

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

**OCCUPACIONAL RISK ASSESSMENT OF WASTE WORKERS' EXPOSURE  
TO BACTERIAL LOAD IN WASTE MANAGEMENT INDUSTRY**

KAREN SIMONE MOTA PIEDADE

UNDER THE SUPERVISION OF PROFESSOR DR. CARLA VIEGAS FROM ESCOLA  
SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

Master's degree in Clinical Laboratory Technologies

(this version includes the criticisms and suggestions made by the jury)

Lisbon, June 2021



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## **Abstract**

The waste management industry comprises all industrial activities related with waste management, waste dumping, waste recycling and waste minimization. Many, if not all, activities carried out during waste management result in the emission and release of hazardous substances such as bioaerosols, therefore posing many health risks to the workers that are directly involved with such activities. Bioaerosol is a viable vehicle of exposure to many pathogenic and non-pathogenic microorganisms, including bacteria and endotoxins and it has been associated with many adverse health effects, mainly affecting the respiratory system. The aim of the present study was to examine and quantify waste collectors' occupational exposure to inhalable bacterial burden during waste management activities using active and passive sampling approaches. Hence, 16 cabin ventilation filters and 25 air samples (SASS filters) were collected for risk assessment through culture-based methods for bacterial count. TSA and VRBA were the selected culture medium to assess cell viability and culturability. Results revealed an increased total bacterial count in TSA, in both matrices compared to Gram-negative bacteria in VRBA. Further analysis of results showed the possible expected microbial burden of waste workers when performing laboral activities, by comparing long-term and short-term exposure. Workers should be properly educated and informed about the results to induce behavior changes for hygienic measures compliance.

Keywords: occupational exposure assessment, waste sorting industry, organic dust, bacteria, bioburden.



## Resumo

A indústria de gestão de resíduos inclui todas as atividades industriais relacionadas com a gestão, eliminação, reciclagem e minimização de resíduos. As atividades realizadas durante a gestão de resíduos resultam na emissão e libertação de substâncias perigosas, como os bioaerossóis, apresentando muitos riscos à saúde dos trabalhadores que estão diretamente envolvidos. O bioaerossol é um veículo viável de exposição a microrganismos patogénicos e não patogénicos, incluindo bactérias e endotoxinas, e tem sido associado a muitos efeitos adversos à saúde, afetando principalmente o sistema respiratório. O objetivo do presente estudo foi examinar a exposição ocupacional dos trabalhadores à carga bacteriana inalável durante as atividades de gestão de resíduos, usando abordagens de amostragem ativa e passiva. Foram recolhidos 16 filtros de ventilação de cabine e 25 amostras de ar (filtros SASS) para avaliação de risco por meio de métodos baseados em cultura para contagem bacteriana. TSA e VRBA foram os meios de cultura selecionados para avaliar a viabilidade e cultivabilidade celular. Os resultados revelaram um elevado número de bactérias totais em TSA, nas duas matrizes, comparando com bactérias Gram-negativas em VRBA. Uma análise mais aprofundada dos resultados mostrou a possível carga microbiana esperada de trabalhadores de resíduos durante a realização de atividades laborais, comparando a exposição de longo e curto prazo. Os trabalhadores devem ser devidamente informados sobre os riscos para promover mudanças de comportamento para o cumprimento das medidas higiénicas.

Palavras-chave: avaliação da exposição ocupacional, indústria de resíduos, poeira orgânica, bactérias, biocarga.



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## Abbreviations

CFU	colony forming unit
HP	hypersensitivity pneumonitis
LPS	lipopolysaccharide
MSW	municipal solid waste
ODTS	organic dust toxic syndrome
PMN	polymorphonuclear leucocytes
SASS	surface air system super
TNF- $\alpha$	tumour necrosis factor alpha
TSA	trypticase soy agar
VBNC	viable but non-culturable
VRBA	violet red bile agar



# 1. Chapter I: Introduction

## 1.1. The waste industry

The waste management industry comprises all industrial activities related with waste management, waste dumping, waste recycling and waste minimization (1,2). It's a highly significant employment sector as it provides the adequate management of the municipal solid waste (MSW) from its conception throughout its disposal. The processing of MSW includes the collection, storage, transportation, treatment and disposal of waste generated, together with monitoring and regulation of the waste management process (1,3).

Managing MSW is necessary, and normally it is of responsibility of local governments and authorities. Worldwide, a great number of individuals are employed in the waste management industry as it is a very intensive service. The activities carried out during waste management are indispensable, as solid waste is considered a harmful local pollutant and can affect negatively both on the environment and the human health if not treated properly (2,3).

The waste management industry holds many health risks to workers that are directly involved in managing procedures. Some, if not all, activities carried out during waste management are performed according to procedures that result in the emission and/or release of hazardous substances in an intricate combination of aerosols, bioaerosols and volatile organic compounds, which can be harmful for both the human health and the environment (2). The workers are continually exposed a diversity of biological and chemical agents, that include toxic chemicals, organic dust and microorganisms, as well as various materials that are infected or could be infected by a wide range of dangerous agents (4,5).

Due to the increased exposure to hazardous microorganisms and other harmful agents, the industry becomes an issue of social concern, thanks to its environmental influence, effects on public health and the occupational health problem it represents (2,4,6,7).

### **1.1.1. Waste sorting and recycling in households**

Waste generation and its management are important both to the environment and overall population (8). A steady and significant increase in population number and resulting urbanization, consumption and changing in lifestyles during the last decades has led to an overproduction of waste in most developing and developed countries, as waste is a by-product of consumer-based lifestyles (1,3). As large quantities of waste cannot be abolished, the focus has been put into reducing the environmental impact of waste overproduction by making a more sustainable use of waste by promoting activities like waste sorting, reducing of waste production, reusing of materials and recycling of materials (1).

With the increase in technology and easy access to information nowadays, local authorities have been attempting to give general households proper education and awareness regarding waste recycling. As waste recycling can be defined as the process of recovery and reuse of resources from remaining materials after their primary use (1), the goal is to help minimize the amount of waste that is released into the environment and reduce the environmental burden associated with the overproduction of garbage (9,10). However, the informal recycling performed by the general population is not controlled and can be flawed and increase waste workers' exposure to biohazard as they collect, transport and process waste (11). Municipal waste can often be ill-labelled or even not properly bagged (or not bagged at all), and those who handle it are directly exposed to hazardous components of waste such as dust and other airborne matter (5).

Waste can be defined as any durable or nondurable substance, container or packaging discarded after primary use (1), broadly classified into organic or inorganic. All the material that remains after recyclable components are separated can be considered residual waste. It may contain organic substances, plastics, glass and other recyclable and non-recyclable components (12).

The waste composition is influenced by many factors and varies widely across many sectors, ranging from the level of economic evolution and development of each country, geographical location, weather and atmospheric conditions, to season of the year, household's composition, living habits, level of education of individuals and household, religious and cultural principals, and social and public behaviour (3,13). Acquiring proper

information about waste composition is crucial for planning and decision making in waste management so that the most efficient methods for waste processing can be applied (14).

One of the main elements of municipal waste management is the separate waste collection of waste sorted by households (15). The waste should be sorted according to local disposal systems. The most common categories are (16):

- Paper and cardboard (including packaging);
- Glass;
- Plastics;
- Textiles or fabric;
- Wood and leather;
- Metal;
- Hazardous waste;
- Residual waste.

After waste collection, all the organic matter (residual waste) is redirected to composting in order to recover the organic fraction by converting it into compost and reduce biodegradable waste in landfills (15).

While composting activities can destroy most microorganisms, as it reaches high temperatures such as 50-60° C, it can also favour the growth of other that are more heat resistant, such as actinomycetes and fungi and the release of endotoxins after the destruction of Gram-negative bacteria outer membrane (15).

Microorganisms also have the ability to grow within waste containers, if waste is stored for longer periods of time. That happens often when waste collection cycles are missed or poorly managed by local authorities (12). With the residual waste that is left after separation and removal of dry recyclables, there is a general perception that it could develop strong odours and become inconvenient and unsanitary. But also, the organic and biodegradable contaminants that remain inside the bins become a rich substrate for microorganisms, causing further microbial growth while awaiting waste collection (10,17). This substrate is consumed by microorganisms, providing nutrients for their survival, as well as favourable growth conditions such as appropriate moisture and temperature (8,18). This poses a real health risk regarding not only the household

members, that will be continually exposed to the elevated hazardous microorganisms concentration as days go by, but also to the waste collection workers, who will move and disturb the waste material during collection (12). This disturbance may cause release of microorganisms to the air, creating proper conditions for these microorganisms to adhere to dust particles and become airborne, thus increasing the risk of inhalation of hazardous microorganisms (and their components) and creating a source of biological hazard (12,15).

### **1.1.2. Organic dust**

Organic dust consists of a mixture of viable and non-viable microorganisms, their metabolites and solid particles of vegetable and animal origin. It is often used synonymously with bioaerosols (19). It is a particulate matter with specific ubiquitous microbiological agents likely to cause specific respiratory diseases (20).

The interest in organic dust/bioaerosol exposure has increased over the last years since it has been properly associated with a wide range of adverse health effects (19). In the waste management industry, organic dust is considered one of the main sources of exposure to many pathogenic and non-pathogenic microorganisms, including live or dead bacteria, bacterial endotoxins, fungi, mycotoxins, high molecular weight allergens, viruses, peptidoglycans,  $\beta(1-3)$  glucans, pollen, plant fibres, etc (19,21). It should be considered an important airborne pollutant, as it is a viable vehicle for bacteria, fungi, and their respective metabolites, leading to an increased occupational exposure of the workers to both biological and chemical agents (22,23).

Workers such as garbage collectors and drivers transporting waste products spend many hours between truck cab and a workplace where a lot of organic material is handled. Handling of organic waste is a direct source of exposure to bioaerosol, therefore, they also are exposed to airborne matter and volatile organic compounds while collecting household waste and further in the waste treatment process (24).

The primary health effects of exposure to bioaerosol are an inflammatory response of the upper airways, which can be attributed specially to the presence of Gram-negative bacteria and endotoxins in the organic material (25,26). Long-term exposure to organic

dust can lead to induction of toxic reactions such as organic dust toxic syndrome (ODTS). ODTS is an acute non-allergic illness characterized by flu-like symptoms, such as fever, shivering, cough, headaches, general muscle and joint pain, dyspnea and fatigue (19,27). These symptoms usually last until the following day but could persist for 5 to 7 days, but if not properly treated and if exposure persists, they can progress to a rapid decline in lung function, causing a chronic impairment of pulmonary function (4,19,25).

### **1.1.3. Health hazards in waste industry**

The work performed in waste management facilities has been related with many adverse acute and chronic health effects, with a broader focus on respiratory problems, since there is a higher risk of exposure to biological agents and the activities performed may obligate the handling of chemical substances and infectious materials (4,20).

In the recent years, the interest in occupational exposure to bioaerosol has increased, as it has been appropriately recognized that the exposure to biological agents is linked to a wide range of adverse health effects with significant impact in public health (17). The collection, sorting, and disposal of municipal waste result in dust and microorganisms present become airborne and be easily inhaled by the workers and cause further health issues (12).

The exposure occurs due to complex mixtures of toxins, allergens, and chemicals present in the organic matter that is handled, as the handling of waste may cause microorganisms and dust to become aerosolized (24). Waste materials generally contain a range of nutrients and are moist, creating appropriate conditions for microorganisms to grow. The age, composition of the waste, the temperature and humidity of the storage site can affect the type and quantity of microorganisms, as well as their pathogenicity and the extent to which these can survive and multiply (28). The large number of microorganisms and their fragments will be quickly inhaled, as they are invisible and not detectable to the naked eye.

As microorganisms become aerosolized, and since waste workers are in direct contact with waste material and organic dust, exposure to biological agents through inhalation of

airborne bioaerosol becomes the most relevant route of exposure to biological agents, in this context (28).

Several studies have revealed generous evidence on respiratory complaints, not only among direct waste handlers but also to waste incinerators and for populations residing near landfills (11,19). Health effects associated to bioaerosol exposure and symptoms most frequently reported include eye and skin irritations, nasal congestion and flu-like symptoms, contagious infectious diseases, acute toxic effects, allergies, toxic response and even cancer (11,19,25).

During waste handling, these workers are also prone to accidents with sharp objects, which harbours the risk of infection from blood borne viruses (28). These can easily happen during the separation of waste, when the correct container is not used to store these hazardous objects, or the content is not properly labelled or disclosed.

### **Infectious diseases**

Infectious diseases can be caused by viruses, bacteria, fungi and other pathogenic microorganisms with the ability of transmission of an infectious agent to a susceptible host by direct contact (19). An infection is classified as occupational when some activity of the work involves contact with a biologically active organism. When an occupation place of a worker has an increased risk of infection by a pathogen, an occupational infectious diseases can occur (29,30).

Infectious diseases in occupational settings are mostly caused by inhalation of airborne pathogens or contact with infected person or surface, mainly in specific contexts of occupational-specific exposures (e.g health workers have higher risk of tuberculosis or measles, or farmers and veterinarians having higher risk of having Q-fever or swine influenza). It can also occur in the scenario of clustering of people in the workplace, facilitating the transmission of infectious airborne bioaerosol among individuals (i.e Legionnaires disease) (19).

### **Respiratory diseases**

Exposure to bioaerosol has been largely associated with an inflammatory response at the airways mucosa's level likely due to the interaction between the microorganism or

cell wall component of the microorganism and the immune system of the host (2). Respiratory symptoms are the most common organic dust-associated and bioaerosol-associated health effect, ranging from acute mild conditions to severe chronic respiratory disease that could require specialized care. These symptoms include mucosal membrane irritation, chronic bronchitis, conjunctivitis, and significant decline in lung function (4,20)

Occupationally related respiratory symptoms result from airway inflammation triggered by specific exposure to certain toxins, pro-inflammatory agents and allergens (19). The small-sized particles that are integrated within the inhalable dust can penetrate the upper and lower airways, invading the lungs and becoming embedded in alveoli (28).

According to inflammatory mechanisms and symptoms, there can be made a distinction between allergic and non-allergic respiratory diseases caused by bioaerosol exposure (19).

**Allergic respiratory diseases** refer to an immune-specific airway inflammation, in which antibodies such as IgE and IgG play a major role in the inflammatory response. They include conditions such as eye and skin irritations, allergic asthma, allergic rhinitis, hypersensitivity pneumonitis (HP). Albeit these conditions are very common in compost facilities, they are more likely to be caused by other agents within bioaerosol composition (i.e. fungi and/or fungal spores) than bacteria or endotoxins (19).

HP is a much serious pulmonary problem, comparing to ODTS, with delayed febrile systemic symptoms, that also occurs due to exposure to organic dust. It is an allergic bronchial and alveolar inflammation caused by an extreme and excessive immunological response to noxious organic particles (19,27). The symptoms are similar to those verified in ODTS, which aggravates to non-necrotizing poorly formed granulomas and cellular bronchiolitis. Chronic patients are presented with chronic interstitial inflammation and alveolar depletion associated with fibrosis (27).

**Non-allergic respiratory diseases** refer to non-immune specific airway inflammation. One of the most common non-allergic respiratory disease is the “toxic asthma-like syndrome” whereas the individual develops work-related asthma-like

symptoms (19). Other diseases include non-allergic rhinitis, mucous membrane irritations, chronic bronchitis, chronic airways obstruction (chronic obstructive pulmonary disease – COPD), ODTs and toxic pneumonitis (19,28). These conditions can be caused by bacteria and their derivatives (endotoxins) and are very common in garbage collection and composting work environment (19).

### **Cancer**

In this particular setting, cancer could be caused by a variety of factors like exposure to oncogenic viruses and other biological agents, and the direct exposure (by inhalation or ingestion) to mycotoxins. The most common established human carcinogenic mycotoxins are aflatoxin from *Aspergillus flavus* and ochratoxin A (19,31,32).

## **1.2. Bacterial Load in Waste Industry**

Bacteria are prokaryotic beings of relatively small size (mostly microscopic organisms), in the order of 1 micrometer in diameter. They are organisms with simple internal structure (33,34). Structurally, a typical bacterium usually consists of a cytoplasmic membrane surrounded by a peptidoglycan cell wall (and maybe an outer membrane), a fluid cytoplasm with no nuclear region, numerous ribosomes and the DNA is located freely in the cytoplasm. Sometimes they may present various external structures that aid cell movement and help with attachment to other structures, such as flagella, and pili (35).

Bacteria have a wide variety of shapes and sizes. Most bacteria come in one of three basic shapes: coccus (spherical), bacillus (rod-shaped), and spiral. But they can also present themselves in other shapes that are less common, such as sheathed, stalked, filamentous, square, star-shaped, spindle-shaped, lobed, trichome-forming, or pleomorphic (36).

The degree of microbial diversity in nature is still unknown. In soil, 80 to 99% of microorganisms remain unidentified. These biological communities are known to play an important role in maintaining a sustainable biosphere (8).

Exposure to bacteria, especially exposure to bacterial components capable of causing harm to individuals, is a known cause of respiratory symptoms (37). Waste workers are often exposed to different airborne bacteria, that can be found specially in food scraps (most commonly in meat and other food leftovers). Most of these bacteria are saprophytic, which means that they can feed of decaying organic matter and continue growing and colonizing. Some of these species can be harmless, but some can turn out being extremely pathogenic and cause severe infections (4).

Bacteria can be found in many different environmental contexts, such as in soil, water, plants, animals, waste, arctic ice and glaciers, and hot springs. Aerobic bacteria can only grow in oxygenated environments. On the contrary, anaerobic bacteria, are organisms that do not require oxygen to grow. In humans, they are mostly present in the gastrointestinal tract. There are also the facultative anaerobes, or facultative anaerobic bacteria, use mainly oxygen for metabolism but when oxygen it's depleted, they survive using anaerobic methods for energy production (38). They are mostly found in soil, water, vegetation and some normal flora of humans and animals.

According to the optimal growth temperature, bacteria can be classified in three main groups. Mesophilic bacteria are bacteria responsible for most human infections. They thrive in moderate temperatures, around 37°C, which matches the average temperature of the human body (39). Thermophiles are bacteria that thrive at relatively high temperatures (ranging from 41°C and 122°C), and they are a well-known source of allergens that contributes to the development of HP, along with some identified fungi species (19,34). Psychrophilic bacteria, in contrast, are active when the temperature is relatively low. Extremophilic bacteria, can withstand conditions considered too extreme for most life forms.

### **1.2.1. Gram negative bacteria**

Bacteria can be classified and distinguished according to several different criteria like the nature of their cell walls, by their shape, or differences in their genetic makeup. The Gram stain is the most common method to distinguish two main types of bacteria, Gram-positive and Gram-negative bacteria. This test identifies bacteria according to the chemical and physical composition of their cell walls (40). This method has been widely

used since 1884, as a preliminary morphologic technique for identification of the two large groups of bacteria in a specimen (40,41).

Gram-negative bacteria can be distinguished from Gram-positive bacteria in regard to the structure of the cell wall, which influences their ability of the penetration and retention of chemical agents and determines the stain colour of each group (42).

The cell of a Gram-negative bacteria generally consists of three essential layers, that constitute the structure called the envelope (43):

1. An outer membrane, that comprises the lipopolysaccharide (the toxic component of the Gram-negative bacteria)
2. A peptidoglycan cell wall with peptide chains;
3. An inner membrane also designated cytoplasmic membrane.

The outer membrane is the distinguishing characteristic of both bacteria category. Gram-positive bacteria lack this membrane. This membrane functions as a permeability barrier, preventing certain drugs and antibiotics from penetrating the cell wall and protecting the bacterial cell of hostile environment, and at the same time allowing selective nutrients passage (42,43).

Gram-negative bacteria are ubiquitous in the environment (26), but most bacteria and bacterial agents are not very powerful allergens, as they are very sensitive to temperature (except thermo-tolerant and thermophilic bacteria) and can easily be degraded. Most of them carry and release a specific substance from their cell walls that is a combination of polysaccharide chains, a lipid unit and a core molecule that connects both components (26), also known as endotoxins or lipopolysaccharides (LPS). These components are agents with pro-inflammatory properties that induce respiratory symptoms (19,25).

### **1.2.2. Endotoxins**

Endotoxins are only identified in Gram-negative bacteria, a biologically active component in most organic dust. Endotoxins, or lipopolysaccharide, are located in the outer membrane of the cell, and are known to induce various biological reactions(28). Endotoxins were first described by Richard Pfeiffer, as they were isolated from *Vibrio*

*Cholerae*, described as cell-associated material, resistant to heat, that's able to instigate toxic reactions (44).

The terms endotoxin and LPS can be used as synonyms, as the endotoxin consists of polysaccharides and a lipid part (the lipid A). However, the term LPS should be used to describe the chemically pure substance. The term Endotoxin should be used to denote LPS as it is found in nature, i. e., on fragments of Gram-negative bacteria cell walls (26).

Being an essential part of the bacterial cell wall, endotoxins pose an important role in the bacterial survival, as they protect the bacteria from host defence strategies and antibiotics. They also play an important role during severe infection of the host, leading to a range of pathophysiological reactions such as fever, tachycardia, hypotension to multi-organ failure, and other adverse effects (44).

Bacteria naturally release small quantity of endotoxins as they replicate, and all of the membrane components are released upon death and following cell lysis (45). The presence of endotoxins (or identification of endotoxin producers) in the air of the places where waste is handled or the operatives can directly touch should be a matter of concern, as they are known to result in numerous health problems (5). They are recognized by the individual's immune system and therefore triggering the release of allergic mediators and production of antibodies (2,26).

After inhalation of organic dust harbouring Gram-negative bacteria (and their compounds), a variety of host's immune cells are able to respond in an attempt to protect the host from external agents and the possible infection. In general, the endotoxin interacts with polymorphonuclear (PMN) leukocytes, monocytes/macrophages, B- and T- lymphocytes and other vascular/epithelial cells (44).

They are also classified as pulmonary immunotoxicants. Individuals exposed endotoxins by inhalation experience adverse reactions such as acute systemic and respiratory symptoms, with clinical effects such as fever, shivering, joint pain, asthma like symptoms (dry cough, dyspnea, chest tightness), blood leukocytosis, neutrophilic airway inflammation, bronchial obstruction and acute lung function changes (46).

Even low levels of exposure to endotoxins concentration (down to nanograms/m<sup>3</sup>) can trigger a response from defence cells and therefore lead to an inflammation of the upper airways, by activation of neutrophils and release of cytokines such as IL6, IL8 and tumour necrosis factor alpha (TNF- $\alpha$ ) (26,28). The production of these cytokines should occur in moderate amounts, as they constitute a beneficial anti-infectious inflammatory reaction from the host when encountering an external agent. When there is an overproduction of such products, such as in cases of over-exposure or prolonged exposure to a pathogenic agent such as endotoxins, these can become harmful to the host and possibly cause fatal pathophysiological effects such as prolonged high fever, hypotension, disseminated intravascular coagulation and cell and organ damage, that could eventually lead to manifestation of septicaemia, multi-organ failure and lethal septic shock (44).

Endotoxins are extremely resistant to heat and can remain even after the bacteria dies, therefore the complete inactivation of these components could be a difficult task (47,48).

### **1.2.3. Microorganisms viability**

A viable entity is capable of enduring in the environment and form new entities to persist in the future (49). Operationally, microorganism viability can be defined as the demonstration of the ability of microbial cells to grow and reproduce in a validated laboratory system (50).

The viability of microorganisms can be affected by numerous variables that all combined can provide favourable conditions for their growth (51). Depending on several physicochemical conditions such as culture media, temperature, pH, incubation period, carbon source, humidity, presence of nutritional substances, moisture, etc, and also meteorological and climatic parameters such as temperature, relative humidity and UV radiation, bacterial growth can vary, affecting the concentration of outdoor bacteria (8,51,52).

Waste products contain various nutrients and provide a favourable environment in which microorganisms can grow and flourish. Combining factors such as age and composition of waste, along with storage temperature and humidity inside the containers determines the type and quantity of microorganisms present and enhances their ability to survive,

reproduce and multiply in such environment. Many microorganisms that are able to adapt and thrive under such conditions can be pathogenic, leading to a higher risk of infection (4).

Seasonal influence should also be taken in account when evaluating bacterial load and viability. The concentration of total microorganisms, culturable species and endotoxin levels are expected to be lower in autumn and winter as temperatures are lower (depending on geographical location of sample collection)(53). With the extremely low temperature conditions, cell membrane fluidity is affected thus microbial activity and reproducibility can be restricted (24,53). In the hotter months, such in summer season, it should be expected to have an increased bacterial load, as the increased atmospheric temperature promotes growth and release of bacteria (53). However, the role of relative humidity counteracts this effect, along with UV radiation. The considerable high UV radiation intensity noted in summer season is more likely to inhibit bacterial growth and reduce outdoor bacterial concentration.

When temperature decreases, an increase in relative humidity is noted and elevated water activities are favourable for bacterial growth and cell clumping (which adds to the odds of cell survival) (53,54).

Other meteorological factors such as rain, snow, and wind velocity should be taken into account as well and analysed thoroughly, as they play two contrasting aspects in regard to influencing bacterial concentration levels. They may resuspend bacteria from surfaces, boosting the concentration of microorganisms in the air. At the same time, it could also diminish ambient bacterial levels due to atmospheric dilution (53,55).

Viable microbes can induce several respiratory health symptoms and affect a variety of biological mechanisms, such as the inflammatory and cytotoxic response of the host (9). As workers in waste industry are continually exposed to organic matter that functions as nutrient substrate to microorganisms for long periods of time, evaluating the viable component of the bioburden becomes crucial in occupational settings, as it determines the infection potential of a microbial agent (56,57).

#### **1.2.4. Antibiotic resistance**

Antibiotic resistance has been widely covered over the past century, as it has been targeted as an aspect of high interest in the field of research throughout the decades. It has emerged as one of the leading public health problems of recent years.

Antibiotic resistance is a natural phenomenon that happens when the microorganisms are exposed to antibiotic agents or drugs. Under this exposure and selective pressure of antibiotics, bacteria that are susceptible to the penetration of these drugs can be killed and those that are more resilient and resistant have greater chance to survive and multiply (58). It is, essentially, a consequence of natural selection and comes as a mean of survival (42). Bacteria without this advantage most likely will die or have their reproductive capacity abolished, and resistant bacteria will be able to multiply with less competition.

As mentioned before, Gram-negative bacteria have an outer membrane that grant protection from hostile environment. Because of this inherent structural and functional feature, Gram-negative bacteria have this intrinsic antibiotic resistance as some antibiotic agents are unable to penetrate the outer membrane (42).

Bacteria can also acquire resistance. Some bacterial populations undergo some mutations that can provide genetic variations that could become beneficial for their survival in the presence of antimicrobial agents (42).

Over the years, the overuse and inappropriate use of antibiotics (including the use without proper indication, inappropriate choices, incorrect dosing, lack of adherence to treatment guidelines, excessive use) has led to the loss of efficacy of these drugs and contributing to the emergence and dissemination of resistance among several bacterial pathogens (58,59). The broad improper use of antibiotic has accelerated the development of an antibiotic resistant pathogens, increasing the risk of severe infectious diseases, if in contact to such pathogens. The lack of knowledge about antibiotics within general population, or even the erroneous knowledge about the mean of action of these products and their adverse effects when poorly administered all contribute for the intensification of the issue. Self-medication has also become very common, in countries with poor regulatory systems. Self-medication is also a way to aid microbes to adapt

rather than being eliminated, since it's oftentimes unnecessary, inadequate, with the wrong timeframe of treatment and incorrect dosing (58).

Certain types of Gram-negative bacteria have become increasingly resistant to available antibiotic drugs, and these drugs are becoming ineffective against a broad spectrum of species, which difficult and limits treatment options. Most common antibiotics include quinolones, colistins (polymyxins), carbapenems, third and fourth generation cephalosporins and other  $\beta$ -lactam antibiotics. Bacteria use different mechanisms to withstand antimicrobial action, mechanisms that are later carried on to next generations by mobile genetic elements upon reproduction (60,61).

The emergence of antibiotic resistance, both in clinical and environment settings, should be addressed. It is a serious concern, leading to limited therapeutic options available and challenging the effective management of many dangerous pathologies.

### **1.3. Methods to assess occupational exposure to microorganisms**

Bioaerosol exposure assessment can harbour many challenges, as some pathogenic microorganisms can be hazardous at extremely low levels and others only can adverse effects at higher concentrations. Some organisms can be very resistant while others can easily be damaged by sampling processing. Due to their size, bacteria can be very difficult to detect with the naked eye. They require techniques and methodologies that are sensitive and specific that allow to distinguish them and other biological agents (19).

With many factors that influence the composition of bioburden in organic dust, it is important to establish and explore several sampling methods for a more accurate exposure assessment and establish a complete picture of exposure to bioaerosol and to adequately assess the risks associated with it (19).

The accurate estimation of microorganisms depends on a wide range of sampling methods and multiple approaches help with the identification of microorganisms. Usually, collection of air samples for airborne microorganisms' analysis is done using suction samplers, that mimics the aspiration of airborne particles by the respiratory system of the

human body (62). The collection of a sample is performed into culture media and the sample follows a series of microscopic, microbiological, biochemical, immunochemical or molecular biological analysis (19,62).

### **1.3.1. Active and passive sampling**

Since organic dust prevails in various occupational environments, including waste industry, these workers have an increased risk of occupational exposure to airborne pathogens that are more prevalent in this industry (21,63,64). To accurately assess the bioburden these workers are constantly exposed to, the method used to collect samples for analysis should best represent the reality of exposure.

Active air sampling provides information about the contamination load, representing the load from a short period of time, whilst passive sampling provides a valid risk assessment as it measures the harmful part of the airborne population which falls onto surface for a longer period of time, being cumulative days, weeks or several months (65–67).

Active air sampling mimics the human respiratory system since only a fraction of airborne particles is inhaled into the human body and only a small fraction of the total air volume present in an occupational environment is collected (62,68). Since air samples are usually collected through aspiration with suction samplers, it can affect the microorganisms viability and DNA integrity if the suction intensity not well controlled (68). It can easily cause loss of cultivability and affect the results.

The collection and analysis by passive sampling should be used as a complement to active air sampling, as it relies on the kinetic energy of molecules to deposit into a surface area in an enclosed space. This method helps rectify the issue of microorganisms variability that active air sampling lacks as this method provides a time-integrated sample of the bioburden varying from days, months or longer and thus allows the verification of contamination levels of a larger period of time (68,69).

Passive and active methods should be applied in jointly to ensure a more accurate and real scenario of the occupational exposure assessment to bioburden, and even

complemented with other environmental matrices to ensure better data findings and perform a better risk characterization (67).

### **1.3.2. Culture-based methods for bacteria detection**

After sample collection, microorganisms can be analysed by culture-based and nonculture-based methods.

Culture-based methods can detect viable microorganisms that are able to grow in a selected culture media. Not all viable microorganism are able to grow in medium, even when presented with the ideal growth conditions so these method can only detect a fraction of bacterial communities, underestimating the real diversity presented in the environment (62,70). Nonculture-based methods are not dependent of microorganisms' cultivability and are able to provide a more realistic understanding of the diversity of the bacterial profile present in the environment (62).

Culture-based methods can provide supplementary valuable information regarding the occurrence of bacterial species in samples. The most used culture-dependent method is agar plating, where the culturable microorganisms can grow on nutrient media, and counted afterwards macroscopically (62). The culture media to be used should provide the most suitable nutrients for microorganism growth, depending on research aims.

Tryptic-Soy Agar (TSA) is a general agar medium used for culturing many kinds of non-fastidious and moderately fastidious microorganisms (18,62). Growth requirements vary widely between each group microorganisms, and the growth can be enhanced or suppressed according to selected settings for culture.

Violet Red Bile Agar (VRBA) is a gram-negative selective media used to detect coliform microorganisms. Coliforms refer to all aerobic and facultative anaerobic, non-spore-forming rod-shaped bacteria that ferment lactose with gas and acid formation when incubated between 35 and 37°C (39). VRBA uses selective inhibitory agents such as bile salts and crystal violet, to inhibit the growth of Gram-positive bacteria and suppress the spread of unwanted microorganisms (71,72).

Idealistically, both culture-based and nonculture-based methods should be used in parallel in order to retain the most information regarding bacterial communities within samples. However, nonculture-based or molecular tools are often too costly and demand highly trained technicians to correctly perform these methods (73).

## **2. Chapter 2: Materials and Methods**

The objective of this study is to examine waste collectors' exposure to inhalable bacterial species during waste management.

This study was conducted between January and October of 2019, as part of an extended study with financial support to assess occupational exposure to microbiologic agents in waste management units (9). Sampling collection was carried in one waste-sorting industry located in Canada during winter season, and sample processing and data analysis were conducted in following months in Portugal.

This study is classified as a descriptive study, since it performs an analysis, registration, and interpretation of the facts, without the direct manipulation of any variables. It is classed as a quantitative cross-section research, since it aims to explain and predict a phenomenon by measuring variables and analysing numerical data (74)

### **2.1. Sample collection**

In order to assess occupational exposure of waste industry workers to bacterial load and contamination, 41 samples from trucks working in waste industry during winter season in Canada were collected.

The samples consisted in 16 Cabin Ventilation Filters (ventilation filters from vehicle cabinets of waste management trucks) and 25 corresponded to airborne microorganisms collected using microbiological air sampler Surface Air System Super (SASS) Filters (Avantor Sciences, VWR). They were sent over for processing in Lisbon, Portugal, under refrigerated conditions (4 °C) until extraction for analysis.

### **2.2. Bacterial extraction and inoculation**

Upon arrival to the laboratory, the samples for culturable counts were extracted in falcon tubes with 10 mL of 0.1% Tween™ 80 saline solution (NaCl 0.9%) for 30 minutes at 250 rpm on an orbital laboratory shaker (Edmund Bühler SM-30, Hechingen, Germany).

Two culture media were used in order to enhance the selectivity of bacterial growth and evaluate microbial contamination: TSA supplemented with nystatin (0.2%) for mesophilic bacteria and VRBA for coliforms (Gram-negative bacteria).

150  $\mu$ L of extracts were inoculated on petri dishes previously prepared with TSA, to assess total bacterial load, and onto VRBA, for Gram-negative bacteria. For total bacteria contamination assessment (TSA), the plates with respective medium were kept at 30 °C, and the plates for Gram-negative bacteria contamination assessment (VRBA) at 37 °C for 7 days.

### **2.3. Bacterial quantification**

Total colony count was performed during 3 points of incubation; on the third (72 hours), fifth (120 hours) and seventh (168 hours) day of incubation. The number of colonies formed during incubation period was counted by visual inspection resulting in a direct quantitative estimate of bacterial density, and results are expressed in colony forming units (CFU) per square meter of air (CFU/m<sup>2</sup>).

### **2.4. Statistical analysis**

All data were collected and analyzed using the statistical software GraphPad Prism 8 (GraphPad Software, San Diego, CA) using methods of descriptive and inferential statistics. Samples obtained by culture-based methods will be considered as CFU/m<sup>2</sup> for prevalence distribution of microbiological species.

To test normality of the data, a Shapiro-Wilk test was performed. Data characterization was determined by calculation of values of minimum, maximum, mean and standard deviation. Since normality of data was not verified, non-parametric Spearman Correlation test was performed to analyze the relationship between bacterial contamination in different culture media. To identify if there is a significant change in bacterial prevalence/count between two sampling methods, Mann-Whitney U test was performed.

### 3. Chapter 3: Results and discussion

This project aimed to quantify waste collectors' exposure to inhalable bacterial species during waste management and overall exposure to bacteria was assessed by quantifying bacterial contamination in two environmental matrices, identifying the most suitable sampling approach for this occupational environment.

To properly evaluate microbial contamination, the comparison of bacterial count in CFU/m<sup>2</sup> between two culture media was performed: TSA for total bacteria and VRBA for Gram-negative bacteria. In order to verify which sampling method would better represent worker's occupational exposure, two different sampling approaches were used: to represent passive sampling long-term exposure to bioburden Cabin Ventilation Filters were used and as representative of active sampling and airborne microorganisms' inhalation SASS Filters were selected.

Bacterial growth curve represents a cycle of events and changes within bacterial population. When plotting a bacterial growth chart, it's possible to observe bacterial growth in a closed and controlled system, i. e. in a petri dish with appropriate nutritive media. The chain of events includes 4 phases: a) lag phase – no growth observed; b) exponential phase – exponential growth of number of cells; c) stationary phase – balance between viable cells and dead cells; and d) decline phase – remarked cell death (49). After exponential growth phase, there is the stationary phase where there is a balance between number of cells being formed and the number of cells that are dying, with translates to a zero growth rate. After a long period of incubation, the saturation of the culture media occurs and subsequent depletion of nutrients, there is a remarkable decline in the number of viable cells. Figures 3.1 and 3.2 represent the bacterial growth after 7 days of incubation of our samples, in each matrix (Cabin Ventilation Filters and SASS Filters, respectively).

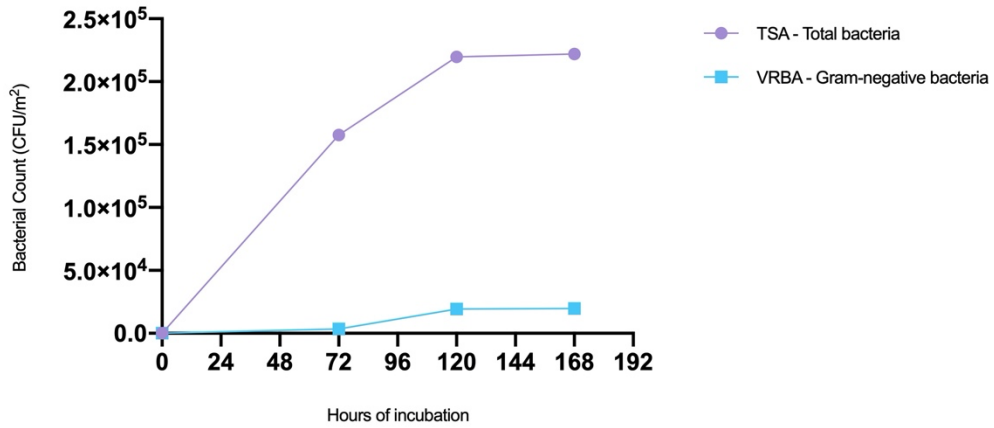


Figure 3.1: Bacterial growth chart for Cabin Ventilation Filter samples

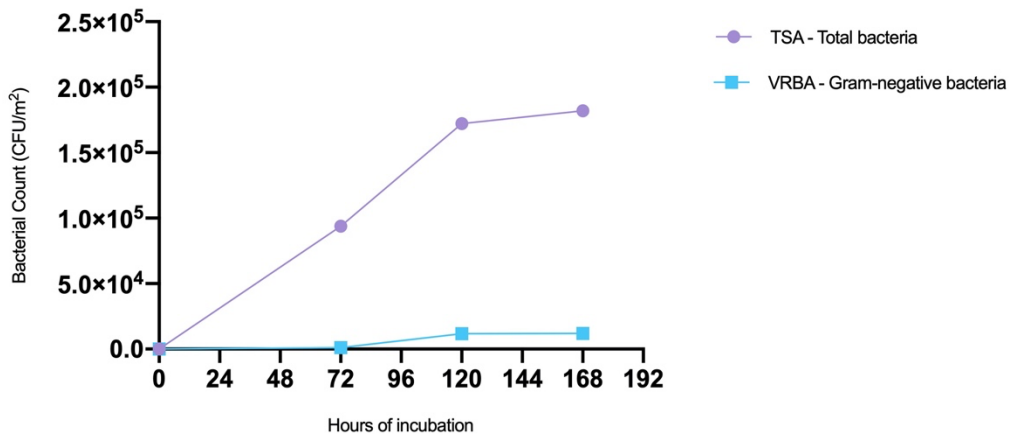


Figure 3.2: Bacterial growth chart for SASS Filter samples

The 7<sup>th</sup> day incubation, corresponding to 168 hours, was the chosen point for analysis, as it represents the max point of the stationary phase before observing any results that indicate the beginning of the decline phase and all bacteria die because of the lack of nutrients within the environment. Not underestimating the importance of studying bacteria in the exponential growth phase, for the purpose of this study the stationary phase was selected as it represents the highest point of viable cells within our samples and measurements, with distinctive properties in terms of virulence factor production and stress susceptibility (49).

Total bacterial contamination ranged from  $1.3 \times 10^4$  CFU/m<sup>2</sup> to  $2.5 \times 10^5$  CFU/m<sup>2</sup> and the Gram-negative bacteria from 0 CFU/m<sup>2</sup> to  $2.5 \times 10^5$  CFU/m<sup>2</sup> in Cabin Ventilation Filters. As for SASS filters, total bacterial contamination ranged from  $5 \times 10^2$  CFU/m<sup>2</sup> to  $2.5 \times 10^5$  CFU/m<sup>2</sup> and the Gram-negative bacteria from 0 CFU/m<sup>2</sup><sup>(1)</sup> to  $2.5 \times 10^5$  CFU/m<sup>2</sup>, as summarized in Table 3.1.

**Table 3.1: Bacterial contamination distribution on Cabin Ventilation Filters and SASS Filters**

		<b>TSA – Total bacteria</b>	<b>VRBA – Gram- negative bacteria</b>
<b>Cabin Ventilation Filters</b>	<b>Min.</b>	$1.30 \times 10^4$	0
	<b>Max.</b>	$2.50 \times 10^5$	$2.50 \times 10^5$
	<b>Mean (SD)</b>	$2.22 \times 10^5$ ( $7.6 \times 10^4$ )	$1.95 \times 10^4$ ( $6.20 \times 10^4$ )
<b>SASS Filters</b>	<b>Min.</b>	$5 \times 10^2$	0
	<b>Max.</b>	$2.5 \times 10^5$	$2.5 \times 10^5$
	<b>Mean (SD)</b>	$1.81 \times 10^5$ ( $1.1 \times 10^5$ )	$1.18 \times 10^4$ ( $4.96 \times 10^4$ )

In both matrices, the highest percentage of bacterial burden was found in TSA media, presenting a mean value of bacterial count of  $2.22 \times 10^5$  CFU/m<sup>2</sup> (with Std. deviation of  $7.6 \times 10^4$  CFU/m<sup>2</sup>) in Cabin Ventilation Filters and a mean value of  $1.81 \times 10^5$  CFU/m<sup>2</sup> (with Std. deviation of  $1.1 \times 10^5$  CFU/m<sup>2</sup>) in SASS Filters (Table 1). The percentage of bacterial contamination was higher in total bacteria (TSA) than Gram-negative bacteria (VRBA) in both matrices, as represented in Figure 3.3. These results suggest a higher contribution of Gram-positive bacteria over Gram-negative bacteria in the bacterial load. This same pattern of contamination was verified on a previous study, in regards to swine production (23).

<sup>1</sup> Considering this value as the lowest number of microorganisms that the method can detect.

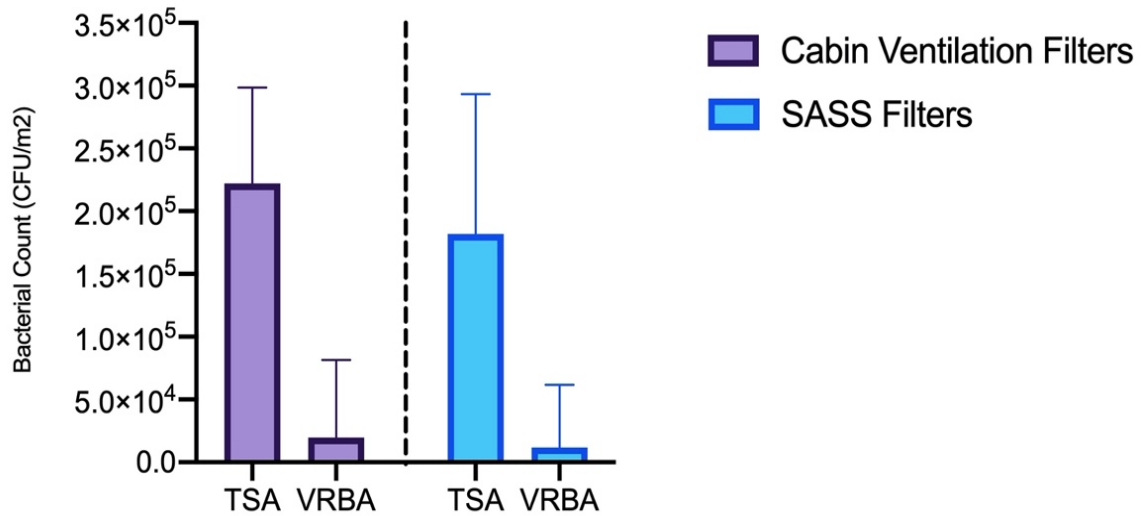


Figure 3.3: Distribution of bacterial contamination on TSA medium and VRBA medium, in both Cabin Ventilation Filters and SASS filters

The importance of using different culture medium can be verified within these results. In both settings of sampling, we can verify that there is a greater contamination load for total bacteria than Gram-negative bacteria. Normally, waste-related bacteria are mainly Gram-positive, which can explain the low frequency of Gram-negative bacteria in these samples. Gram-positive bacteria are present in larger quantities in atmosphere since they present greater resistance and survival capabilities (18). The use of selective media allows the isolation of certain organisms and provide a growing environment conducive to some types of organisms but not to others. The VRBA media used to inhibit the growth of Gram-positive bacteria, but it can also give false-negative results. One of the selective inhibitory agents that constitute VRBA media, bile salts, can suppress the growth of some Gram-negative bacteria that are more sensitive to additional selective agents (71), which could help explain the low Gram-negative bacterial count comparing to total bacterial count, in both matrices.

Culture-based methods have been shown to be crucial to identify viable microorganisms, however viable microorganisms only represent a percentage of the real bioburden that workers are exposed to. However, results based only on the capacity of viable bacteria to thrive under certain growth condition will only give an underestimation of the real burden, as there are many microbial and sampling process variables that can affect

bacterial viability (75). Not all viable microorganisms are able to grow in controlled *in vitro* systems and the viability of microorganisms is not mandatory for the occurrence of health effects (62). Growth and cell division are the main parameters considered when determining cell viability. Culture-based methods have been shown to be efficient in exhibit bacterial growth in form of propagation in liquid or solid culture media until forming visible colonies. The absence of bacterial growth after artificial contamination could be attributed to many factors: a) incorrect culture media; b) stress or damage of the cells; c) low population density (76).

In recent years, an important discussion has arisen about bacterial species that are found to exist in a viable but non-culturable (VBNC) state (49,76). Bacteria in this state pose a real new threat to medical and environmental microbiology, as they are characterized by the loss of culturability in general culture medium and with a reduced chance of detection by conventional bacterial count techniques, which could lead to a underestimation of total viable cells in samples (76). Bacteria in a VBNC state could be confounded as non-viable when using only culture-based methods, when in reality they enter a dormant phase as mean to survival under stressful conditions. They won't grow when inoculated in either general medium but they remain metabolic activity and may return to their virulent viable state when conditions are more favorable (49,50,76).

Non-culturable methods should be also used for bioburden analysis, as they can identify or specify organisms without considering their viability and do not depend on the culturability of microorganisms (62). Culture-based methods have proven to be of limited use for quantitative exposure assessment, as errors during manual counting of culturable microorganisms can occur such as poor reproducibility (19). Therefore, using only culture-based methods can lead to an underestimation of exposure risk. Non culture-based methods, such as microscopy methods (light microscopy, fluorescence microscopy and scanning electron microscopy) and assays that measure of bioaerosol components originating from microorganisms, can be very useful for the accounting of viable and non-viable microorganisms and giving more information contributing for a better understanding of bacterial diversity. The use of both culture-dependent and independent methodologies in a complimentary way for microbial burden assessment in order to achieve a better risk characterization in occupational settings (75).

Still in regard to Figure 3.3, it is also possible to analyse that overall the highest bacterial count was verified in Cabin Ventilation Filters, both for the total number of bacteria and for the number of gram-negative bacteria, comparing to SASS Filters. Keeping in mind that truck drivers spend many hours inside the vehicle, Cabin Ventilation Filters represent the sampling method with higher prevalence of bacterial contamination, as well as more representative of a long-term exposure. The risk of microbial contamination is thus increased when working shifts inside the truck comparing to shifts where the workload is mainly outside, and may contribute to diverse effects in humans health (77).

As temperature affect bacterial growth rate significantly, that could explain why the total bacterial count from SASS filters was slightly lower, comparing to Cabin Ventilation Filters. Cabin Ventilation Filters are located indoors, i. e. inside the vehicle, where the temperature is better controlled. SASS Filters represent the outdoors and airborne microorganisms with the risk of being inhaled. Considering that samples were collected during winter months (December and January) in Canada, where the winter season is very heavy, with extremely low temperatures, limited bacterial growth would be expected. Additionally, Gram-negative bacteria are more susceptible to aerosolization and sampling processes than other bacteria (6). Due to sample collection methods and consequent sampling stress, more sensitive bacteria can lose their cultivability.

Another point to have in consideration is the fact that low Gram-negative bacteria count does not mean lower exposure risk. During cell growth and upon cell death of Gram-negative bacteria during waste-related activities occurs the release of endotoxins, and may result in a higher prevalence of various adverse respiratory effects, as indicated in previous studies (73). Even though the Gram-negative bacteria presence was relatively low in all analysed matrices, we should consider further analysis of occupational exposure to endotoxins

A non-parametric Spearman Correlation test examined the relationship between different culture media and bacterial load, in both filters. A low negative correlation between total bacteria and Gram-negative bacteria was found in Cabin Ventilation Filters ( $r_s = -0.33$ ,  $p = 0.233$ ), whereas total bacteria count is positively correlated with Gram-negative bacterial counts in air samples ( $r_s = 0.42$ ,  $p = 0.033$ ). It is important to remember that correlation does not mean causation, so this data shows how it is important to combine different sampling methods and various selective media for a better risk assessment.

In order to evaluate if there were any significant difference between active and passive sampling when influencing bacterial viability, a Mann-Whitney test was performed. The test revealed no statistically significant difference between the use of Cabin Ventilation Filters and SASS Filters ( $p > 0.05$ ). There is a wide range of environmental variables, such as work practices that may affect bacterial load. Also, it is important to take into consideration that the correlation was tested only using culture-based methods results, since we can not disregard the fact that viable bacteria only account for a small percentage of total concentration of contamination. Therefore, any of the sampling methods performed are valid for a proper risk characterization of airborne, and if not possible to combine both methods, either method can be applied. Although it is recommended to assess both air and surface contamination load to ensure a complete exposure assessment, it should be taken into consideration that passive methods can be more convenient and help overcome some active sampling limitation. Active air sampling best represents the contamination load that can be inhaled by individuals in a short period of time, but methods used to collect air samples can affect cell viability and lose culturability. Also, with active air sampling the loss of microbial diversity can be induced because of the filter saturation and limited period of sampling (78). Passive air sampling can help overcome these problems since the sample collected comprises of airborne microorganisms that has fallen into a surface with no mechanical disturbance. Also, since these samples comprise of microorganisms that has deposited into a surface during longer periods of time, allowing a cumulative sampling, it is a better representation of the variability of bacterial species that these workers could possibly be exposed to.

## 4. Chapter 4: Conclusions

Waste management facilities are usually considered critical in regard to occupational exposure to airborne microorganisms. Workers in waste industry are exposed to a higher risk of biological hazards due to the work characteristics. As waste materials is sorted in waste sorting units to produce raw material, waste workers become directly exposed to their contaminants. Organic residues enhance workers' exposure to microbial contaminants as they provide substrate and nutrients for various microorganisms (9,79).

The results of this cross-sectional study may give some insight into the possible expected exposure of the waste workers to inhalable bacterial species during waste management, showing a high prevalence of bacterial load. This study highlighted the importance of use of different culture media as a way to enhance the growth of specific types of microorganisms as a first step for a complete risk assessment. It also shows the differences between two different sampling approaches. Although no significant difference was verified between using passive or active air sampling, the importance of the combined use of these methods in this setting emphasized

Bioaerosol exposure is underlying to waste handling and the risk of developing adverse health effects. The focus should be on finding measures to minimize exposure, such as implementing good hygiene routines, ventilation systems, educating workers to be aware of the risks to which they are exposed daily and how they can act to reduce it. The lack of knowledge about the health hazard and risks associated with exposure makes workers more vulnerable, combined with the lack of proper safety clothing and poor work performance (80). Workers should be properly educated and informed about the results to induce behavior changes for hygienic measures compliance.

When the risk of exposure can be translated to households and this issue poses a real public health threat, local authorities should also provide general population and households further and proper education regarding health risks associated with extended periods of waste stored in house and help minimize exposure to bioaerosol emission. It is also important to keep reinforcing the importance of ecologic activities such as reducing waste production, reuse of long-lasting materials and increment waste sorting for maximizing proper recycling within households, in order to help minimize occupational exposure of workers to bioaerosols during waste management.

#### 4.1. Recommendations for further work

There are some notes to take in consideration that should be incorporated or improved in further studies, that might be crucial helping acquire more information for a more complete and accurate risk assessment. It is important to consider that in highly contaminated environment such as waste management industry, workers are most likely exposed to all the components of bioaerosols, which can result in different and more severe health effect comparing to the exposure to single components.

- **Microscopical identification combined with biochemical properties:** qualitative composition of the bioburden helps establishing the toxicological profile and health effects. Characterizing the bacterial isolates based on their microscopic morphology (cellular morphology), combined with macroscopic features (pigmentation, texture, shape) and biochemical characteristics (Gram staining, catalase and oxidase activities) can provide valuable and better understanding of individual microbial diversity (18).
- **Molecular detection of bacterial species:** a detailed risk characterization in this occupational environment should include not only the quantification of bacterial load in samples but also incorporate the identification of relevant bacterial species. A multi-approach strategy, combining culture-based methods with molecular tools will better assess the bacterial contamination in occupational environments. As the culture-based methods provide valuable information regarding the sample viability, the molecular tools allow to determine the qualitative composition and microbial biodiversity of the bioburden, as they also impact the toxicological profile of the sample and contribute to link specific bacterial species to a potential health effect. The complementary use of culture-based method and molecular tools is becoming necessary, almost unavoidable, for a more complete and accurate risk characterization to occupational exposure to bioburden.
- **Measurement of outdoor relative humidity and temperature as additional data for analysis:** the knowledge of the determinants of bacterial bioburden is important. These variables are crucial for determination of microbial prevalence and dispersion. Detailed information combined with a valid registration of these

variables during working periods can contribute to a more meticulous evaluation of the exposure (81).

- **Endotoxin analysis:** airborne exposure to bioaerosol occur via organic dust inhalation during occupational settings. As endotoxin is one important component of organic dust, and it has been established that the inhalation of this agent can have many acute and chronic health effects. The presence of endotoxins in the air is related to the presence of Gram-negative bacteria or fragments of cell wall from these bacteria in airborne organic dust. Many investigations have used the amount of airborne endotoxin to characterize and assess occupational exposure. Sometimes endotoxins can be detected among environmental samples whereas gram-negative bacteria are not detected through culturable methods. That can provide additional information regarding the bioburden, as we can conclude that Gram-negative bacteria is in fact present but not culturable in the selected culture media or conditions of growth are not ideal (12).
- **Screening for antibiotic resistance:** environmental burden often comprises of gram-negative bacteria, which are naturally more resistant to antibiotics due to the nature of the cell wall. With the increase of drug resistant human pathogenic microbial species and reports of therapeutic failure against infections, there is the need to perform surveillance of bacterial resistance and implement microbial monitoring, preventative, and protective measures in order to protect the health of both individuals that are directly exposed to resistant bioburden in waste industry and also for the general public.

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