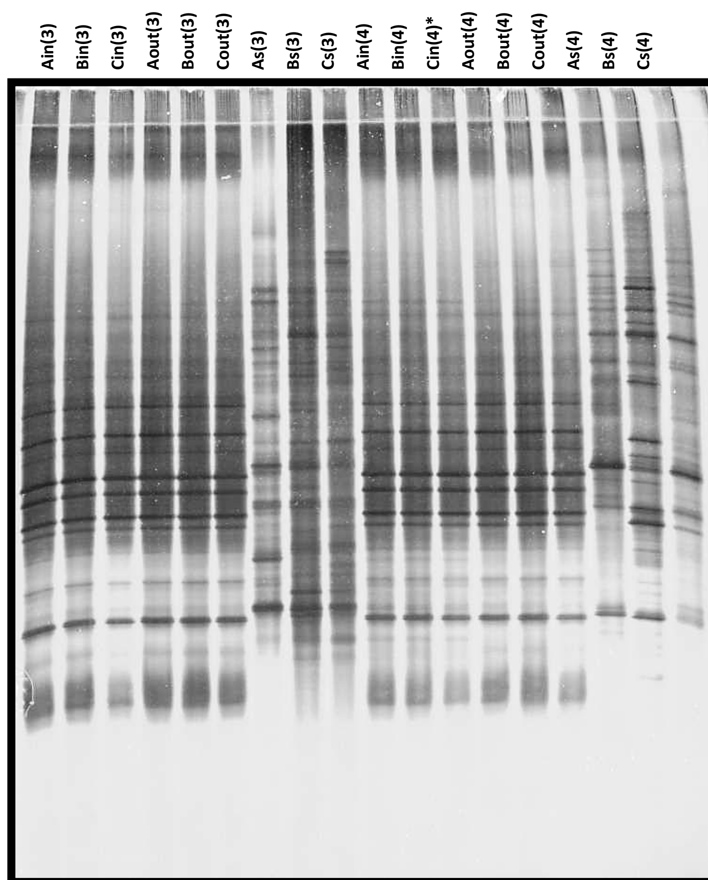

Figure S3.2 DGGE fingerprints of 16S rRNA amplified from various indoor and outdoor air samples obtained with gelatin filters, as well surface samples. Number "3" indicates samples collected at the Lecture theatre, while index "4" corresponds to the university common room site. (*Sample Cin(4) was used as a marker in previous gel).

Appendix S4

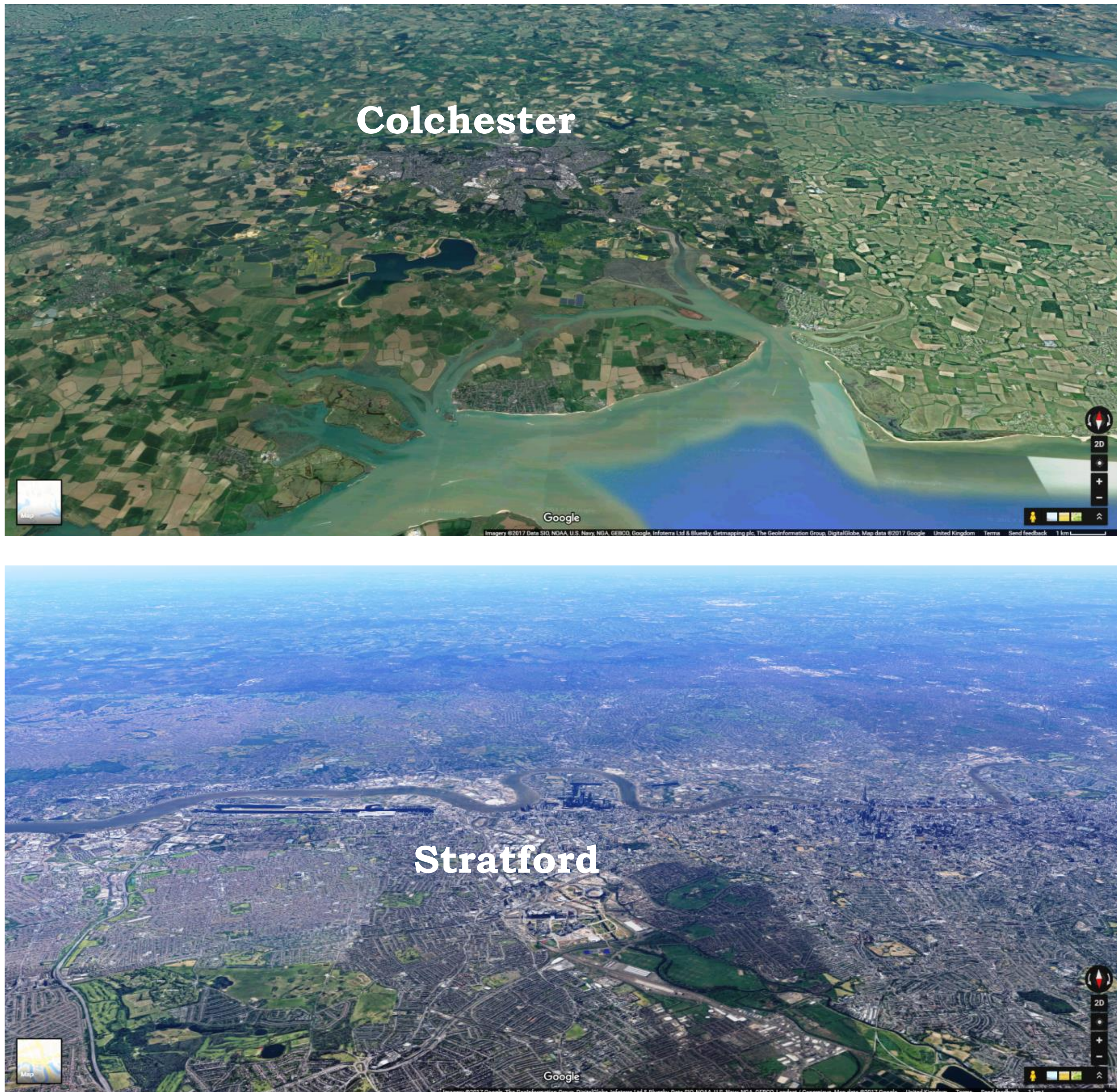

Figure S4.1 Aerial views (Google Earth images) of Colchester (semi-urban) and Stratford (urban) areas.

Real-time instruments data correction

The real-time measurements for the quality assurance of the data obtained with the particle counters in this study were conducted in an open plan living room and kitchen area of a residence. The instruments were placed side by side approximately 1 m from a periodically opening balcony door and the measurements lasted approximately nine days.

The PM_{10} and $PM_{2.5}$ mass concentrations measured with Grimm 1.108, Met One Aerocet 531 and two Speck sensors (Fig. S4.1) were compared against a Grimm 1.109 instrument, which was used as the reference sampler (Fig. S4.3 & S4.4). All instruments were in reasonable agreement with the Grimm 1.109, as demonstrated by the coefficients of determination (R^2), and the formulas derived from the linear regression were used for adjusting the samplers' data (Fig.S4.5).

Instruments used (Fig. S4.2):

- **Grimm models 1.108 and 1.109** (Aerosol Technik GmbH & Co. KG) are optical aerosol spectrometers that are based on light scattering of single particles using a laser diode as light source. The wavelength used for model 1.108 is 780 nm (infrared range), whereas for 1.109 is 655 nm (visible range). Grimm 1.108 provides particle concentrations in sizes between 0.3 µm and 20 µm in 15 size channels, while Grimm 1.109 detects particles from 0.25 µm up to 32 µm over a size range of 31 channels. The internal pump samples air at a flow rate of 1.2 LPM. Both models report data in particle number and mass concentration mode and data can be logged in intervals from 6 seconds up to 60 minutes. Instruments also possess a filter chamber that can be used for gravimetric control of the optical gained measurement results.

- **Met One Aerocet 531** (Met One Instruments Inc., USA) is a laser-diode-base (780 nm) optical aerosol mass profiler that counts particles larger than 0.5 microns and converts them to the equivalent particle mass using a proprietary algorithm. Mass concentrations are displayed for the fractions of PM_1, $PM_{2.5}$, PM_7, PM_{10} and TSP (total suspended particles) in mg/m^3, within a concentration range of 0 - 1 mg/m^3. The sample air is sucked at a flow rate of 2.83 LPM (0.1 cfm) and data are logged every 2 minutes.
- **Speck air quality monitor** (Airviz Inc., USA) is an infra-red LED-based particle counter that employs a Syhitech DSM501A sensor. The instrument contains a fan to

draw air through the sensor and can detect particles in the size range of 0.5 to 3 μm at a sampling interval from 5 seconds to 4 minutes. Data are reported in both counts and mass units per cubic meter. Two models are currently available, the classic Speck and Speck 2.0 that includes a humidity sensor.

Figure S4.2 Real-time particle counters used in this study.

For the parallel measurements the sampling interval was set at 1 minute for both Grimm models and at 2 minutes for Met One Aerocet 531 (default logging interval) and Speck sensors. As "Speck-1" is referred the classic model, while "Speck-2" is the Speck 2.0 model. An average every two minutes was calculated for the Grimm values and used for the comparison analysis.

Grimm model 1.108 gravimetric correction

Prior to data correction using adjustment formulas obtained from the side-by-side comparisons of the instruments against Grimm 1.109, data correction of the Grimm 1.108 optical light scattering gained measurement results was performed using the gravimetric factor - the so-called C-factor - method, as suggested by the manufacturer. Briefly, a new 47-mm PTFE filter was inserted in the filter chamber of the instrument and was weighed before and after sampling on a high precision microbalance, after 24 h of equilibration to constant relative humidity and temperature conditions. The gravimetric factor was determined as the ratio of the collected particle mass, calculated as the difference in weight of the filter before and after sampling, to the dust weight shown by instrument and was found to be 1.19. All Grimm 1.108 mass concentration values measured in this study were multiplied by this factor.

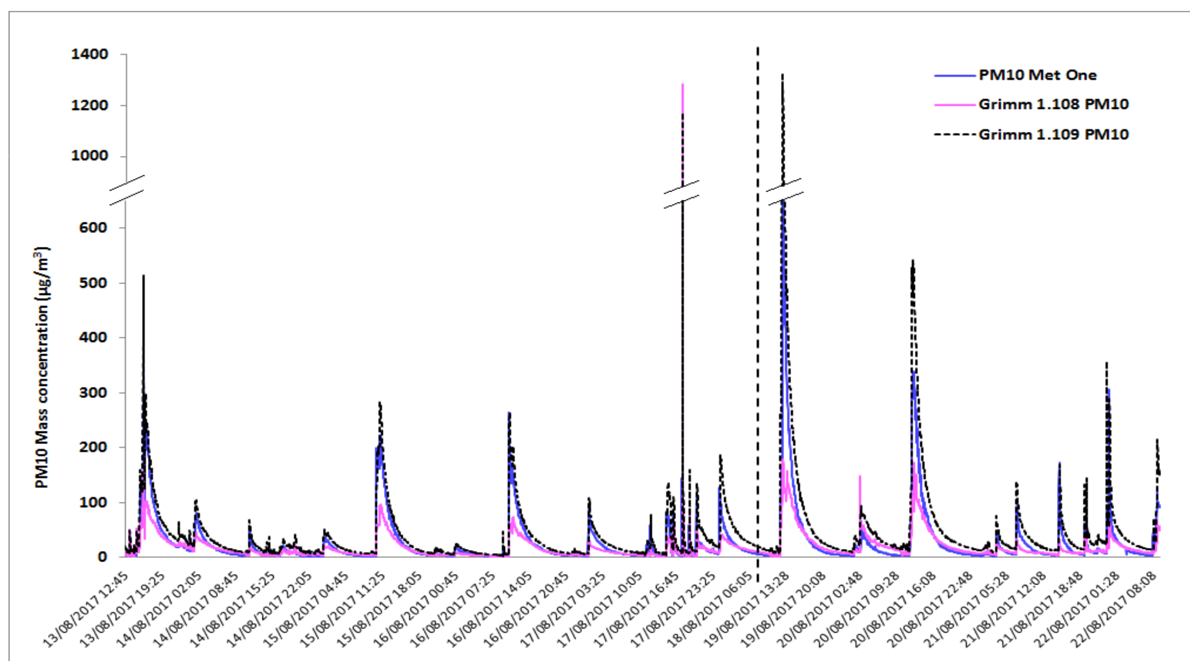

Figure S4.3. Time series of PM_{10} mass concentrations (μg/m^3) from the Met One Aerocet 531, Grimm 1.108 (C-factor corrected) and Grimm 1.109 intercomparisons. Dotted line indicates a break period between the two sets of continuous measurements.

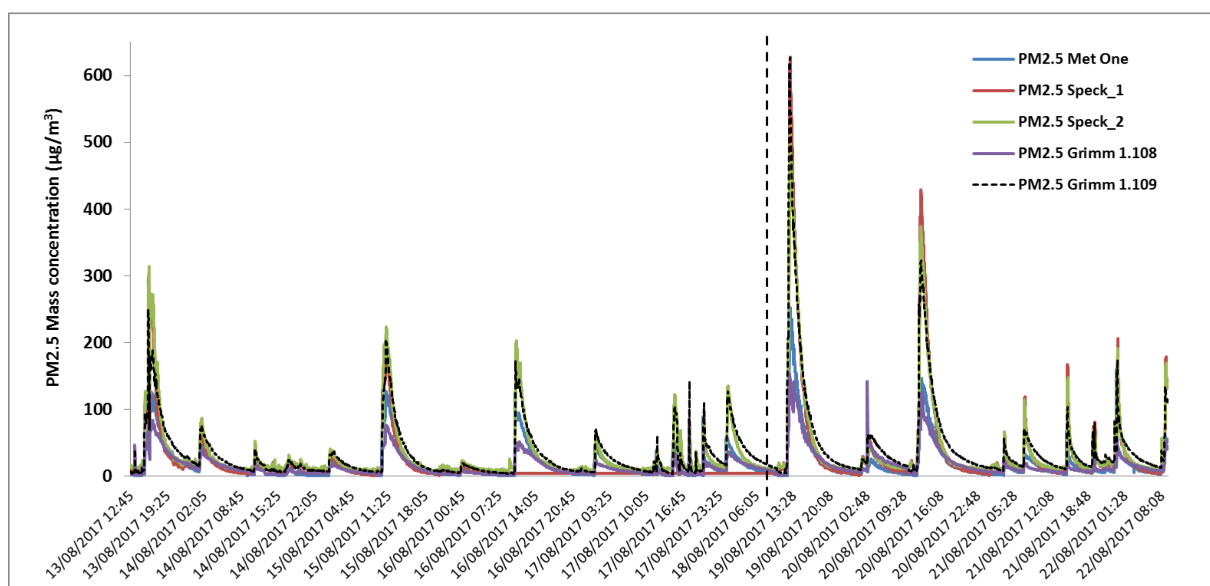

Figure S4.4. Time series of $PM_{2.5}$ mass concentrations (μg/m^3) from the Met One Aerocet 531, Speck-1, Speck-2, Grimm 1.108 (C-factor corrected) and Grimm 1.109 intercomparisons. Dotted line indicates a break period between the two sets of continuous measurements.

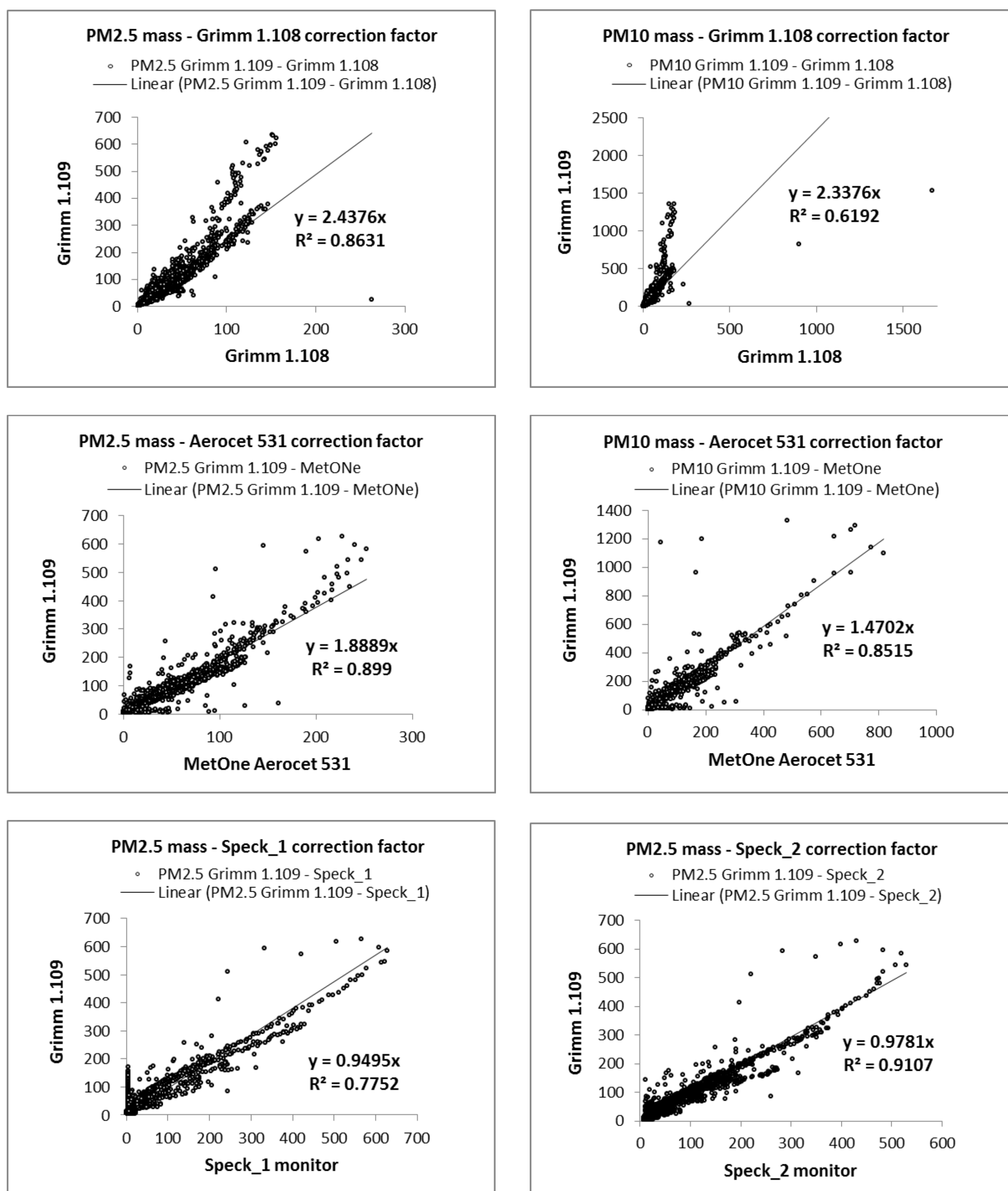

Figure S4.5 Linear regression between Grimm 1.109 and Grimm 1.108 (C-factor corrected), Met One Aerocet 531 and Speck-1, 2 monitors for $PM_{2.5}$ and PM_{10} real-time mass concentrations. All data are reported in μg/m^3. The y-intercept for the regressions was set to equal zero.

References

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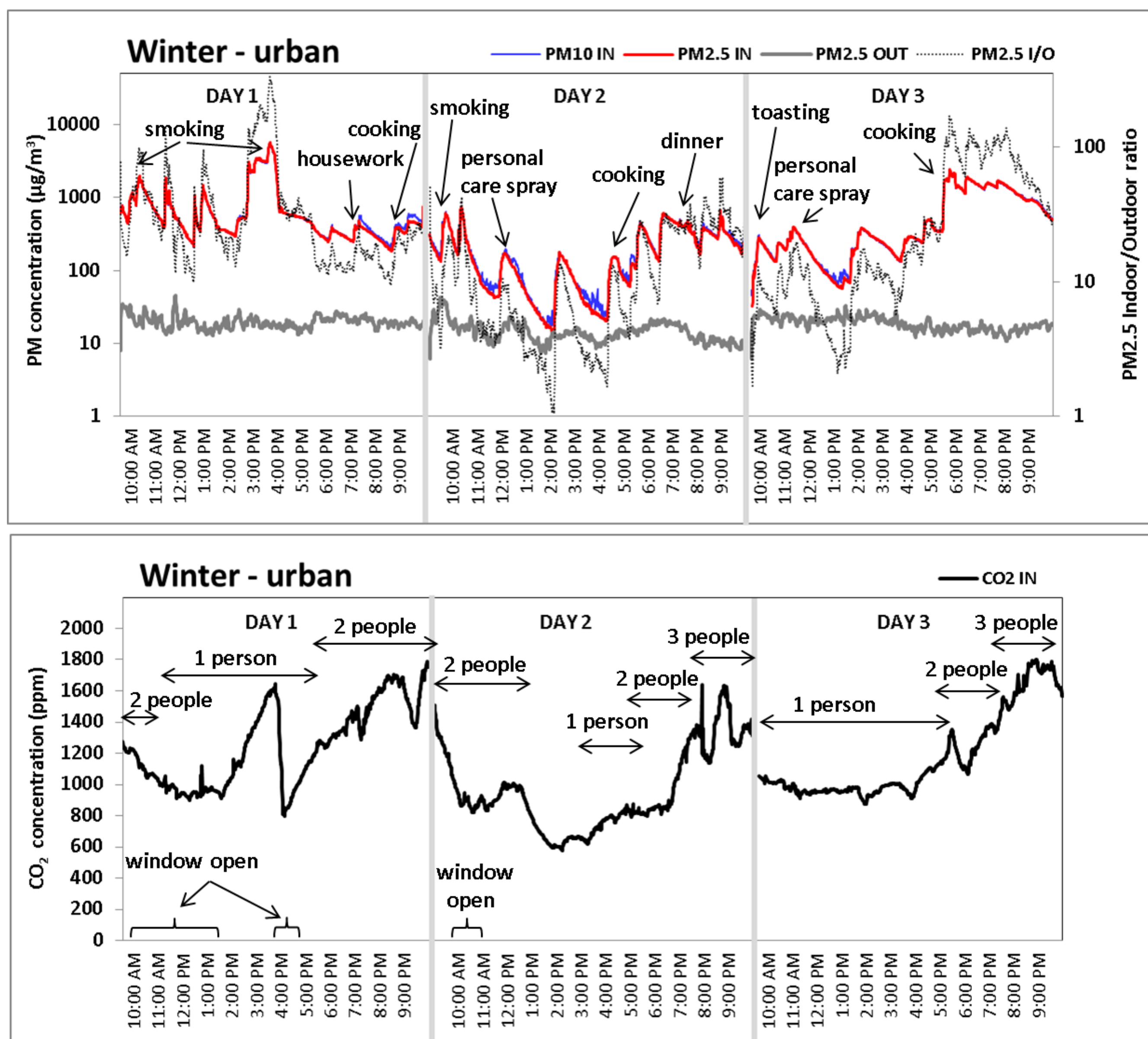


Figure S4.6 Top panel: Time series of PM mass concentrations (μg/m^3) measured indoors (PM_{10}, $PM_{2.5}$) and outdoors ($PM_{2.5}$) with selective indoor activities resulting in elevated levels (based on time-activity diary) indicated. Axis y is on log scale. Real-time Indoor-to-Outdoor (I/O) ratios for $PM_{2.5}$ are also presented (secondary y-axis). Bottom panel: Time series of CO_2 concentration (ppm) with marked periods of time that windows were kept open and levels of occupancy. All measurements were obtained at the urban site during 3 day-time periods in winter.

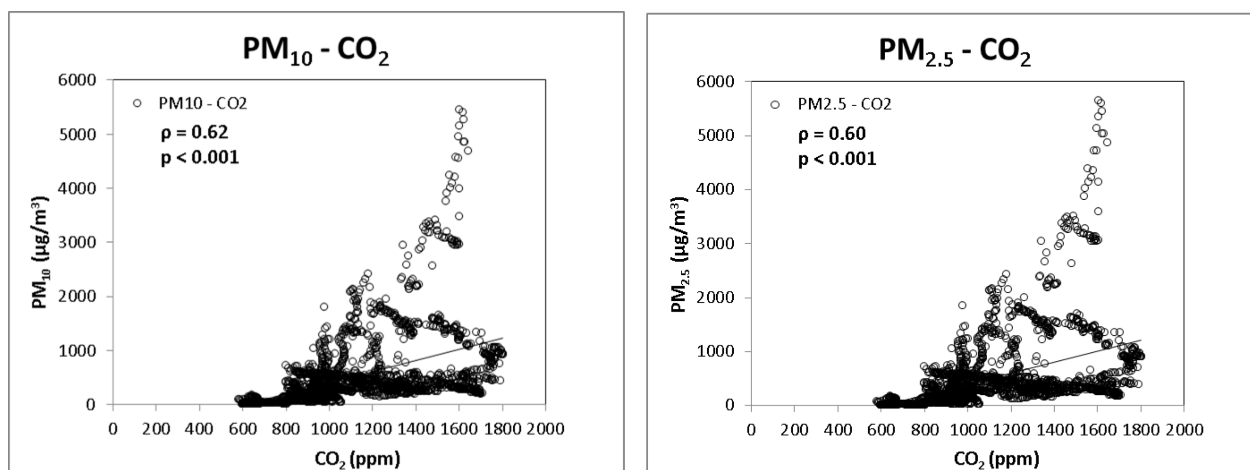


Figure S4.7 Scatterplots showing the relationship between PM_{10}/$PM_{2.5}$ mass concentrations (μg/m^3) and CO_2 concentration (ppm) measured simultaneously indoors at the urban flat during 3 day-time periods in winter. The association between the two variables was estimated using Spearman's rank correlation coefficient (ρ).

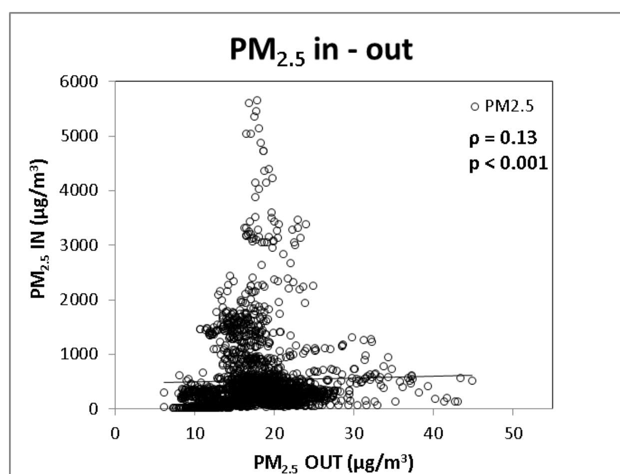


Figure S4.8 Scatterplot showing the relationship between $PM_{2.5}$ mass concentrations measured simultaneously indoors and outdoors at the urban site during 3 day-time periods in winter. The association between the two variables was estimated using Spearman's rank correlation coefficient (ρ).

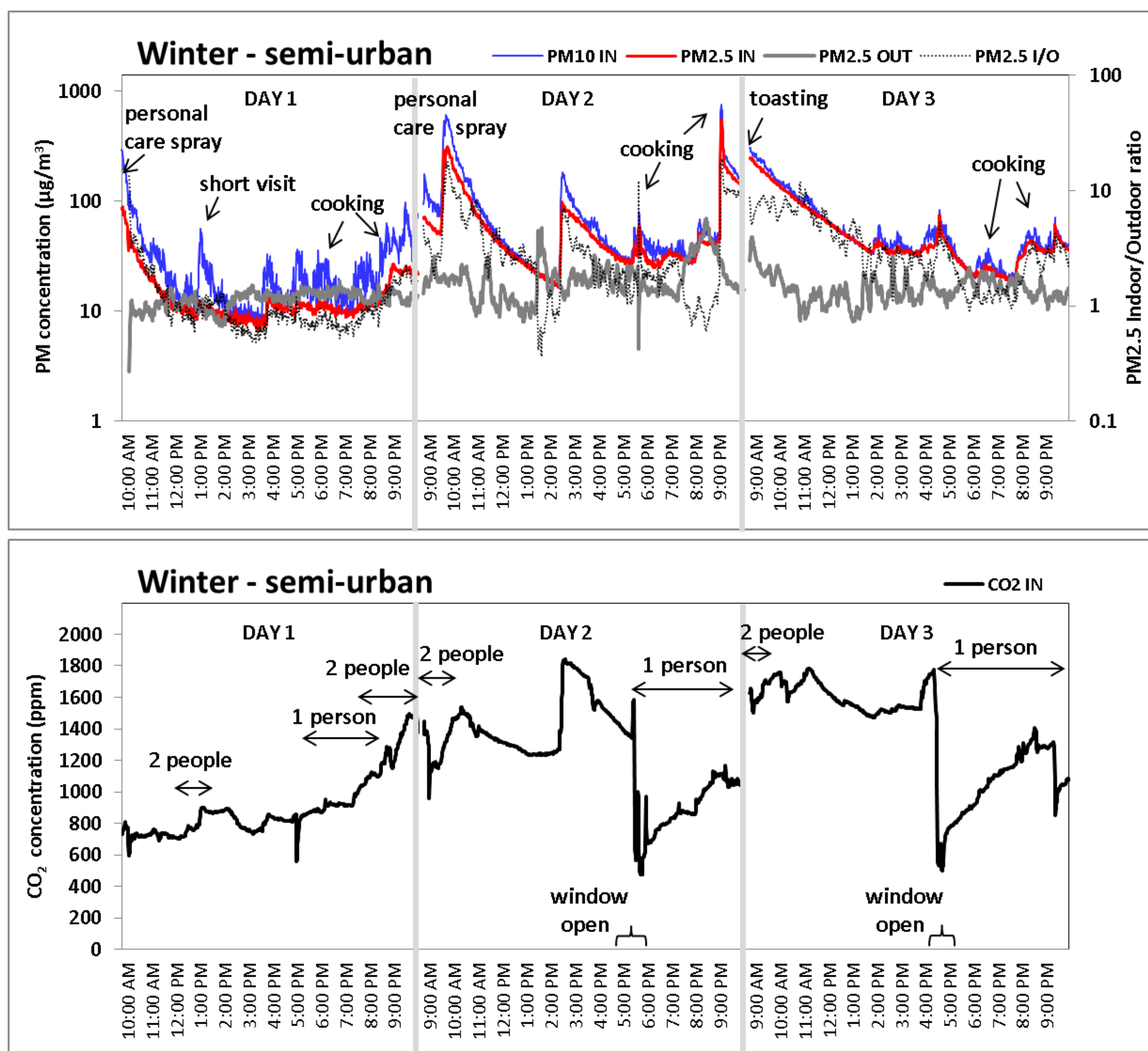


Figure S4.9 Top panel: Time series of PM mass concentrations (μg/m^3) measured indoors (PM_{10}, $PM_{2.5}$) and outdoors ($PM_{2.5}$) with selective indoor activities resulting in elevated levels (based on time-activity diary) indicated. Axis y is on log scale. Real-time Indoor-to-Outdoor (I/O) ratios for $PM_{2.5}$ are also presented (secondary y-axis). Bottom panel: Time series of CO_2 concentration (ppm) with marked periods of time that windows were kept open and levels of occupancy. All measurements were obtained at the semi-urban site during 3 day-time periods in winter.

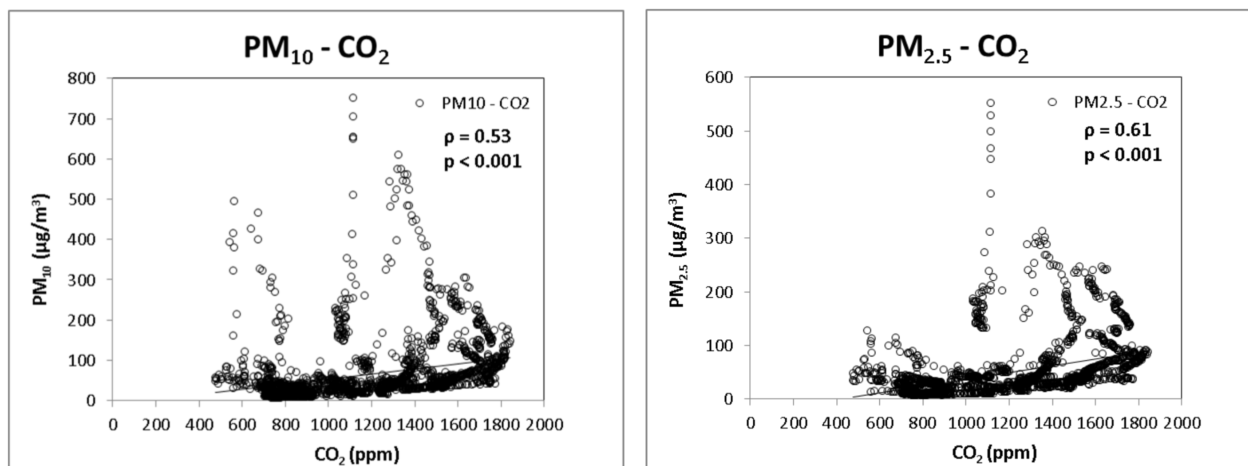


Figure S4.10 Scatterplots showing the relationship between PM_{10}/$PM_{2.5}$ mass concentrations (μg/m^3) and CO_2 concentration (ppm) measured simultaneously indoors at the semi-urban flat during 3 day-time periods in winter. The association between the two variables was estimated using Spearman's rank correlation coefficient (ρ).

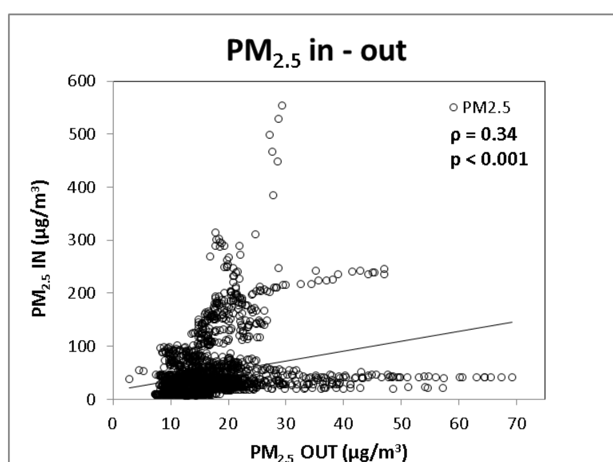


Figure S4.11 Scatterplot showing the relationship between $PM_{2.5}$ mass concentrations measured simultaneously indoors and outdoors at the semi-urban site during 3 day-time periods in winter. The association between the two variables was estimated using Spearman's rank correlation coefficient (ρ).

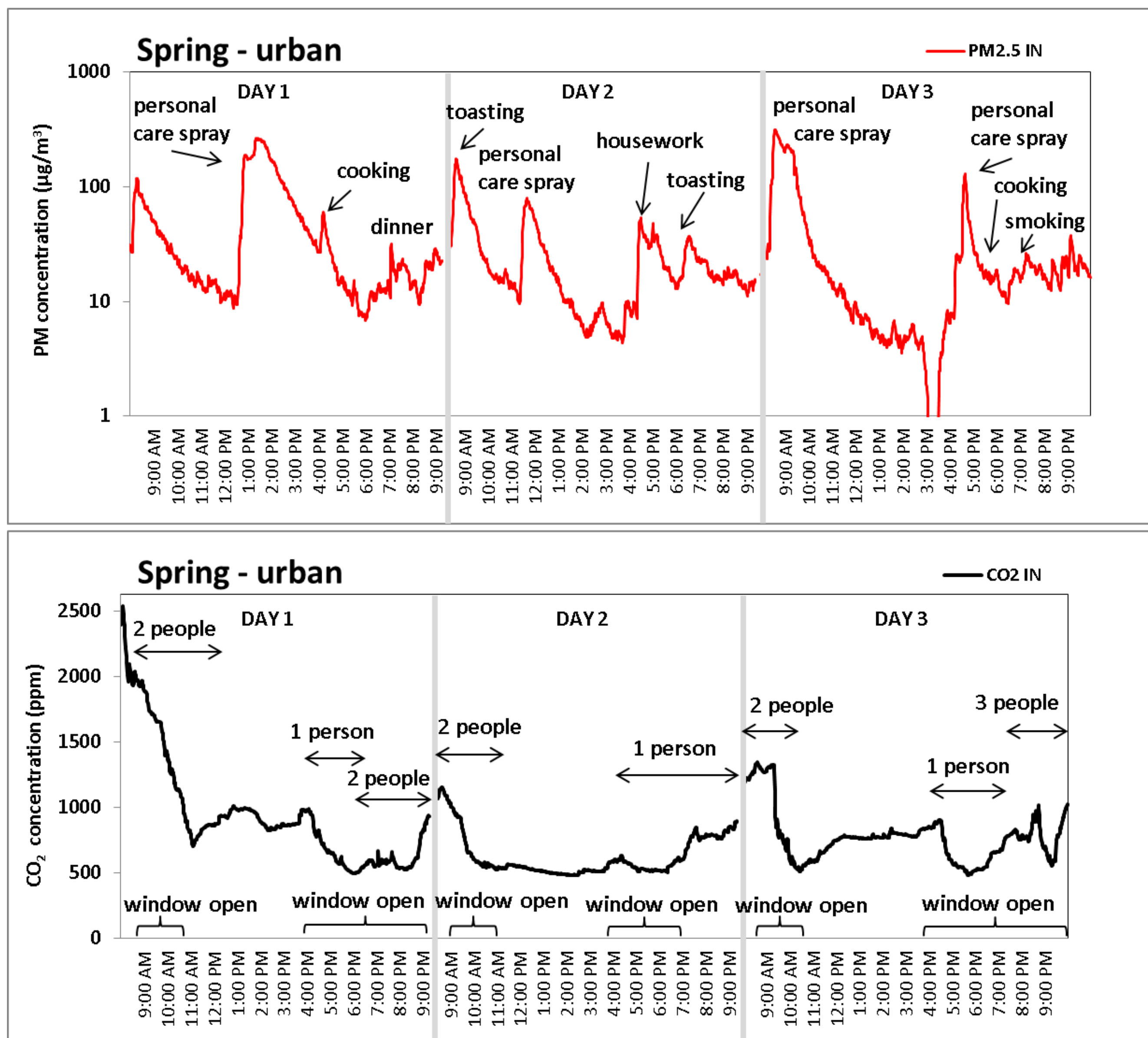


Figure S4.12 Top panel: Time series of $PM_{2.5}$ mass concentrations ($\mu g/m^3$) measured indoors with selective indoor activities resulting in elevated levels (based on time-activity diary) indicated. Axis y is on log scale. Outdoor $PM_{2.5}$ were not measured due to equipment limitations. Bottom panel: Time series of CO_2 concentration (ppm) with marked periods of time that windows were kept open and levels of occupancy. All measurements were obtained at the urban site during 3 day-time periods in spring.

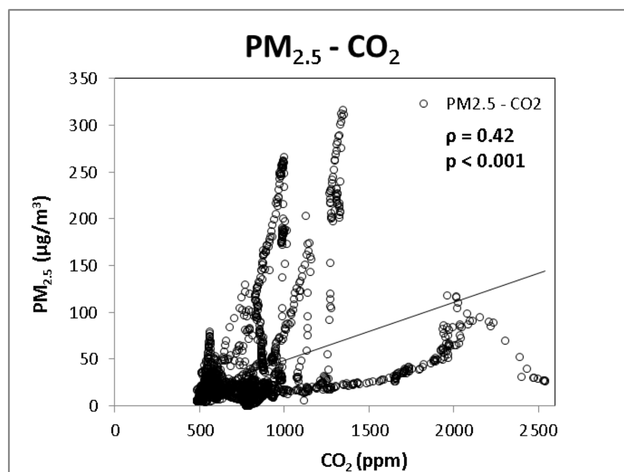


Figure S4.13 Scatterplot showing the relationship between $PM_{2.5}$ mass concentration (μg/m^3) and CO_2 concentration (ppm) measured simultaneously indoors at the urban flat during 3 day-time periods in spring. The association between the two variables was estimated using Spearman's rank correlation coefficient (ρ).

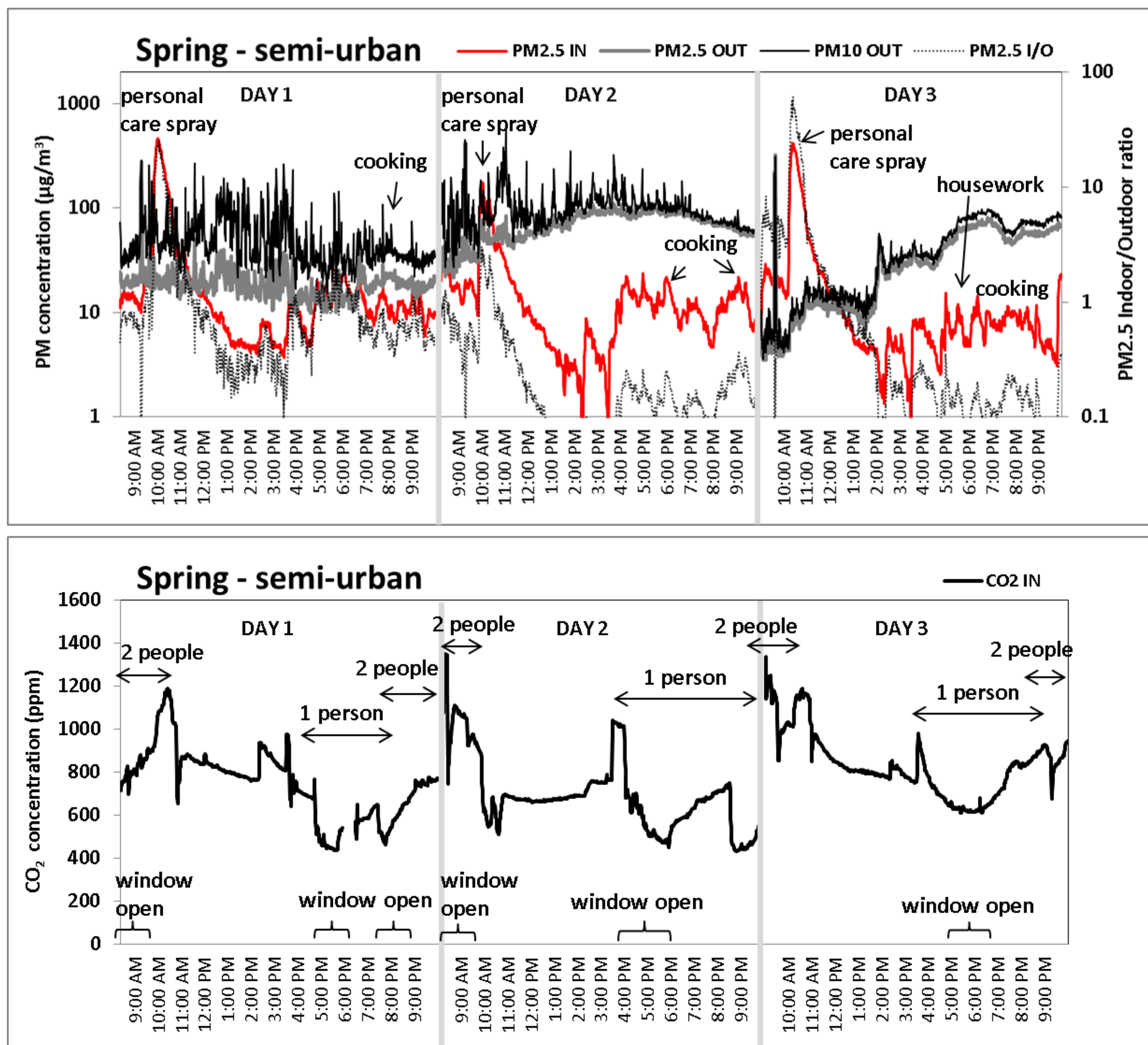


Figure S4.14 Top panel: Time series of PM mass concentrations (μg/m^3) measured indoors ($PM_{2.5}$) and outdoors (PM_{10}, $PM_{2.5}$) with selective indoor activities resulting in elevated levels (based on time-activity diary) indicated. Axis y is on log scale. Real-time Indoor-to-Outdoor (I/O) ratios for $PM_{2.5}$ are also presented (secondary y-axis). Bottom panel: Time series of CO_2 concentration (ppm) with marked periods of time that windows were kept open and levels of occupancy. All measurements were obtained at the semi-urban site during 3 day-time periods in spring.

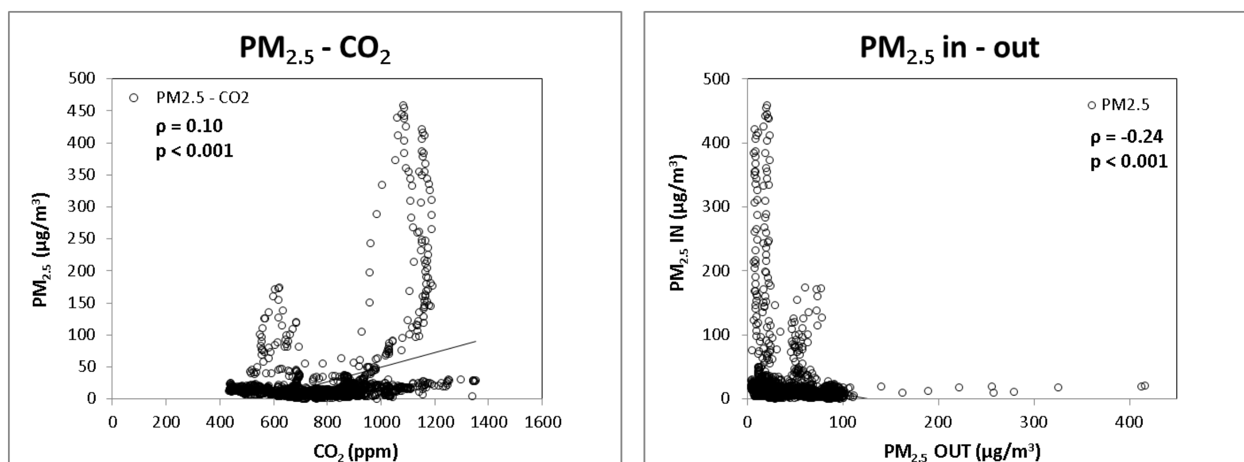


Figure S4.15 Scatterplots showing the relationship between $PM_{2.5}$ mass concentrations (μg/m^3) and CO_2 concentration (ppm) measured simultaneously indoors (panel on the left) as well as between $PM_{2.5}$ mass concentrations measured simultaneously indoors and outdoors (panel on the right) at the semi-urban during 3 day-time periods in spring. The association between the variables was estimated using Spearman's rank correlation coefficient (ρ).

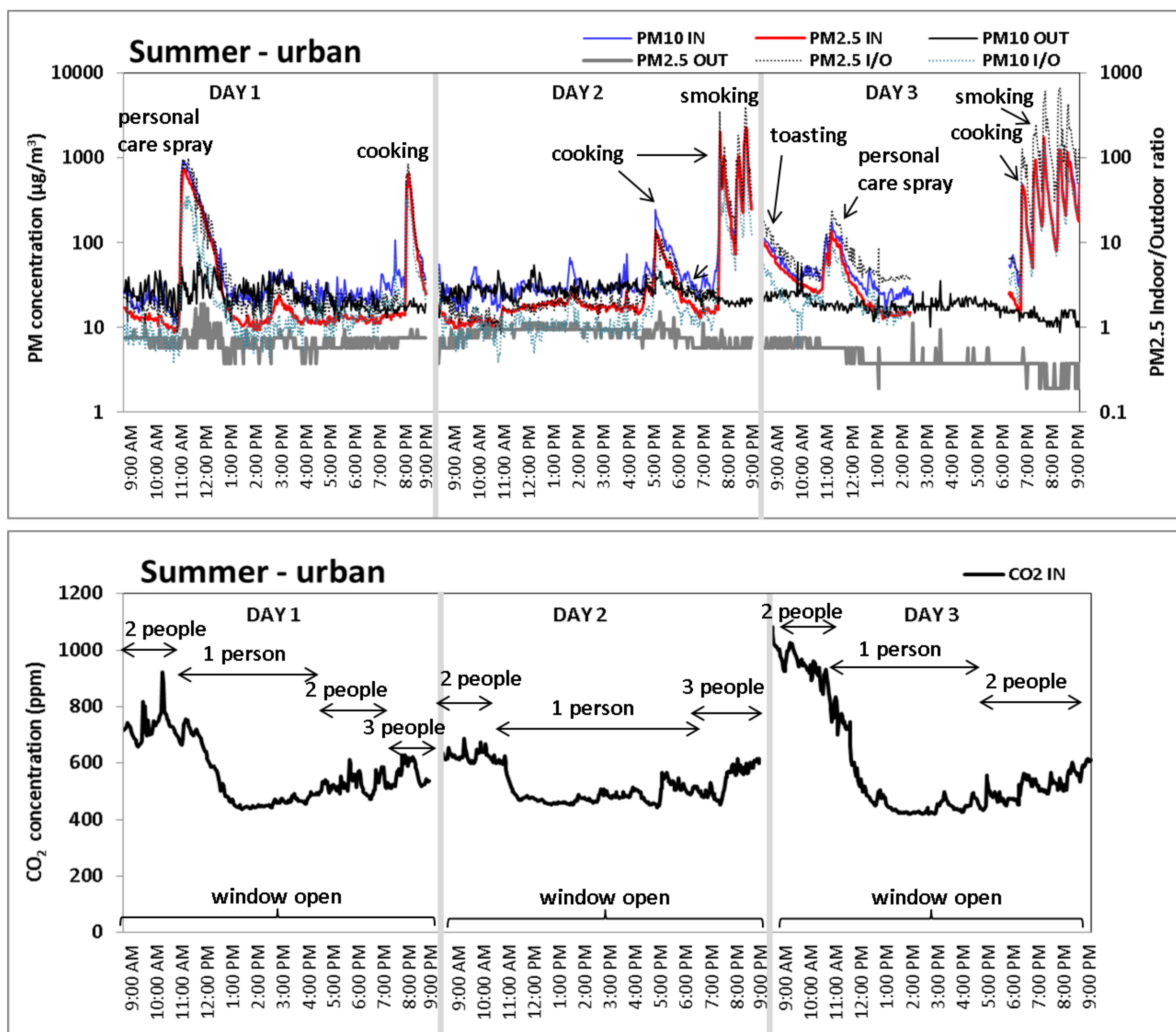


Figure S4.16 Top panel: Time series of PM mass concentrations (μg/m^3) measured indoors (PM_{10}, $PM_{2.5}$) and outdoors (PM_{10}, $PM_{2.5}$) with selective indoor activities resulting in elevated levels (based on time-activity diary) indicated. Axis y is on log scale. Real-time Indoor-to-Outdoor (I/O) ratios for $PM_{2.5}$ and PM_{10} are also presented (secondary y-axis). Bottom panel: Time series of CO_2 concentration (ppm) with marked periods of time that windows were kept open and levels of occupancy. All measurements were obtained at the urban site during 3 day-time periods in summer.

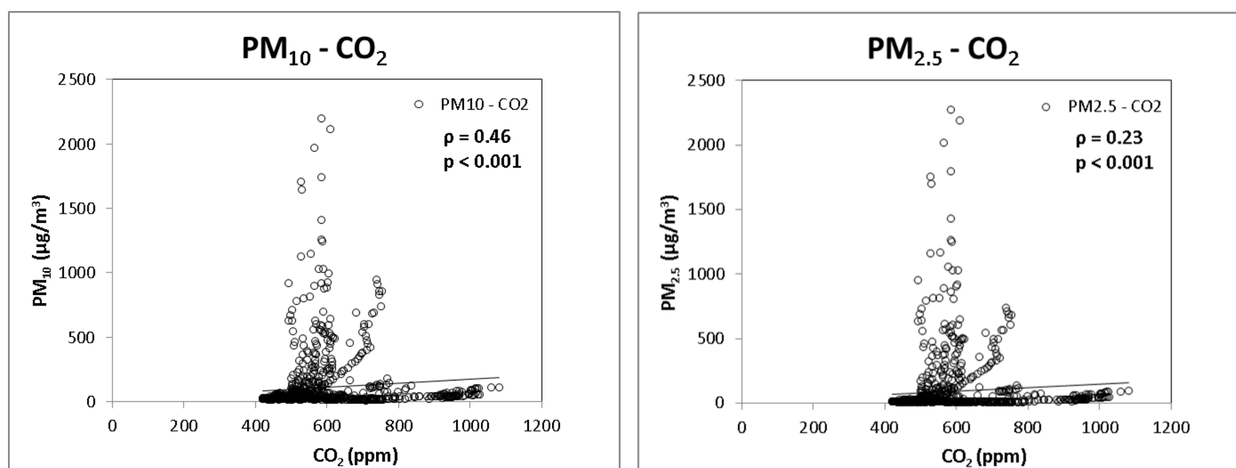


Figure S4.17 Scatterplots showing the relationship between $PM_{10}/PM_{2.5}$ mass concentrations ($\mu g/m^3$) and CO_2 concentration (ppm) measured simultaneously indoors at the urban flat during 3 day-time periods in summer. The association between the two variables was estimated using Spearman's rank correlation coefficient (ρ).

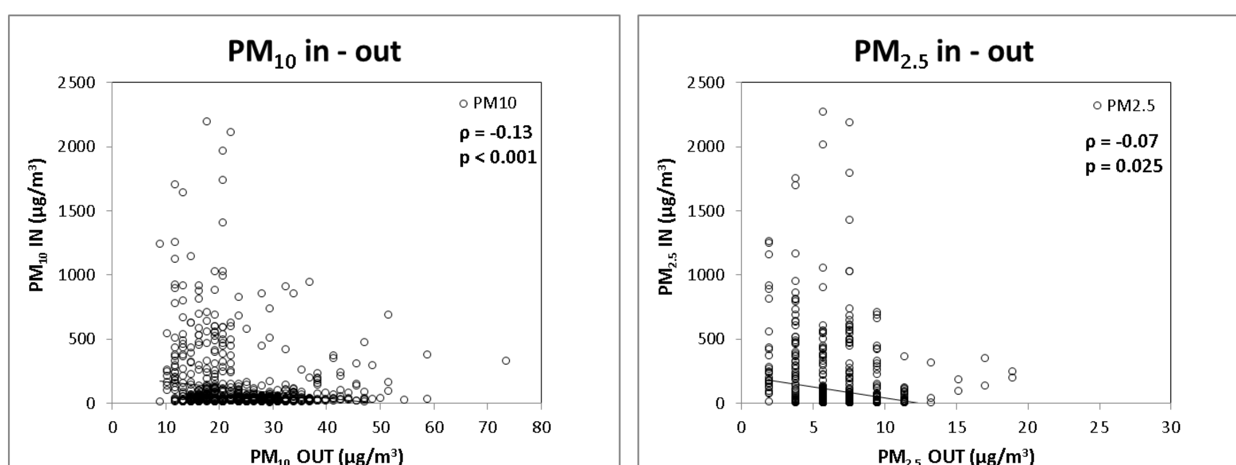


Figure S4.18 Scatterplots showing the relationship between $PM_{10}/PM_{2.5}$ mass concentrations measured simultaneously indoors and outdoors at the urban site during 3 day-time periods in summer. The association between the two variables was estimated using Spearman's rank correlation coefficient (ρ).

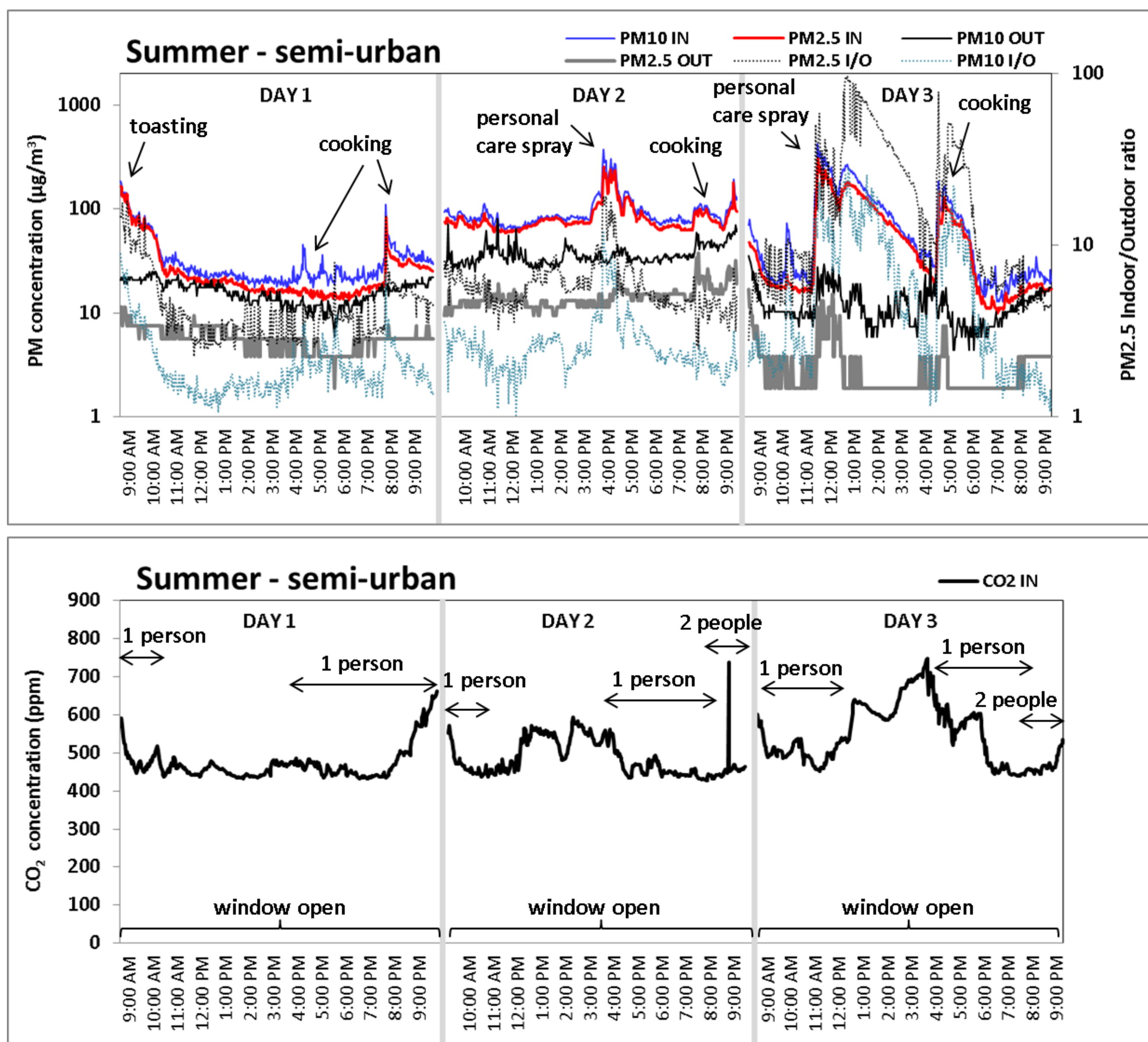


Figure S4.19 Top panel: Time series of PM mass concentrations (µg/m^3) measured indoors (PM_{10}, $PM_{2.5}$) and outdoors (PM_{10}, $PM_{2.5}$) with selective indoor activities resulting in elevated levels (based on time-activity diary) indicated. Axis y is on log scale. Real-time Indoor-to-Outdoor (I/O) ratios for $PM_{2.5}$ and PM_{10} are also presented (secondary y-axis). Bottom panel: Time series of CO_2 concentration (ppm) with marked periods of time that windows were kept open and levels of occupancy. All measurements were obtained at the semi-urban site during 3 day-time periods in summer.

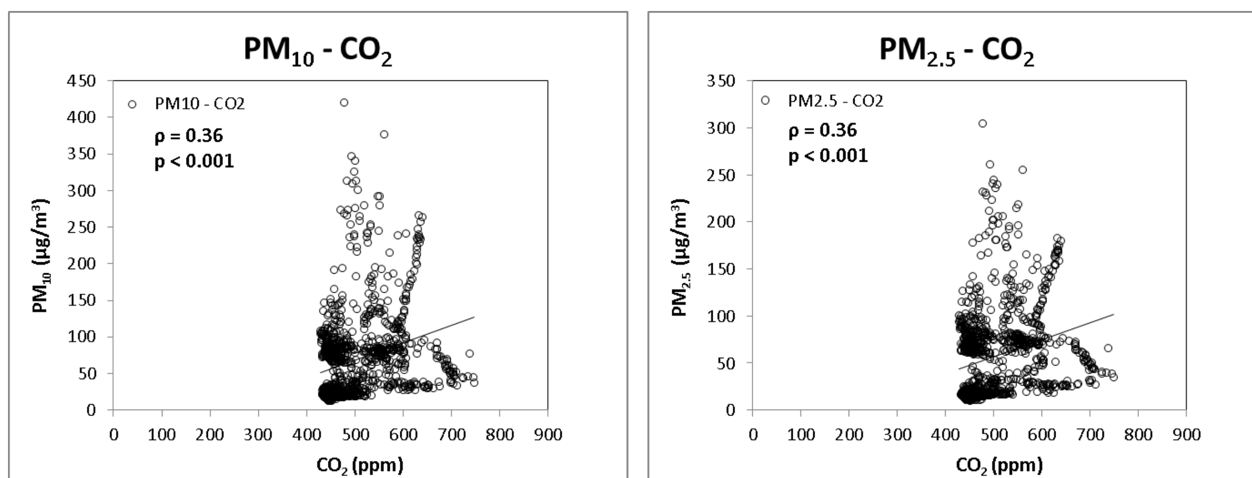


Figure S4.20 Scatterplots showing the relationship between PM_{10}/$PM_{2.5}$ mass concentrations (μg/m^3) and CO_2 concentration (ppm) measured simultaneously indoors at the semi-urban flat during 3 day-time periods in summer. The association between the two variables was estimated using Spearman's rank correlation coefficient (ρ).

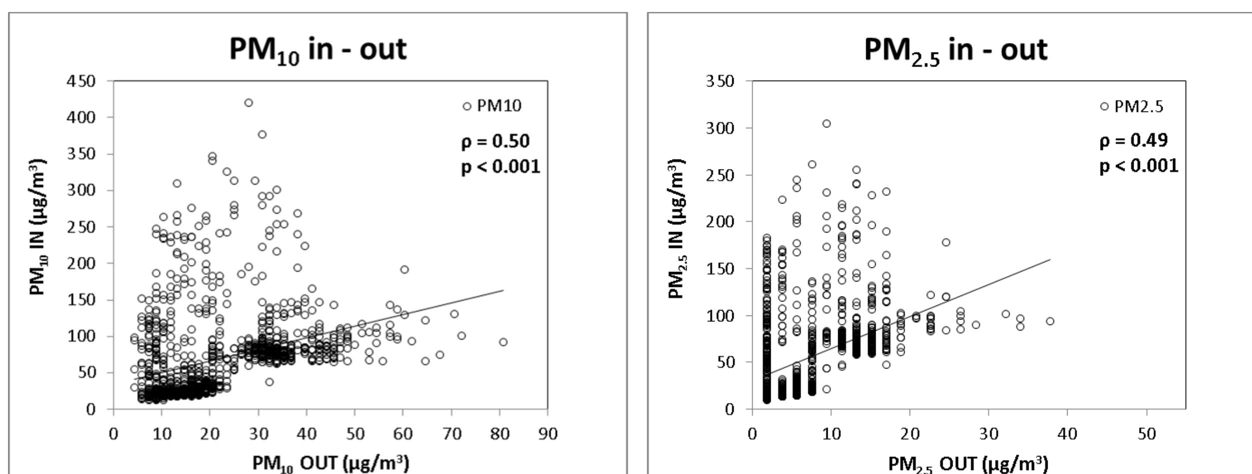


Figure S4.21 Scatterplots showing the relationship between PM_{10}/$PM_{2.5}$ mass concentrations measured simultaneously indoors and outdoors at the semi-urban site during 3 day-time periods in summer. The association between the two variables was estimated using Spearman's rank correlation coefficient (ρ).

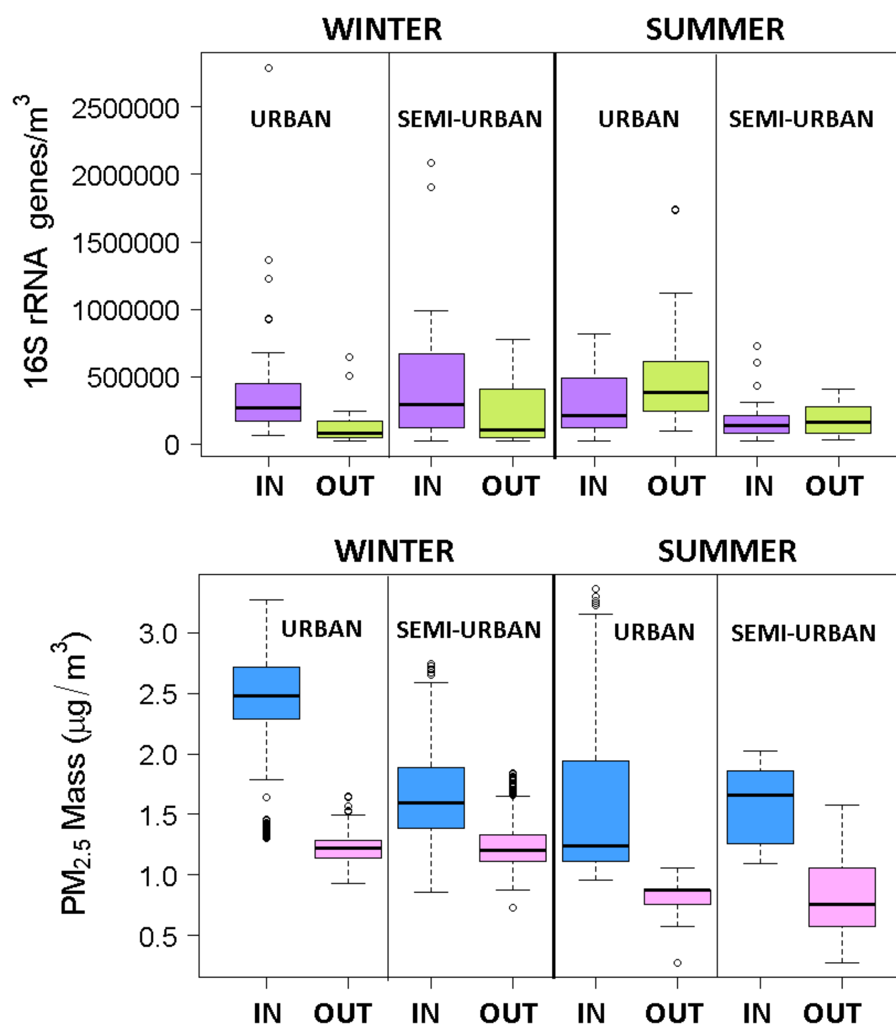


Figure S4.22 Summary boxplots of total indoor and outdoor airborne bacterial concentrations (16S rRNA genes per m^3) and $PM_{2.5}$ mass concentrations ($\mu g/m^3$), per house (urban, semi-urban) and season (winter, summer), based on 1-h filter samples collected during different times of day (outliers included).

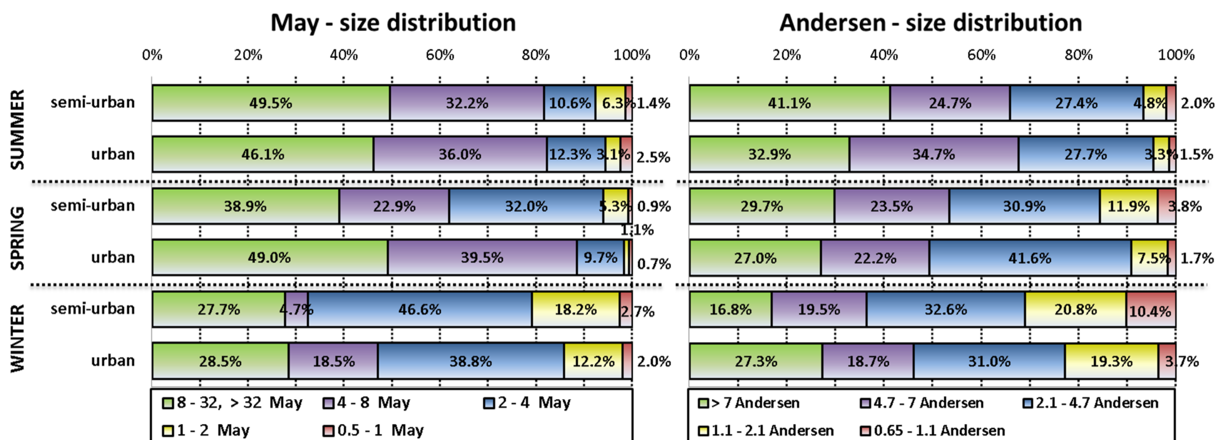


Figure S4.23 Average percentages of indoor bacterial abundance (16S rRNA genes /m^3) recovered for each size fraction for both May and Andersen impactors per each site (urban, semi-urban) across three seasons. Dissimilar impactors size bin ranges have been grouped for the purpose of comparison.

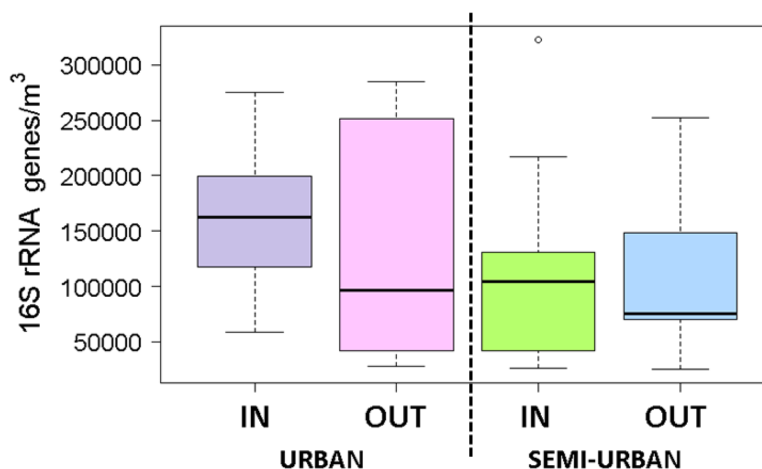


Figure S4.24 Summary boxplots of total aerosol bacterial abundance, indoors and outdoors, per house (urban, semi-urban), based on 12-h samples collected by May impactor. All measures of bacterial abundance are in 16S rRNA genes per m^3 of air.

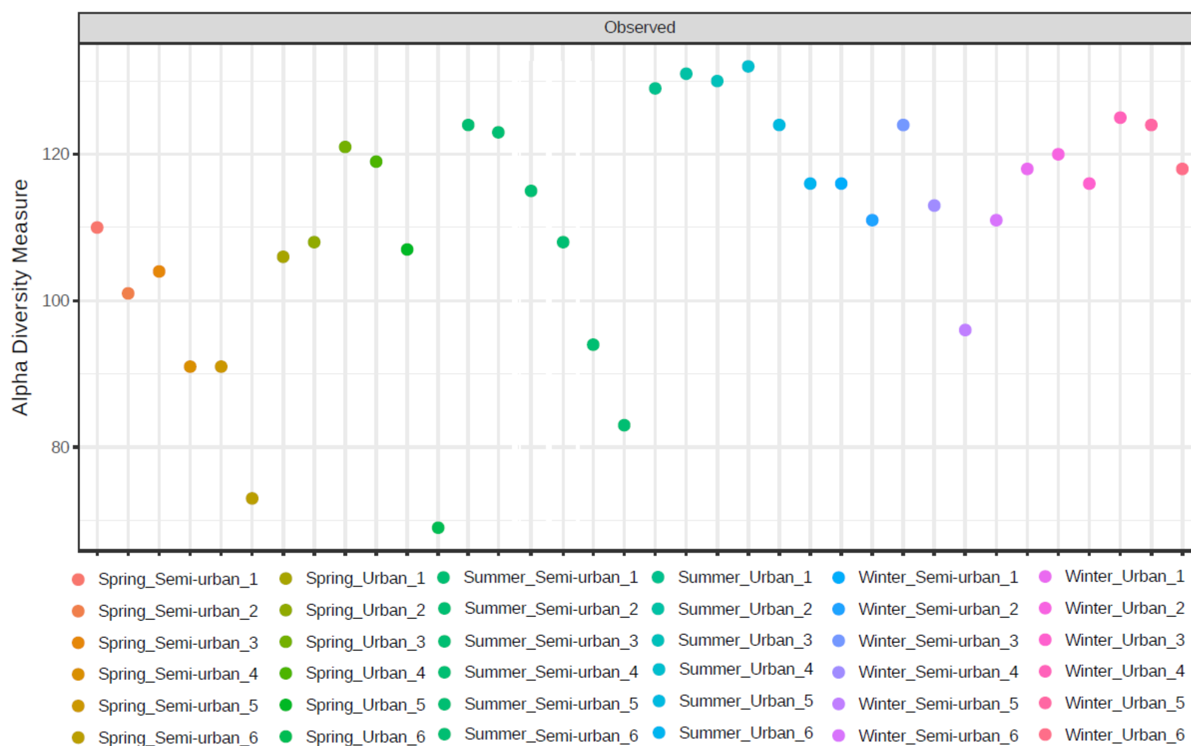


Figure S4.25 Numbers of indoor air bacterial OTUs recovered per each stage, sampling site (urban, semi-urban) and season (winter, summer, spring). Size-resolved samples were obtained with the Andersen impactor. Stages 1, 2, 3, 4, 5 and 6 correspond to particle size ranges: > 7 μm, 7 - 4.7 μm, 4.7 - 3.3 μm, 3.3 - 2.1 μm, 2.1 - 1.1 μm and 1.1 - 0.65 μm.

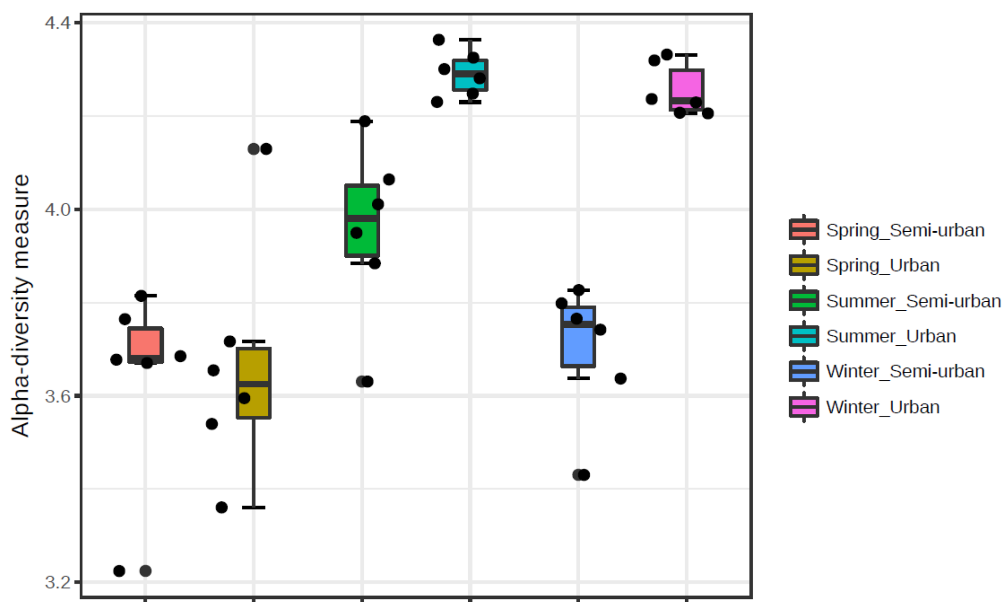


Figure S4.26 Boxplots of Shannon index per sampling site and season of 12-h day-time size-resolved aerosol samples collected indoors with the six-stage Andersen impactor.

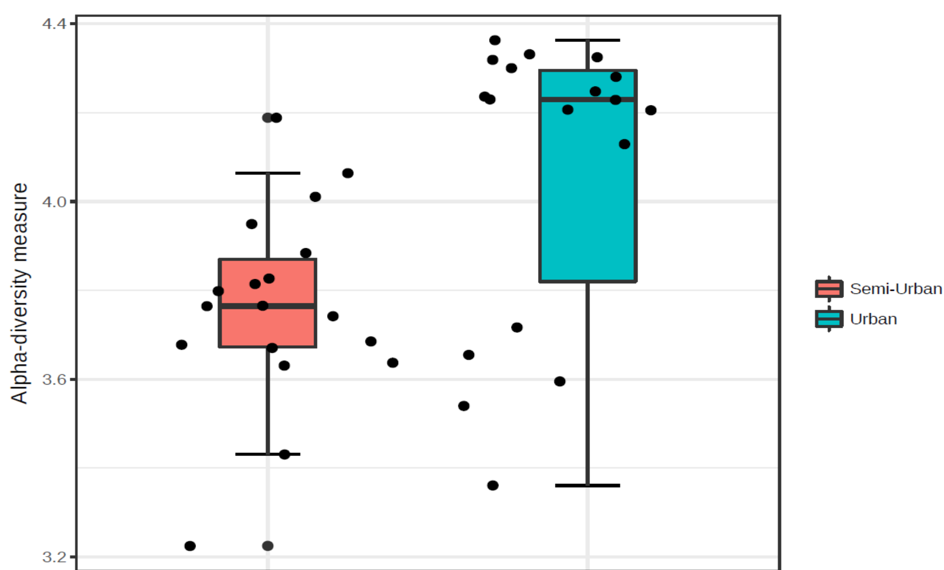


Figure S4.27 Boxplots of Shannon index per sampling site of 12-h day-time size-resolved aerosol samples collected indoors with the six-stage Andersen impactor.

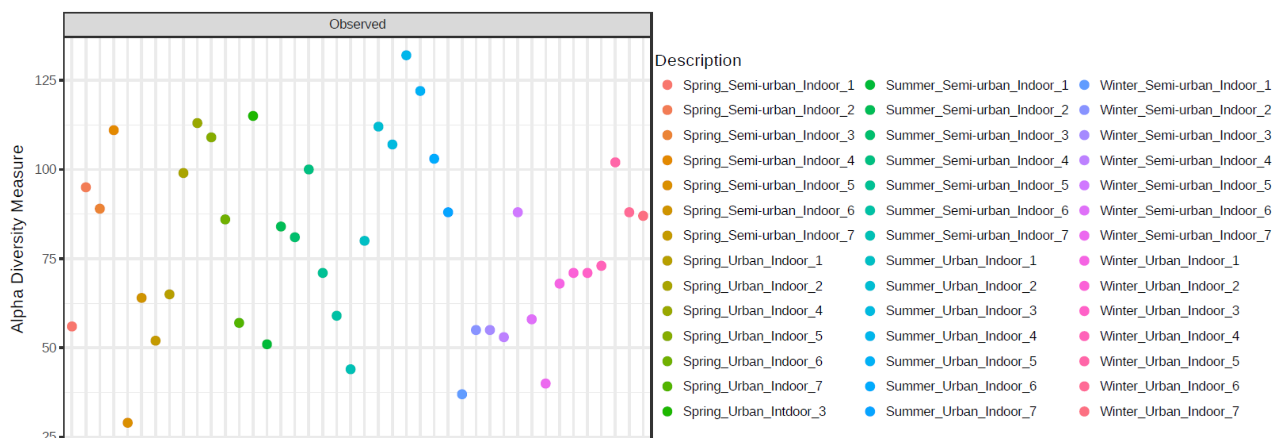


Figure S4.28 Numbers of indoor air bacterial OTUs recovered per each stage, sampling site (urban, semi-urban) and season (winter, summer, spring). Size-resolved samples were obtained with the May iimpactor. Stages 1, 2, 3, 4, 5, 6 & 7 correspond to particle size ranges: >32 µm, 32 - 16 µm, 16 - 8 µm, 8 - 4 µm, 4 - 2 µm, 2 - 1 µm and 1 - 0.5 µm.

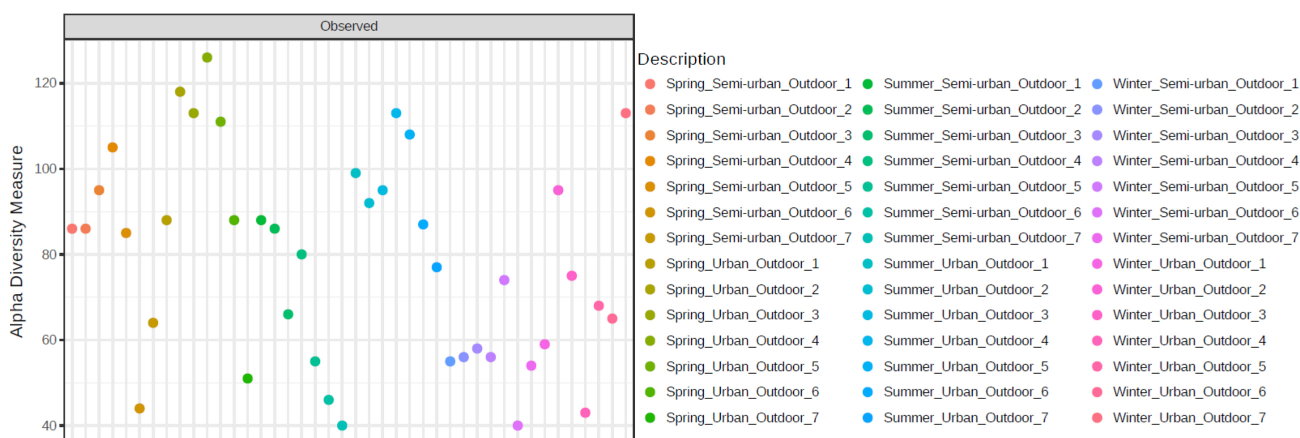


Figure S4,29 Numbers of outdoor air bacterial OTUs recovered per each stage, sampling site (urban, semi-urban) and season (winter, summer, spring). Size-resolved samples were obtained with the May iimpactor. Stages 1, 2, 3, 4, 5, 6 & 7 correspond to particle size ranges: >32 µm, 32 - 16 µm, 16 - 8 µm, 8 - 4 µm, 4 - 2 µm, 2 - 1 µm and 1 - 0.5 µm.

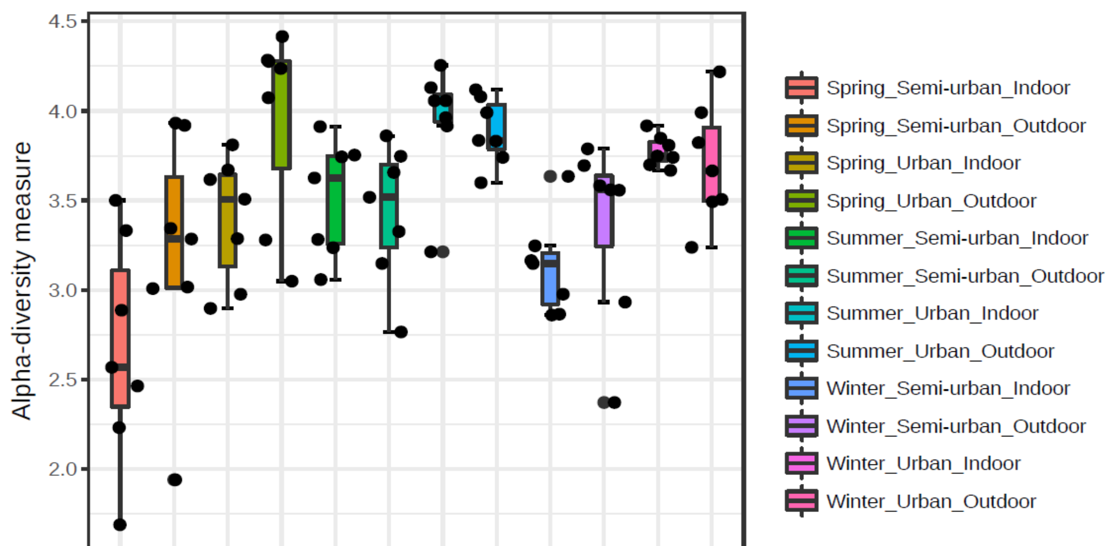


Figure S4.30 Boxplots of Shannon index per sampling site and season of 12-h day-time size-resolved aerosol samples collected indoors and outdoors with the seven-stage May impactor.

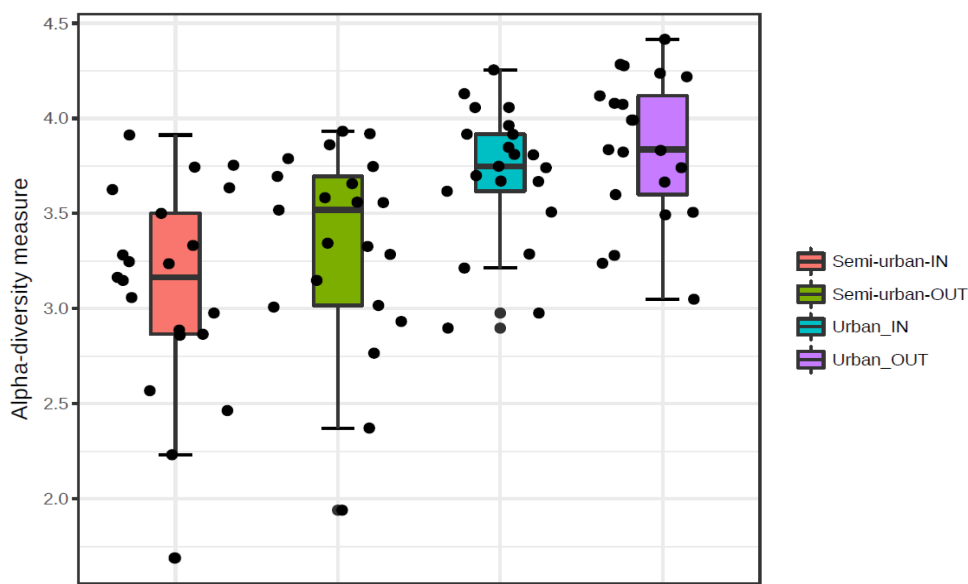


Figure S4.31 Boxplots of Shannon index per sampling site of 12-h day-time size-resolved aerosol samples collected indoors and outdoors with the seven-stage May impactor.

Figure S4.32 Numbers of indoor air bacterial OTUs recovered per each replicate for each sampling site (urban, semi-urban) and season (winter, summer, spring). Samples were obtained with whole-month passive collection using suspended (empty) petri dishes indoors.

Figure S4.33 Boxplots of Shannon index per sampling site of aerosol samples collected indoors with passive collection using suspended (empty) petri dishes.

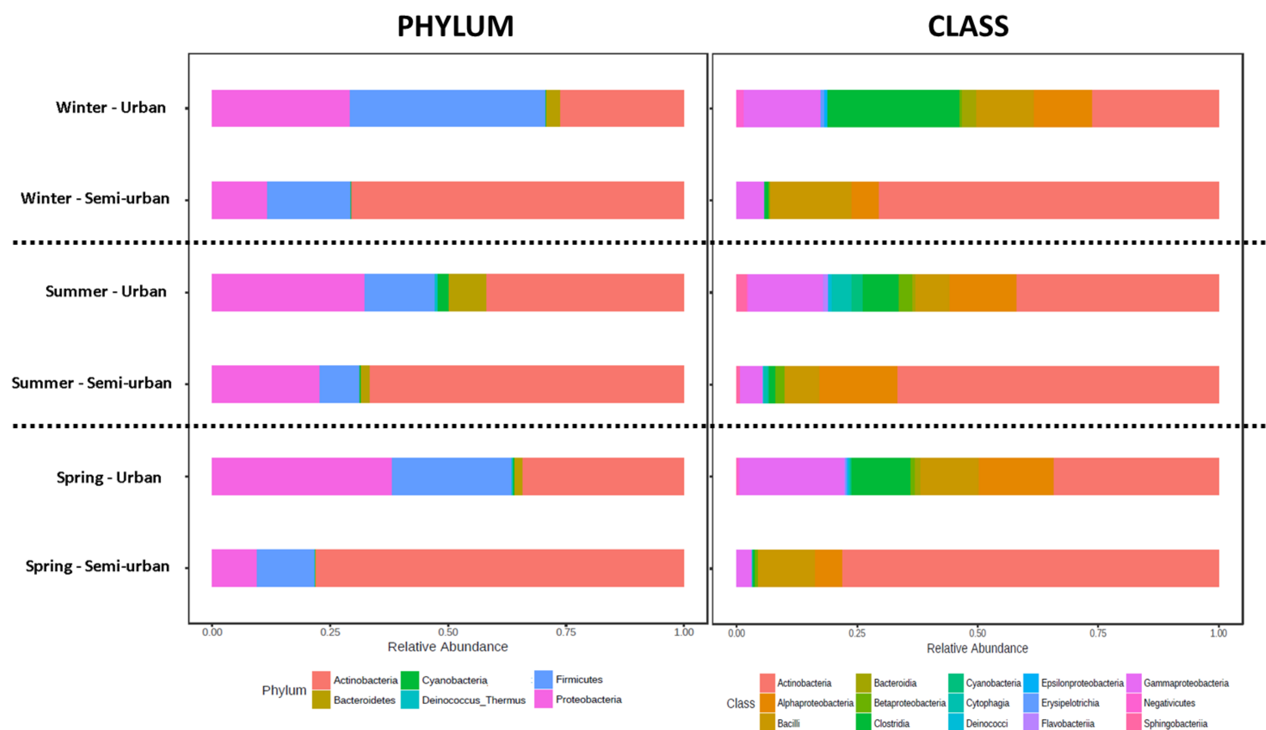
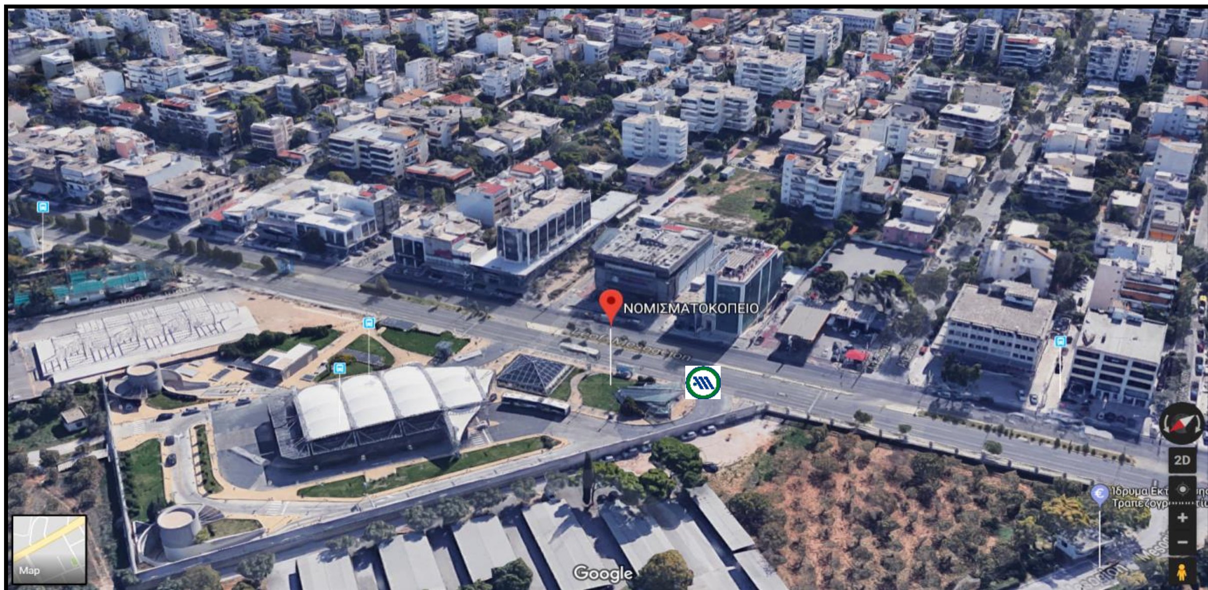


Figure S4.34 Relative abundance of indoor air bacterial OTUs at the phylum and class level per each sampling site (urban, semi-urban) and across seasons (winter, summer, spring). Samples were obtained with passive collection using suspended petri dishes. Each barplot represents the average relative proportion over three replicates.

Appendix S5



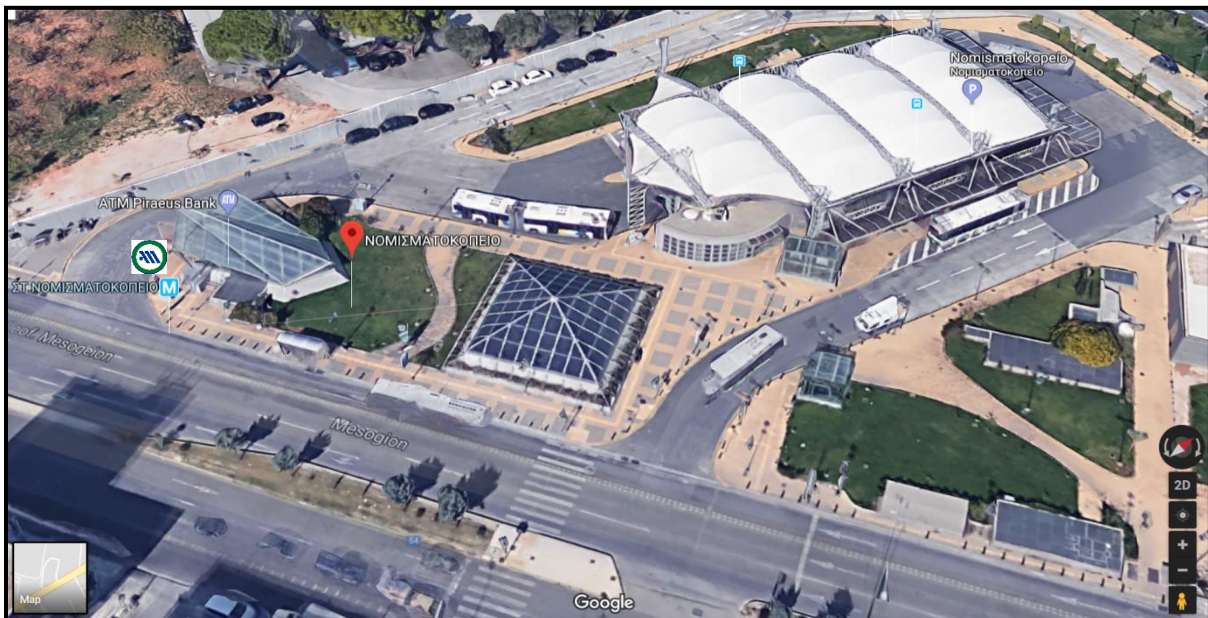


Figure S5.1 Google Maps 3D images of the surrounding area of station Nomismatokopio ("ΝΟΜΙΣΜΑΤΟΚΟΠΕΙΟ", Athens Metro Line 3, Mesogion Str., Athens, Greece, Google Maps, 2015). The Metro station is located in a densely populated residential area in northeast Athens, under a highly trafficked major motorway, part of the Greek National Road network.

Table S5.1 Primers used in the current study.

Primer name	Primer sequence (5' – 3')	Reference
ITS1F with Illumina overhang forward adapter sequence	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns (1993)
ITS2 with Illumina overhang reverse adapter sequence	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCGTTCTTCATCGATGC	White et al. (1990)

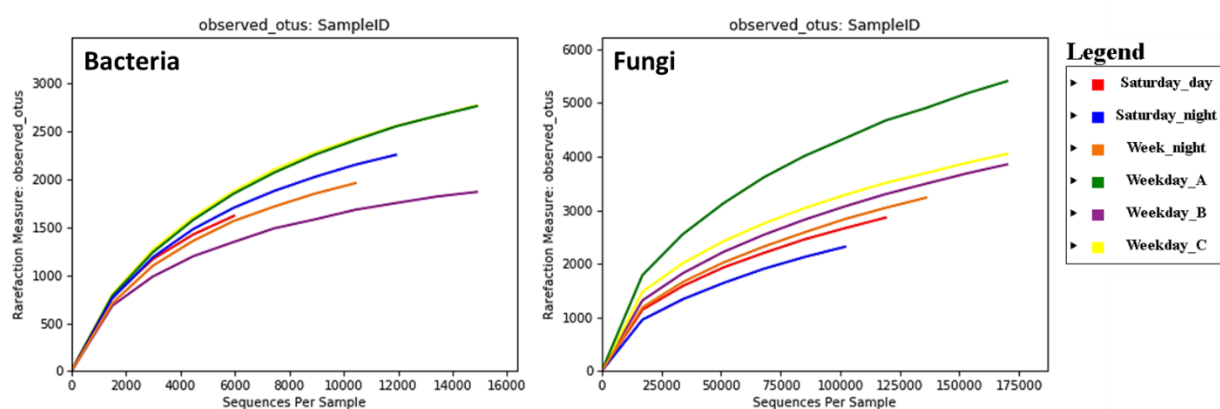


Figure S5.2 Rarefaction curves of observed bacterial and fungal OTU richness based on 97% similarity, prior normalisation and low-abundance OUT filtering. The alpha rarefaction analysis showed that curves for each sample started to level-off approaching the plateau of number of OTUs, indicating that the sequencing effort was adequate to detect most of the OTUs.

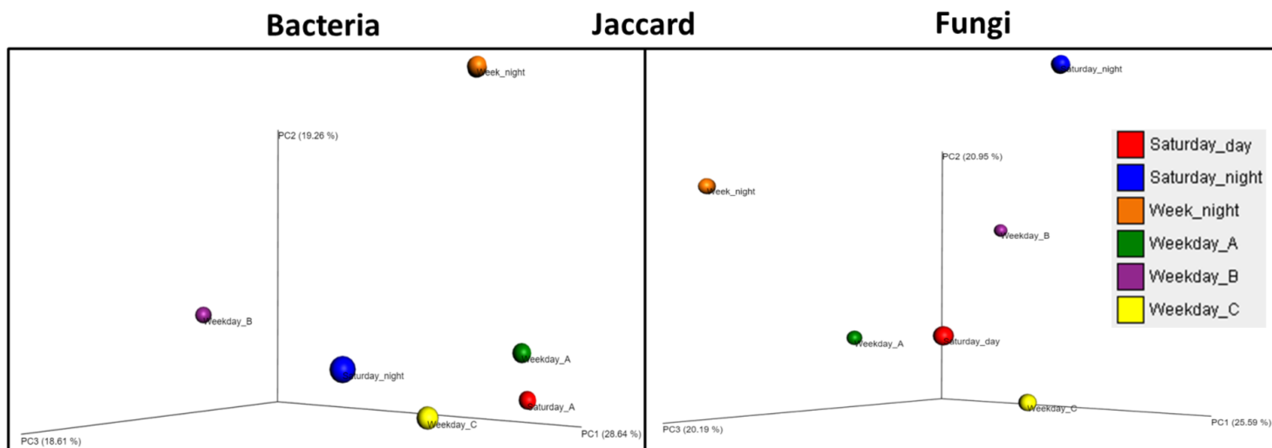


Figure S5.3 Principal coordinate analysis 3D-plots of bacterial and fungal beta diversity based on Jaccard index.

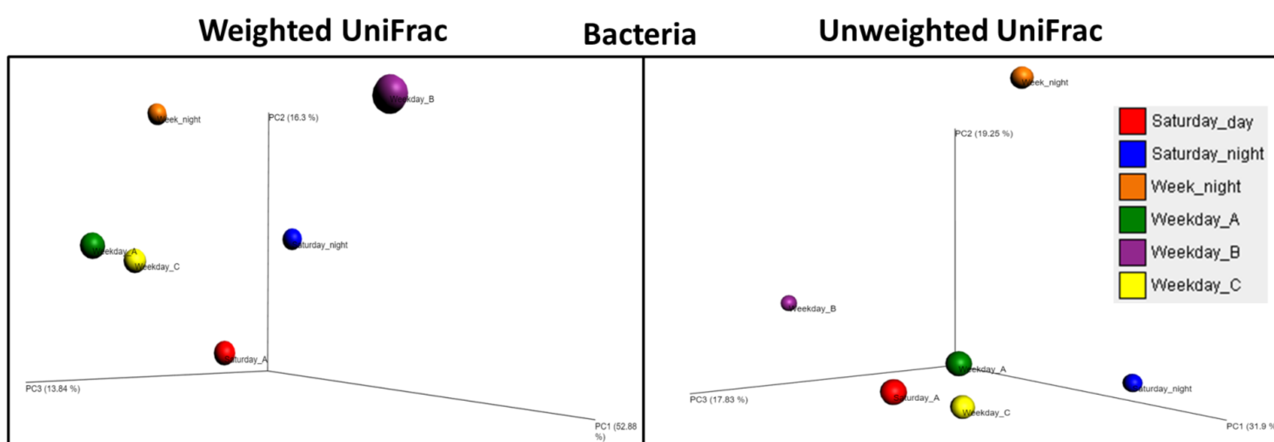


Figure S5.4 Principal coordinate analysis 3D-plots of bacterial beta diversity based on weighted and unweighted UniFrac distance metric.

Appendix S6

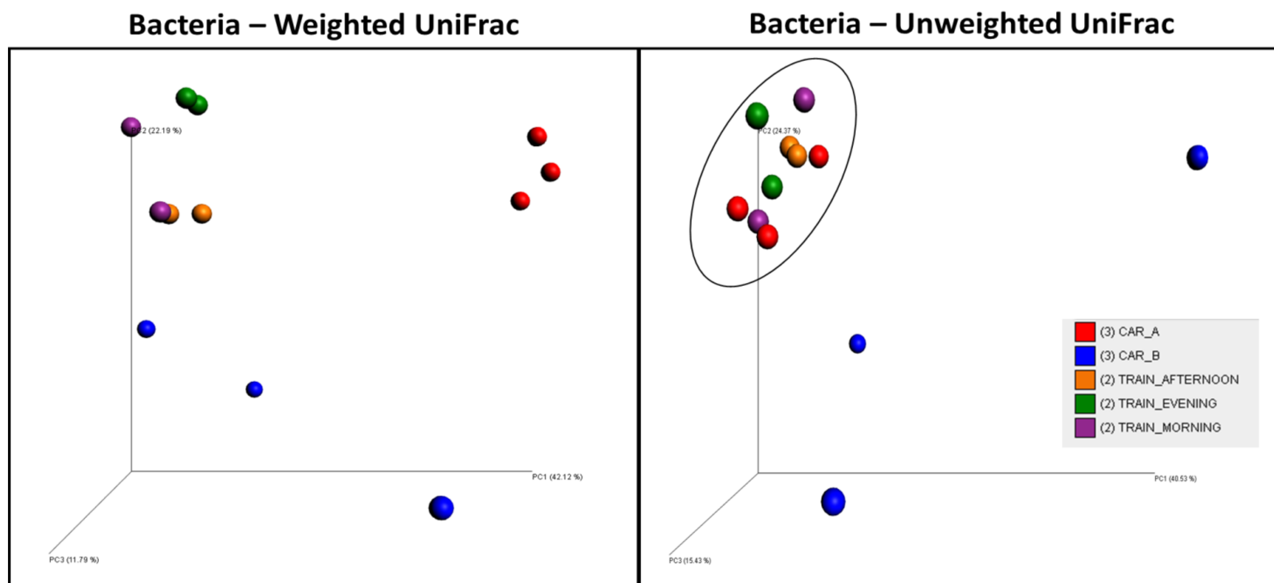


Figure S6.1 Principal coordinate analysis plots of bacterial beta diversity based on UniFrac, weighted (panel on the left) and unweighted (panel on the right), distance matrix.

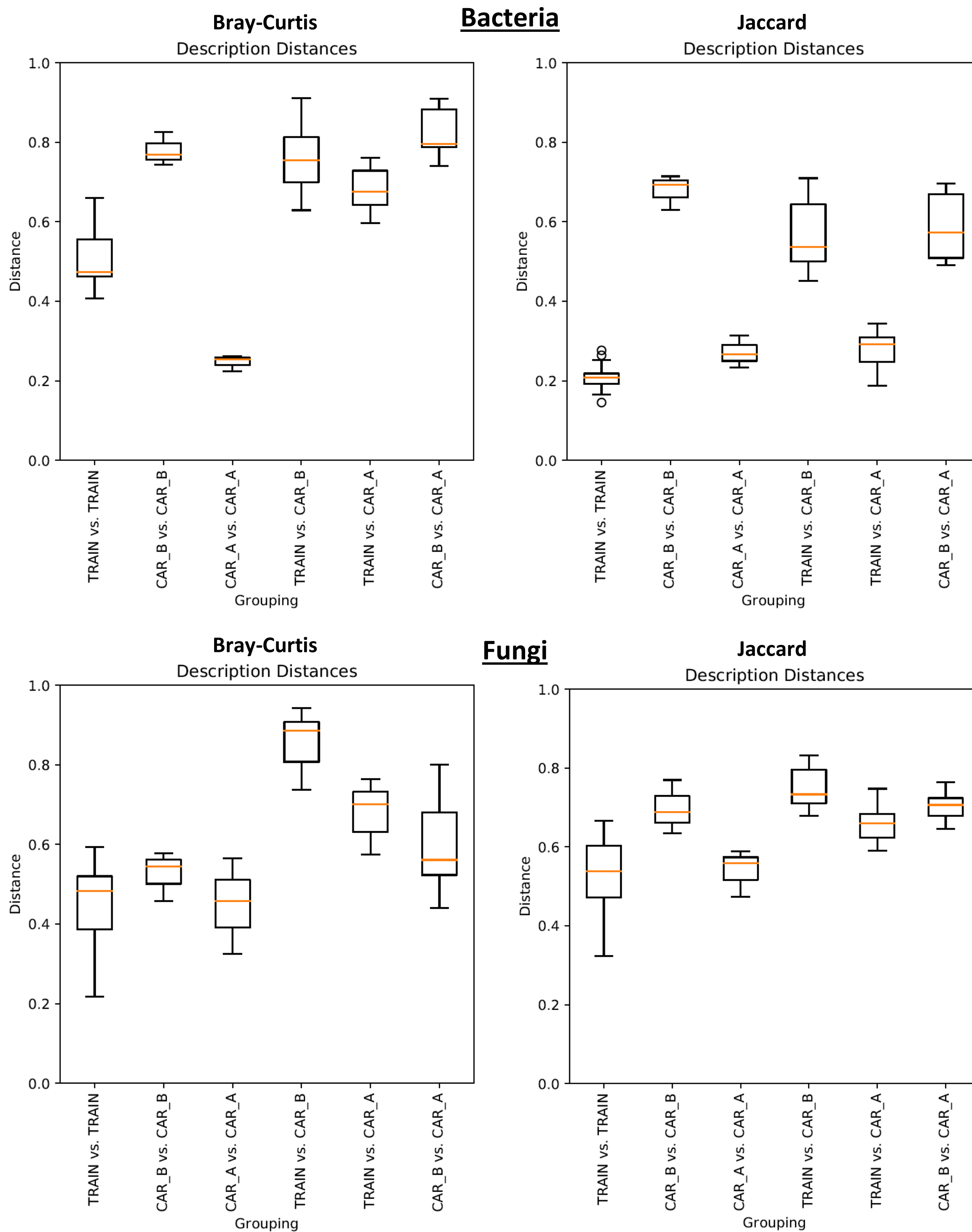


Figure S6.2 Boxplots of Bray-Curtis dissimilarity (panel on the left) and Jaccard distances (panel on the right) within and between bioaerosol bacterial and fungal composition obtained during different types of commuting trips.