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Bacterial analysis of polluted air in Antwerp and surroundings

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List of abbreviations

BC	Black Carbon
CFU	Colony Forming Unit(s)
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	Dimethyl Sulfoxide
FCM	Flow Cytometer
FISH	Fluorescent *In Situ* Hybridization
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
PAH	Polycyclic Aromatic Hydrocarbons
PBAP	Primary Biological Aerosol Particles
PCR	Polymerase Chain Reaction
PM_{10}	Particulate matter with an aerodynamic diameter of 10 µm or less
UFP	Ultrafine Particles
VMM	Vlaamse Milieumaatschappij (Flemish Environment Agency)

Samenvatting

Luchtvervuiling is sterk verweven met het alomtegenwoordige bacteriële leven in de atmosfeer. Microbiologisch onderzoek van deze omgeving is echter relatief beperkt. Door hernieuwde interesse en technologische vooruitgang wordt de kennis snel uitgebreid, maar er is nog veel dat onderzocht moet worden. In deze masterthesis werden moleculaire en functionele eigenschappen van de bacteriële gemeenschappen afkomstig uit vervuilde lucht in en rond Antwerpen onderzocht. Cultuurafhankelijke methoden toonden aan dat ongeveer 10^2 tot 10^3 'kolonievormende eenheden' of CFU/m^3 lucht aan cultiveerbare bacteriën aanwezig zijn in de onderzochte stalen. Daarnaast toonden tellingen van DNA-bevattende partikels door middel van flowcytometrie aan dat 9 x 10^5 tot 7 x 10^6 per m^3 biologische partikels aanwezig zijn in de onderzochte lucht. Dit geeft aan dat cultiveerbare fractie van bacteriën in de lucht slechts ca. 0,008 tot 0,254% bedraagt van de totale gemeenschap. Dergelijke schattingen zijn in overeenstemming met waarden geschat voor andere milieus. Specifieke functionele eigenschappen van de cultiveerbare bacteriën die onderzocht werden, zijn onder andere groei bij 4°C, tolerantie aan vriestemperaturen, pigmentatie en adaptatie voor zware metalen. Deze experimenten toonden aan dat een grote fractie, namelijk 65% - 92%, aan gepigmenteerde bacteriën voorkwam in de buitenlucht, mogelijk gerelateerd aan de bescherming van deze pigmenten tegen UV straling. De bacteriële gemeenschappen in de lucht vertoonden eveneens opmerkelijke aanpassingen aan lage temperaturen. Verder toonden de testen voor adaptatie aan zware metalen aan dat er potentiële verschillen zijn in aanpassing voor bacteriën uit een industriële, stedelijke en residentiële locaties (respectievelijk de haven, het stadscentrum en een groene woonwijk van Antwerpen). Dit kan mogelijk gekoppeld worden aan de verschillende metaalhoeveelheden in de lucht van deze locaties, maar de ICP-MS analyses van de metaalconcentraties zijn nog niet afgerond. Enkele geselecteerde kolonies werden ook geïdentificeerd met behulp van sequencing van het 16S rRNA gen en vastgestelde soorten behoorden tot de genera *Arthrobacter, Sphingomonas, Microbacterium, Herbaspirillum* en *Paenibacillus.* Deze cultuurafhankelijke analyses leverden vooral preliminaire data en verder onderzoek is nodig in toekomstige studies. Als aangegeven door de beperkte cultiveerbare fractie, kunnen DNA-gebaseerde gemeenschapsanalyses, zoals denaturerende gradiënt gelelektroforese (DGGE) en metagenomisch sequencen (Illumina HiSeq), belangrijke

complementaire inzichten leveren. Om deze reden werden verschillende DNA extractiemethoden getest tijdens deze masterthesis. Uit deze experimenten bleek dat het bekomen van voldoende en kwalitatief DNA van luchtgemeenschappen een hele uitdaging is, voornamelijk door contaminatie en de aanwezigheid van PCR inhibitoren, waardoor verdere optimalisatie noodzakelijk is. Een geïntegreerde studie van luchtbacteriën in de toekomst zou kunnen leiden tot interessante nieuwe toepassingen voor biomonitoring, bioremediatie en andere biotechnologische toepassingen.

Abstract

Air pollution is inevitably intertwined with the ubiquitous bacterial life in the atmosphere. However, this field of microbiology is relatively underexplored. Due to renewed interests and technological improvements, the knowledge gap is slowly being decreased, but much is still to be learned. In this master thesis, molecular and functional properties of bacterial communities, originating from polluted in and near Antwerp (Belgium) were assessed. By using culture-dependent techniques, it was observed that the concentration of culturable bacteria appeared to be in the range of 10^2 – 10^3 CFU/m^3 air. In contrast, DNA-staining of particles followed by flow cytometry indicated that the biological particles in the air ranged between 9 x 10^5 and 7 x 10^6 per m^3 air. This suggests that the culturable fraction is only between 0.008-0.254 %, which is a range that is also common in other environments. Specific functional properties of the culturable bacterial fractions that were assessed included growth at 4°C, freeze tolerance, pigmentation and adaptation to heavy metals. These experiments showed that an unusual large fraction of 65% - 92% of pigmented colony forming units (CFU) occurred in the outdoor air, which might be related to the protection of these pigments offer against UV radiation. In addition, the airborne communities showed a remarkable adaptation to cold temperatures. Moreover, heavy metal resistance of the culturable fraction was not equal between the industrial, urban, and residential location (the harbor, the city centre, and a green suburb of Antwerp), which might be related to the different metal content in the air in these environments. Selected colonies were also subject to further identification based on 16S rRNA gene sequencing and included *Arthrobacter, Sphingomonas, Microbacterium, Herbaspirillum* and *Paenibacillus* species. Nevertheless, the culture-dependent analyses only provided preliminary data and need to be further explored in future studies. Furthermore, DNA-based community analysis of airborne bacteria shows high promise, including denaturing gradient gel electrophoresis (DGGE) and metagenomic sequencing (Illumina HiSeq). Therefore,arious protocols for DNA extraction were explored in this master thesis. These experiments indicated that obtaining sufficient and high quality DNA from airborne communities is a considerable challenge, especially due to the presence of unknown contaminants and PCR inhibitors, and further optimization is necessary. Future perspectives include the exploration of biomonitoring , bioremediation and other biotechnological applications based on airborne bacteria.

1 Introduction and objectives

The atmospheric air, the air we breathe, consists of far more than meets the eye. You begin to realize this if you shine a ray of light in a darkened room, illuminating the once invisible particles. What is often overlooked, is that air does not only consist of gases and dust, but it is teeming with life forms. There are numerous microorganisms present, of which only little is known, despite their versatile impact on our lives. The atmosphere has been described as one of the last frontiers of biological exploration on Earth (Rothschild & Mancinelli, 2001). Our knowledge about the functional potential of airborne microbes (both beneficial and pathogenic) is therefore scant and, much remains to be discovered. Only recently, next-generation sequencing has allowed the detailed study of the biodiversity and biogeography of this largely unknown ecosystem. In addition, airborne microbes have been found to be more than just passive inhabitants of the atmosphere. At least some of them are metabolically active and well adapted for the harsh atmospheric conditions (Vaïtilingom *et al.*, 2012).

Furthermore, the European Commission has declared 2013 as 'the year of air' to promote better care and knowledge of the atmosphere. This master thesis fits congruously within these general objectives, by aiming to explore the microbial communities of the near-surface atmosphere at locations with different degrees of air pollution in and near the city of Antwerp (Belgium). Antwerp is centrally located in Western Europe and has one of the largest sea ports of entire Europe, making it a hot-spot for traffic-related and industrial air pollution. Ongoing research at the Department of Bioscience Engineering of the University of Antwerp, where this master thesis takes place, aims to provide information on the spatiotemporal occurrence of various air pollutants in relation to airborne microbes.

The objective is to study the microbial communities by using both culture-dependent and independent techniques, aiming to gather functional information on specific characteristics of airborne bacteria and to identify the microbial members present. Of all the various microorganisms, we choose to focus on bacteria. For the functional characterization of the bacteria, we focus on stress resistance, as the atmosphere is a harsh environment for microorganisms, especially when the air is polluted. UV radiation, desiccation, pollutants, and other stressors are common. Potential correlations between concentration of fine

particulate matter (PM_{10}) or black carbon (BC) in the atmosphere and the presence of functional properties such as pigmentation and DNA concentrations will be investigated. In addition, heavy metal stress resistance of airborne bacteria will be explored in areas with different levels of air pollution as a possible future biomonitoring tool for air pollution. These experimental objectives will be preceded by a detailed study of the available literature, and followed by a comparison of our data with previous studies of airborne bacteria in other locations.

2 Literature study

2.1 Aerosols

Many different kinds of particles are small enough to have a significant atmospheric residence time, so that they become an integral part of the atmospheric aerosol (Jones & Harrison, 2004). This includes, among others, a wide range of pollutants, fine dust particulates, water droplets and bioaerosols. The latter represent the biological fraction of aerosols. Atmospheric bioaerosols can include pollen, spores, fractions of organisms, or complete microorganisms like bacteria, fungi, viruses, amoebae, and archaea (Eduard *et al.*, 2012). Alternatively, bioaerosols are designated with another term in literature, namely 'Primary Biological Aerosol Particles' or PBAP (Després *et al.*, 2012). Aggregation of biological matter, water, dust, and pollutants often occurs (Maron *et al.*, 2005; Tong & Lighthart, 2000; Tringe *et al.*, 2008). These particles or aggregates, suspended in the atmosphere make up the total aerosol.

2.2 Airborne bacteria

Due to low humidity, scarce nutrients, variable temperatures, and UV exposure, the atmosphere is considered an oligotrophic and harsh environment and therefore challenging, not only for the survival, but especially for the growth of microorganisms (Franzetti *et al.*, 2011; Peccia & Hernandez, 2006). Despite this, bacteria continue to be ubiquitously present in ambient air (Jaenicke, 2005) and in significant cell concentrations, expected to be of the magnitudes 10^3 to 10^5 per m^3 air in Western Europe (Burrows *et al.*, 2009b; Maron *et al.*, 2005). The airborne communities even appear to show high diversities, comparable to soil and water communities (Brodie *et al.*, 2007; Maron *et al.*, 2005). This apparent contradiction can be explained by various bacterial adaptation mechanisms such as temperature tolerance, DNA-repair mechanisms, and other means for UV protection like embedding in particles (that can contain liquid) or producing a wide range of pigments (Polymenakou 2012; Tong & Lighthart, 1997; Womack *et al.*, 2010). Furthermore, Tringe and colleagues (2008) used DNA shotgun metasequencing on air samples and found that genes coding for

membrane proteins responsible for secretion, cell aggregation, and iron transport and metabolism were overrepresented in sampled air communities. They hypothesized that these genes indicate that not only oxidative damage and desiccation are major stresses for airborne microbiota – as could be expected –, but also that iron availability is a limiting factor, to which adaptation occurs. Another protection mechanism of bacteria is to enter a non-dividing state (dormancy), where they morphologically transform to endospores or undergo other cell wall modifications (Dewi Puspita *et al.*, 2012). This is a typical strategy for bacteria exposed to stresses such as lack of nutrients, water scarcity, and fluctuations of other environmental parameters (Bär *et al.*, 2002), which are very common characteristics of airborne life.

Bacteria can also be in a metabolic active state in the air and even cell multiplication in fog or cloud water occurs (Amato *et al.*, 2007a; Amato *et al.*, 2007b; Dimmick *et al.*, 1979; Sattler *et al.*, 2001). The moisture in fog and clouds offers protection against desiccation and contains organic molecules (C1 to C4) to metabolize (Vaïtilingom *et al.*, 2010). However, information on the growth and activity of airborne bacteria is still fragmentary, because the atmosphere is often being overlooked as a habitat for microorganisms (Morris *et al.*, 2008). All of these adaptations, and possibly many more, help to explain the ubiquitous presence of microorganisms in the atmosphere.

Another important facet of aerobiology of microbes is their transport and dispersion through the atmosphere. Some studies have tried modeling of this bacterial transport. Nguyen and colleagues (2006) have attempted to predict disease outbreak of *Legionella pneumophila* by using a Gaussian dispersion model that usually serves for modeling of pollutants from a point source. They managed to obtain a good fit, based on data from an outbreak, but bacterial concentrations could not be determined. Burrows and colleagues (2009b) have modeled world-wide bacterial aerosol transport and concentrations, by using models for atmospheric circulation, weather, and emission from different ecosystem classes (Figure 1). They concluded their model still needs a lot more experimental data to overcome its limitations, but this modeling not only allows prediction of bacterial concentrations world-wide, but also holds interesting applications for microbial biogeography by allowing insight into the dispersion of genetic material between ecosystems.

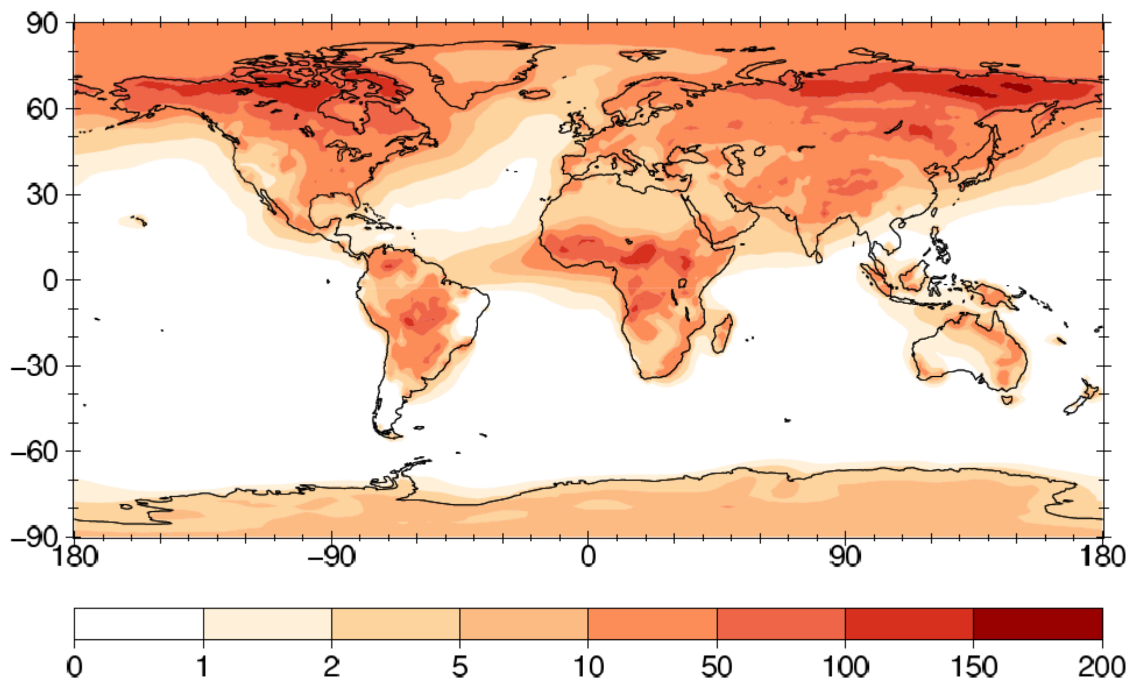

Figure 1: Near-surface concentrations of bacteria (10^3 m^{-3}) based on the model of Burrows and colleagues (2009b). In this case, bacteria were assumed to be of a size of 1 µm. Bacteria larger than 1 µm are expected to be transported over shorter distances (Burrows *et al.*, 2009b).

Bacteria enter the near-surface atmosphere by continuous aerosolization from various surfaces such as those from plants, animals (including humans), soil and water (Figure 2) (Burrows *et al.*, 2009b and Jones & Harrison, 2004). Aerosolization of bacteria might occur on bacteria per se or on particles with bacteria (Jones & Harrison, 2004). Droplets containing microorganisms have been shown to aerosolize from water surfaces (Burrows *et al.*, 2009b; Woolf, 1997). The phyllosphere (plant and leaf surfaces) is also thought to be an important source of airborne bacteria, because it is one of the largest microbial habitats on earth with a highly exposed surface area to the air (Redford & Fierer, 2009). In addition, many airborne bacteria were identified as species that also occur within bacterial soil communities, implying the soil as a very likely contributor (Bowers *et al.*, 2012). Apart from aerosolization, cell multiplication may contribute to the bacterial concentrations in the air, though it is not known to what extend (Amato *et al.*, 2007b; Morris *et al.*, 2008). Thus, replenishment of

bacteria in the atmosphere comes from many surface-related sources and to a certain unknown extend from cell division.

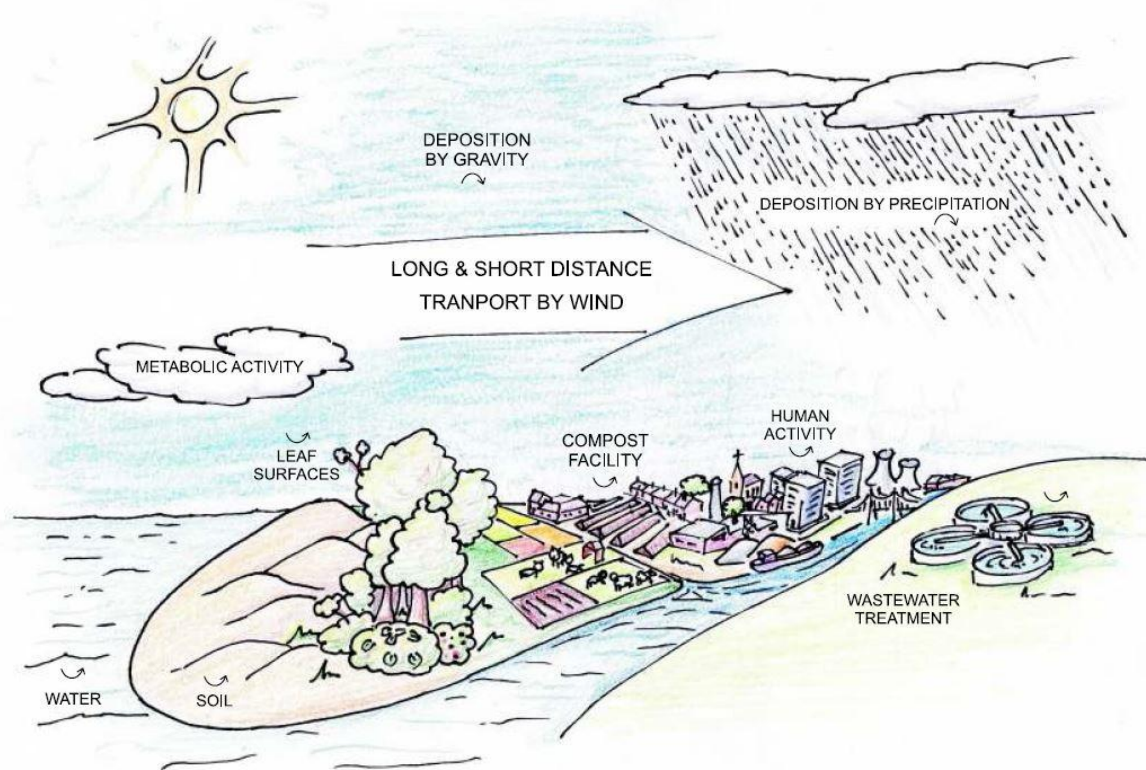

Figure 2: Schematic of typical processes that determine composition of local airborne bacterial communities. Abundant sources of aerosolized bacteria are marked with an upward arrow, while deposition is indicated with a downward arrow. Airborne bacteria are often transported and may occur in a metabolic active state in cloud droplets. This scheme has been depicted based on Albrecht *et al.*, 2007; Amato *et al.*, 2007a; Amato *et al.*, 2007b; Bowers *et al.*, 2011b; Burrows *et al.*, 2009b; Favet *et al.*, 2013; Grammatika & Zimmerman, 2001; Han *et al.*, 2012; Lindemann *et al.*, 1982.

After bacteria spend an undetermined time in the atmosphere, conditions for deposition may occur. Particulate matter and bacteria may eventually settle back on available surfaces. The deposition velocity depends on environmental parameters and particle characteristics like the aerodynamic diameter (Jones & Harrison, 2004). Precipitation can also deposit airborne bacteria (Figure 2), because considerable fractions of bacteria are found in cloud water and snow (Amato *et al.*, 2007a; Bauer *et al.*, 2002). It has also been observed that rainfall washes bacteria out of the air (Ravva *et al.*, 2012). The growth, aerosolization, and wind-driven import are hypothesized to compensate for the death, deposition, and wind

export of bacteria (and other organisms), but also cause the bacterial composition to show strong variations in space and time.

Despite spatiotemporal variations, most metagenomic studies of the near-surface atmosphere attribute the following phyla as dominant. *Actinobacteria* are Gram-positive, filament-forming bacteria that usually play an important role in decomposition of organic material, but may include pathogenic *Actinomyces* species that can cause pulmonary actinomycosis (Waksman, 1950; Mabeza & Macfarlane, 2003). For example, Bowers and colleagues (2012) found the fraction *Actinobacteria* of the total airborne bacteria varying between 9 and 16% at a high-elevation site. This range may apply for lower altitude locations too, as at a rural site 12% *Actinobacteria* were found (Maron *et al.*, 2005). *Firmicutes* (including *Bacilli*) are also gram-positive bacteria. Many of them are capable of forming endospores (Onyenwoke *et al.*, 2004), which indicates how they survive in the atmosphere. Bowers and colleagues (2012) found that *Firmicutes* made up 5 to 20% of the airborne bacterial fraction at a high-elevation site. The phylum of the *Bacteroidetes* is a Gram-negative, phenotypically variable phylum, containing some opportunistic pathogens (e.g. in the human gut) and periodontal disease inducers (Gupta & Lorenzini, 2007). Their numbers in airborne samples are usually rather rare, although Bowers *et al.* (2011) found for instance that *Bacteroidetes* are dominant in winter samples, suggesting that dog feces are likely the dominant source of aerosolized bacteria in the winter months in the sampled area (Cleveland, Detroit, and, Chicago). For analyzing airborne samples, the dominant subphyla of the *Proteobacteria* are *α-Proteobacteria*, *β-Proteobacteria*, and *γ-Proteobacteria* (e.g. Bowers *et al.*, 2012; Brodie *et al.*, 2007). This subdivision gives more insight in the large bacterial fraction of *Proteobacteria*, which was observed to be 60% in a rural location studied by Maron and colleagues (2005) and up to ca. 40% in urban areas in in the Midwestern United States (Bowers *et al.*, 2011). They are Gram-negative and many use flagella (Woese, 1987), which may be linked to the high motility gene occurrence found in airborne communities (Tringe *et al.*, 2008). Combined with the fact that many factors influence bacterial communities, it is hard to come up with general percentages for these most abundant phyla.

The large spatiotemporal variations in total bacterial concentration, diversity, and abundance of microbial species is a common theme for airborne analyses of microbial

communities (Bowers *et al.*, 2012; Fierer *et al.*, 2008). Altogether, the microbial profile in a certain region is dependent on the land use (Figure 2) or ecosystem type (determining amount of surface of phyllosphere, soil, water, and specific point sources potentially from human activity), the season, the climate, and different weather factors like wind speed, wind direction, relative humidity, temperature, and precipitation (Amato *et al.*, 2007a; Bowers *et al.*, 2012; Brodie *et al.*, 2007; Burrows *et al.*, 2009b; Chi *et al.*, 2007; Fang *et al.*, 2007; Han *et al.*, 2012). The amount and size of aerosolized particles have also shown to play an important role in the bacterial population dynamics (Bertolini *et al.*, 2012; Brodie *et al.,* 2007). Furthermore, complex air currents in the atmosphere affect the local bacterial populations (Burrows *et al.,* 2009b). Not all of these determining factors show similarly strong correlations with cell concentrations (Bowers *et al.*, 2012; Chi *et al.*, 2007). For example, of the meteorological factors, rainfall, temperature, and UV radiation were shown to contribute the most to bioaerosol concentrations (Chi *et al.*, 2007). Another thing to consider is that the abundance of different types of cells is determined by different factors. For example, Amato and colleagues (2007a) found that Gram-negative bacteria were more abundant during summer because of their resistance to UV damage compared to Gram-positive bacteria. Thus, it is not self-evident to find strong causal correlations between the proposed causes of variability and the general bacterial concentrations. To gain better insight in the bacterial community and its functions, it makes more sense to look for correlations with abundance or metabolic state of one microbial cell type (e.g. one particular species, phylum, Gram type) and the interplay of most relevant environmental parameters. Bowers and colleagues (2012) observed no diversity differences between the seasons, but they discovered that the airborne community's composition does differ. This implies that considering a more complex model can yield better results. For example, Brodie and colleagues (2007) were able to set up a model explaining almost 90% of the variance of the phylum dominance of their air samples.

2.3 Technical challenges associated with analyses of airborne microorganisms

The collection of airborne microbes is the first challenge, which is followed by downstream analysis. The latter can be roughly divided in two main groups, either culture-dependent or molecular-based techniques. Other techniques include microscopy, flow cytometry, and immunological or biochemical assays. Each of these techniques offers a unique perspective in data interpretation.

2.3.1 Collection methods

Various methods for the sampling of airborne microorganisms exist. The organisms are preferably captured and suspended in a liquid to make them suitable for further study (Langer *et al.*, 2012). One of the older methods, however, is the use of settle plates, where Petri dishes with media are simply left open to the air at the location of interest (Russell *et al.*, 1984). More recently, filters became widely used for air sampling. After sample collection, they can be placed in a liquid or rinsed to suspend the microorganisms in a liquid. However, many confounding processes can occur on the filter, like bacterial proliferation (Möritz *et al.*, 2001). Also, dehydration of particles or cells and low extraction efficiency from the filter material are weaknesses of filtration (Martinez *et al.,* 2004; Muilenberg & Burge, 1994). These problems could lead to a distorted representation of the bacterial population in the air. An impactor is a sampler of which the operation principle is based on air suction through holes and subsequent impaction of particles and microorganisms on medium (Martinez *et al.*, 2004). The problems with this technique are desiccation of the cells and their rebound off the medium surface (Juozaitis *et al.*, 1994). Impactors are nevertheless widely applied for airborne microbial sampling. They are preferred for monitoring hospital air (Gangneux *et al.*, 2006). Another type of samplers are liquid impingers. They suck air through a tube and into a stirred liquid in which the airborne particles are suspended (Figure 3). They are, among others, used for bacterial aerosol sampling (May & Harper, 1957). The AGI-30 impinger is a popular type (Lin *et al.*, 2000). It showed a better bacterial recovery than filters (Li *et al.*, 1999) and requires less sampling time. Nevertheless, other studies have shown that microbial cells can get structurally damaged by impingement (e.g. Terzieva *et al.*, 1996) or they get reaerosolized, which decreases efficiency (Lin *et al.*, 1997). As an

improvement of the impinger, Willeke and colleagues (1998) introduced the Swirling Aerosol Collector, also called BioSampler. The particles are drawn into a swirling motion and impacted under an angle on a solid or liquid collection surface (Figure 3). This method showed an improved efficiency. A more recent invention is based on the Coriolis effect, which has also proven to show better efficiencies than an AGI-30 impinger (Ahmed *et al.*, 2013; Langer *et al.*, 2012). A vortex of sampling liquid in a cone makes airborne particles, sucked into this cone, move outward due to centrifugal force, so they can get suspended in the liquid (Carvalho *et al.*, 2008) (Figure 3). This method will be discussed in more detail later on due to its use in this study.

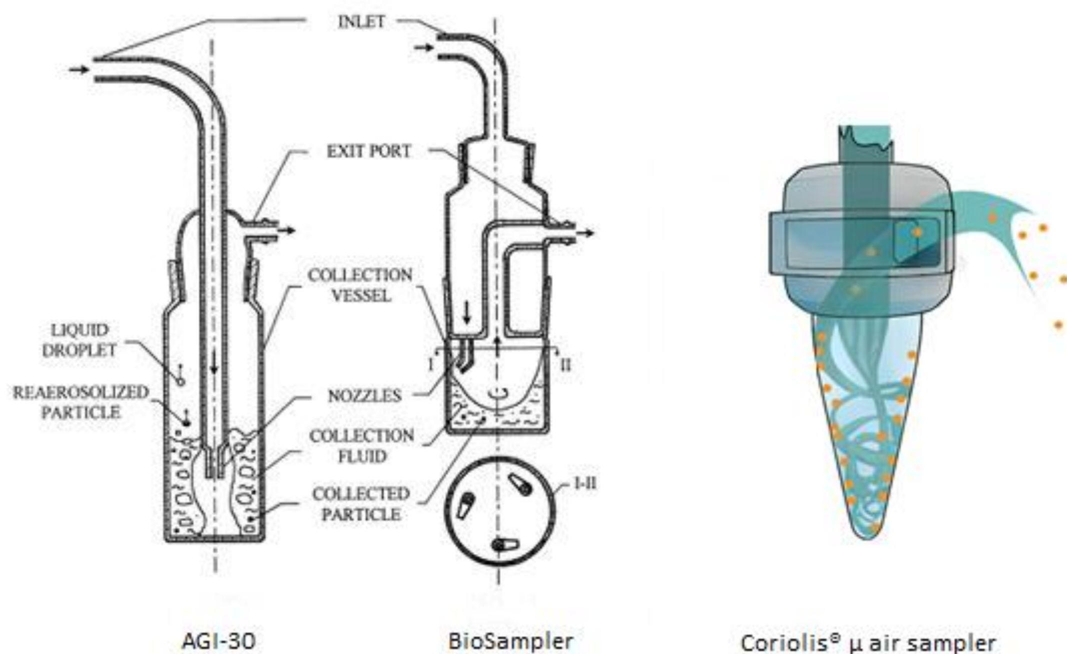

Figure 3: Schematic diagrams of some air sampling principles (Adapted from Lin *et al.*, 1999; Bertin technologies, "Coriolis® Principle", 2013).

2.3.2 Culture-dependent analysis

The 'older' culture-dependent methods are still highly valid to show that airborne microorganisms are indeed alive and to execute further functional studies with these organisms (Giongo *et al.*, 2013). It is the logical next step after sampling with settle plates or

impactors. However, for airborne analyses, these methods are extremely biased due to the presence of 'viable but non culturable' (VBNC) organisms. Hyvärinen and colleagues (1991) tried to find optimal conditions for culturing airborne bacteria and concluded that a poor but complex medium such as R2A yields good results. This medium serves as a broad-spectrum medium and is often used for culturing environmental bacteria (Durand *et al.*, 2002). Up to now, it is still a widely used medium for culturing airborne bacteria (e.g. Dybwad *et al.*, 2012; Kim *et al.*, 2013; Wang *et al.*, 2012). Hyvärinen and colleagues (1991) also concluded that incubation temperatures for growth of outdoor atmospheric bacteria were optimal between 10°C-20°C. Nevertheless, the culturable fraction of all bacteria of well-studied habitats (fresh water, sea, soil, etc.) is estimated to range from 0.001% to 3% (Amann *et al.*, 1995). For air samples, this fraction is also very variable, but the average is estimated at around 1% (Chi *et al.*, 2007; Lighthart, 2000; Peccia & Hernandez, 2006). Such a large fraction of unculturable bacteria highlights that culture-dependent methods largely underestimate the real bacterial diversity (Fierer *et al.*, 2008). Additionally, cultivation techniques may not always accurately represent the dominant *in vivo* bacterial species. They rather favor growth of species most adapted to cultivation conditions.

2.3.3 DNA-based molecular analysis

The bias of culture-dependent techniques can be avoided by the use of molecular techniques, that look at, for example, DNA of all organisms in a sample. A widely used molecular tool is polymerase chain reaction (PCR), which does not require any prior cultivation. PCR is often the basis for some widely used molecular techniques including cloning, fingerprinting, hybridization assays, and sequencing of DNA. This method is based on the direct detection of the organism's nucleic acid. It is advantageous because of its sensitivity, ease, rapid turnover and relatively inexpensive application, and, most importantly, PCR may eliminate the need to isolate and culture. In practice though there is still an incomplete view on the community, because DNA extractions do not extract all present DNA due to incomplete lysis of cells and loss or damage of DNA during DNA extraction and PCR primers can show different affinity for different DNA molecules (Roose-Amsaleg *et al.*, 2001; Suzuki & Giovannoni, 1995). The incomplete lysis will be an important

issue for air samples, because many cells are expected to be in a dormant and sometimes endospore state (which means a thicker cell wall), as explained previously. An example of processing air samples using molecular methods is given by the study of Maron and colleagues (2005), who extracted DNA (after a spore-germinating step), used PCR to amplify 16S rRNA gene fragments of all present species using universal bacterial primers. They made a 16S clone library by inserting the PCR products in vectors and transformed these into *Escherichia coli*. After growing the *E. coli*, they amplified the plasmid insert and used Sanger sequencing. These sequences were subsequently identified by comparing them to databases of previously known DNA. Additionally, PCR was used to amplify the 16S-23S intergenic spacer regions of the DNA samples, followed by electrophoresis on a polyacrylamide gel to obtain an ARISA (Automated Ribosomal Intergenic Spacer Analysis) fingerprint. This technique generates unique profiles for comparing different bacterial communities. The use of Sanger sequencing can be very labor intensive though. With the invent of pyrosequencing, methods started to become a lot faster, more affordable, and practical for high-throughput applications, because pyrosequencing allows sequencing of hundreds or thousands of different DNA molecules at once without requiring previous cultivation of clones. However, the less abundant species can easily be overlooked because of the overpowering presence of the more dominant ones (Kuske, 2006). This effect is increased when a PCR step with universal bacterial primers is necessary to amplify DNA prior to analysis (Ravva *et al.*, 2012). This effect is also limiting fingerprinting. With DGGE (Denaturing Gradient Gel Electrophoresis), for example, DNA concentrations lower than 1% of the total DNA will show no visible band on the gel (Marzorati *et al.*, 2008). Specific PCR and hybridization assays can compensate by detecting less abundant species, but only partly because they are based on previously known sequences (Kuske, 2006). In addition, different methods for DNA extractions can yield very different views of the actual community (Martin-Laurent *et al.*, 2001). Thus, limitations are still evident with regard to the total bacterial community when using molecular techniques.

2.3.4 Other techniques

Microscopy and flow cytometry are also used to count bacterial cells, determine live-dead ratios, and to characterize important functional characteristics of bacterial cells (Despréz *et al.*, 2012). Microscopy includes many possible applications. However, it is difficult to use a bright-field compound microscope for bacterial observation, because bacteria are usually too small. Only pollen grains and fungal spores can be counted with this method (Stetzenbach *et al.*, 2004). FISH (Fluorescent *in situ* hybridization) has been successfully executed on laboratory-generated bioaerosol of *E. coli* and *P. aeruginosa* by Lange and colleauges (1997). After sampling, they used fluorescent labeled DNA probes to hybridize to the two bacterial species for detection and quantitative enumeration with a laser scanning confocal microscope. Though, FISH can be used for more than just enumeration of one species. Aller and colleagues (2005) managed to determine the fraction of *α-Proteobacteria* and the amount of active bacteria and virus-like particles in marine aerosols. They also gained insight in how many of the airborne bacteria were associated with particles in this specific aerosol (Figure 4).

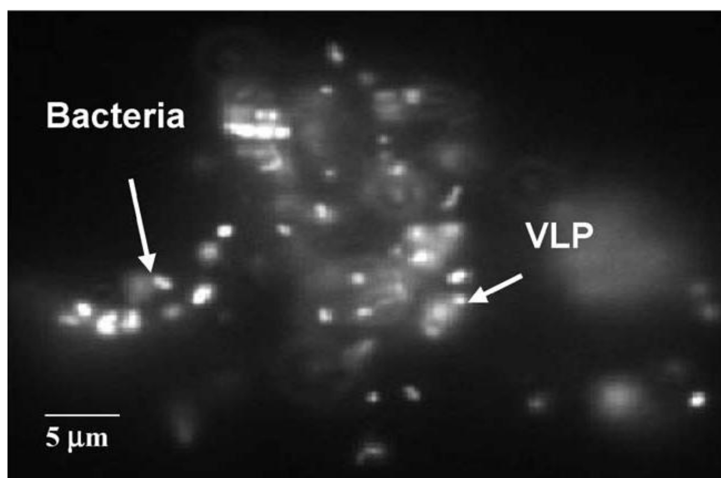

Figure 4: Marine aerosol sample, stained with DAPI. About 60% of the bacteria and 35% of the virus-like particles (VLP) were associated with particles. The particles of marine aerosol are usually transparent exopolymer particles and proteinaceous particles (Aller *et al.*, 2005).

Epifluorescence, which detects fluorochrome stains, is commonly used for enumeration of bacteria in samples of all kinds of environments, including air (e.g. Maron *et al.*, 2005; Rinsoz

et al., 2008). Giongo and colleagues (2013) have performed SEM (scanning electron microscopy) on a desert dust sample in view of using this technique on intercontinental airborne dust originating from deserts. They managed to reveal the microlocation of the microbes, which can also be interesting to study how and where bacteria are associated with airborne dust or possibly other particles.

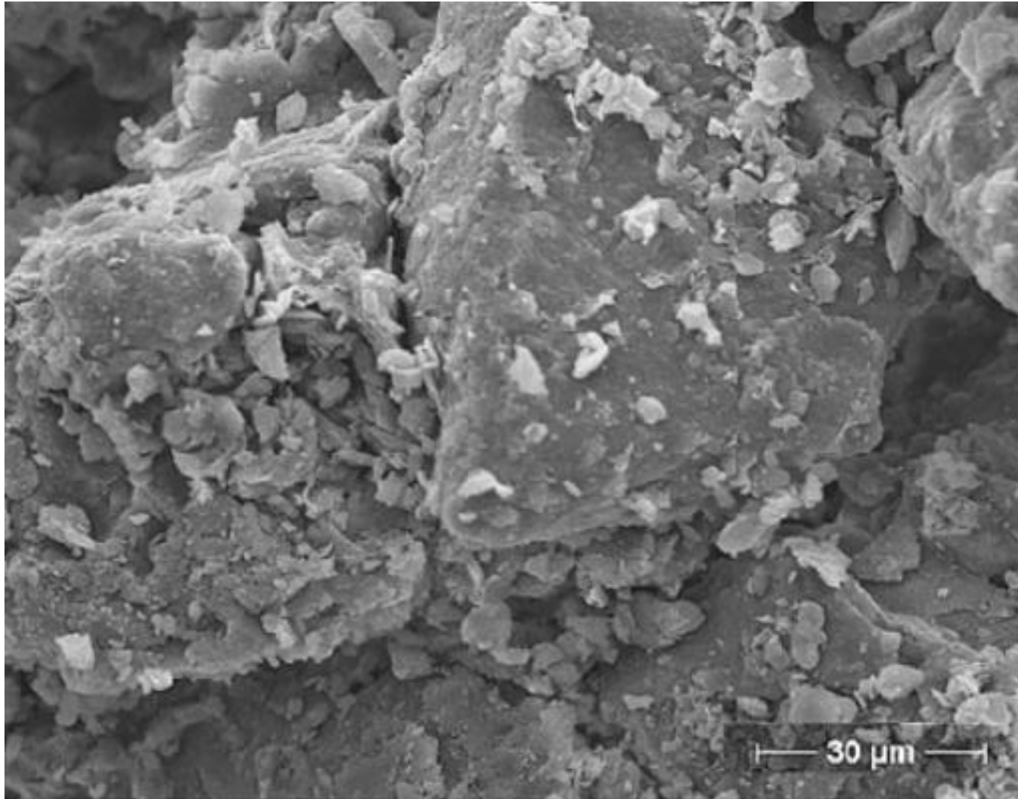

Figure 5: SEM picture of a desert dust sample. Sand grains are held together by microbial biomass and small clay particles. The sample was air-dried before analysis, so the structure of extracellular substances was not preserved. (Giongo *et al.*, 2013)

Lange and colleagues (1997) used flow cytometry for the first time on agricultural aerosol and succeeded in enumeration of total microbial counts by using the DNA-specific-DAPI stain, which showed to be similar to microscopic counts. Finally, popular tools for pathogen detection include immune-based assays. They are based on recognition of immune-response-provoking components of pathogens (i.e. antigens). For airborne pathogens such assays are not yet widely used, but detection and quantification down to 4 x 10^3 cells m^{-3} of *Legionella pneumophila* has been done using microarray sandwich immunoassay analysis on

air samples (Langer *et al.*, 2012). All these techniques each offer advantages and disadvantages and should be selected and interpreted carefully based on their application.

2.4 The knowledge gap

In 1860 already, Louis Pasteur reported the first observations of airborne microorganisms (Pasteur, 1860). However, for a long time, this research has been culture-based and therefore knowledge on the microbial ecology of the air is still limited (Bowers *et al.*, 2012). When DNA-based molecular tools became available, there was an apparent preference for the study of soil and marine environments (Simon & Daniel, 2011), whereas microbial analysis of the air, also called aerobiology, caught less research attention (Burrows *et al.*, 2009a; Maron *et al.*, 2005; Pósfai *et al.*, 2003). Some authors suggest that the lack of research in this field was a consequence of the underestimation of the importance of bioaerosols (e.g. Després *et al.*, 2012). Recently though, interest for airborne microorganisms is growing in view of the expanding awareness of global climate change (Franzetti *et al.*, 2011). New questions arise about the potential roles of the airborne microorganisms as knowledge about them is increasing. Amato and colleagues (2007a) and Vaïtilingom and colleagues (2010) found airborne bacteria are converting certain substances, therefore wondering about the impact of bacterial metabolism on the total atmospheric chemistry. Technical limitations, as described in the previous section, also significantly contribute to the fact that the field of aerobiology is lagging behind.

2.5 Airborne microorganisms: relevance

Some topics of high interest are related to the postulated roles of airborne organisms in human health, economy, ecosystems, and the weather (Bowers *et al.,* 2011b; Fierer *et al.*, 2008). Firstly, airborne pathogens can be a threat to public health. These include, for an important part, viruses such as the rhinoviruses causing a common cold (Kirkpatrick, 1996). The most commonly identified bacterial pathogens are *Streptococcus pneumoniae, Haemophilus influenza, Legionella pneumophila,* and *Moraxella catarrhalis.* They can cause upper and lower respiratory tract infections or sometimes worse and they can use airborne

transmission. For example, a person infected by *H. influenzae* spreads viable pathogens in the air by coughing and sneezing (Nicas *et al.*, 2005). This airborne transmission is an important source of secondary infections (Nicas *et al.,* 2005; Ladhani *et al.*, 2009). Other important pathogens are *Streptococcus pyogenes,* which can cause pharyngitis and tonsillitis (Cappelletty, 1998), and *Bacillus anthracis,* which have already been used for bioterrorist attacks (Takahashi *et al.*, 2004). In addition, bacteria occurring in hay that can be aerosolized, such as *Saccharopolypsora rectivirgula* and *Thermoactinomycetes vulgaris*, are involved in the pathogenesis of Farmer's lung (Dowling *et al.*, 1998; Lecours *et al.,* 2012; Pepys *et al.,* 1990). These species, as well as many others, can pose serious health risks to susceptible people.

Not only live microorganisms, but also microbial components such as endotoxins can cause unwanted immune responses. These endotoxins include the lipopolysaccharides (LPS) and lipoteichoic acid (LTA) embedded in the cell wall of Gram-negative and Gram-positive bacteria respectively. Inhalation is thought to be the major route of exposure and is associated with respiratory and systemic inflammatory responses (Liebers *et al.*, 2006; Rietschel *et al.*, 1994; Rylander & Jacobs, 1997). LPS exerts its activity by binding to Toll-like receptor 4 (TLR4), together with the LPS-binding protein (LBP), CD14, and MD2 as coreceptors. CD14 and TLR4 are present in several immune cells (including macrophages and epithelial cells). This interaction triggers a signaling cascade leading to the release of a variety of inflammatory mediators, particularly IL-1ß, TNF-α and IL-6 (Liebers *et al.,* 2008). The chromogenic limulus amoebocyte lysate (LAL) assay is the standard for detecting and quantifying LPS, however, the strength of the correlation between results of this method and the effects on human health is still under study. LTA, an important constituent of the Gram-positive bacterial cell wall, can also cause inflammation (Morath *et al.*, 2002; Schröder *et al.*, 2003), but less is known about this endotoxin.

Not only human pathogens, but also plant or animal pathogens may use airborne transmission to spread and infect other individuals (Beattie & Lindow, 1999; Pillai & Ricke, 2002). This can cause ecological or economic losses. Agricultural practices and farmers will, sooner or later, contend with airborne bacteria, pathogenic to crops or live-stock animals (Bowers *et al.*, 2011b). Besides that, airborne bacteria are playing an essential role in ecosystems: their presence (or colonization by wind transportation) or absence can

influence the balance of ecosystems. Kellog and Griffin (2006) discovered that bacteria can travel very long distances when associated with desert dust. They suggest these bacteria can have a serious impact on health and ecosystems, such as coral reefs. Shinn and colleagues (2000) already proved that the coral pathogen *Aspergillus sydowii* transported during such a dust event is causing significant damage.

Researchers proposed many more effects of airborne bacteria, such as significant physical and possibly chemical influence on the natural matter and energy cycles. Airborne bacteria, higher in the atmosphere, influence precipitation and cloud processes, because they can serve as ice nucleators or cloud condensation nuclei (Christner *et al.,* 2008; Möhler *et al.,* 2007). As explained before, an undetermined amount of bacteria is metabolically active and therefore they could be changing the chemical composition of the environment in which they are converting different chemical structures. These processes will have to be studied more closely to determine their impact (Morris *et al.*, 2008).

Besides these known effects of airborne bacteria, an interesting aspect is their potential as a tool for biomonitoring of the atmosphere. Certain parts of plants, animals, and even ecological communities are already studied to gain information on the level of local environmental pollution. Examples are polar bear health that has been studied for 30 years (Sonne *et al.,* 2012), heavy metal concentration in feathers from certain birds (Martínez *et al.,* 2012), tree leaf wettability (Kardel *et al.,* 2012), microbial communities of bryophytes in moss bags (Meyer *et al.,* 2010), and the communities of river benthos (Hajibabaei *et al.*, 2011). In a similar way, airborne microorganisms could be used as a measure for air pollution, but this is currently underexplored.

Methods for biomonitoring of environmental pollution are often divided in two categories: passive and active biomonitoring. Passive biomonitoring indicates the study of organisms present in the environment. Active biomonitoring stands for the introduction of one or more organisms into the environment and their subsequent analysis after a set period of time. It is tempting to explore the application of passive biomonitoring of the airborne bacterial community in relation to pollutant concentrations is the air, because it is known that bacterial communities adapt very quickly to their environment (Roth *et al.*, 2011). Adaptation of bacteria to the presence of pollutants is demonstrated in previous studies of

soil (Héry *et al.*, 2003; Müller *et al.*, 2001), waste flows (Stepanauskas *et al.*, 2005), and air (Ahmed *et al.*, 2000). So, heavy metal abundance at a certain location could be estimated by for example, testing for tolerance to heavy metals as previously done for soils (Díaz-Raviña & Bååth, 1996) or by looking at community composition as was done in a study by Meyer and colleagues (2010) where communities in moss bags exposed to polluted air were compared. Population dynamics of airborne bacteria could also reveal pollution problems from nearby sources (Bowers *et al.*, 2011a) and provide more information on the bio-available pollutants in a certain area. This seems to be of high interest, currently, due to a lack of methods monitoring the combined effect of (the whole range of) pollutants. Little is yet known about how the different air pollutants interact in a biological system: they could have additive, synergetic or antagonistic effects to one another (Guerreiro *et al.*, 2012).

An enormous amount of genetic and metabolic biodiversity is found in the biosphere (Vakhlu *et al.*, 2012). As in other environments, the study of airborne bacterial communities can discover new processes, new enzymes, and new genes that can become useful for biotechnological applications. These kinds of discoveries are very likely, because, for example, Bertolini and colleagues (2012) found that up to 39% of the sequences (of V5-V6 hypervariable regions of 16S rRNA genes) of their atmospheric samples were still unidentifiable and that up to 39% of the airborne bacteria they sampled were new species, which implies that many unknown genes are still out there. Airborne bacterial communities are thus not only interesting because of their importance for public health, economy and ecology, but also because the potential use of, for example degrading abilities of the airborne bacteria themselves can be very interesting for future applications.

2.6 Air pollution

An important part of microbial ecology are the interactions between the microorganisms and their environment. In this thesis, the focus lies on the relationship between communities of atmospheric bacteria and the degree of air pollution.

Almost all economic and societal activities cause emission of pollutants into the atmosphere. There are many different kinds of air pollutants and they are often exceeding air quality

standards, even though emissions in Europe already show a downward trend (Guerreiro *et al.*, 2012). This is an important problem to society, because when air-pollutant levels are too high, they cause undesirable effects, among which the most important are: damage to human health (directly through airway uptake or indirectly by pollutant deposition and accumulation in food), acidification and eutrophication of ecosystems, yield losses in agriculture, damage to forests and other plants, toxicity of metals and persistent organic pollutants in ecosystems, contribution to climate change, reduced atmospheric visibility, and damage to cultural heritage (Guerreiro *et al.*, 2012). Therefore, air quality is of major concern.

Particulate matter (PM) is an important air pollutant and a risk to public health (Dockery *et al.,* 1993; Pope *et al.,* 1991). It can be of different size, composition and origin. PM is generally divided in different fractions based on size: the coarse particulates have an aerodynamic diameter smaller than 10 µm, the fine particulates are smaller than 2.5 µm and the ultrafine particulates or nanoparticles are smaller than 0.1 µm. Their mass fractions are indicated by PM_{10}, $PM_{2.5}$, and $PM_{0.1}$ or UFP (ultrafine particles), respectively (Terzano *et al.,* 2010). The different sizes are a danger for human health in different ways. UFP's, for example, are so small they can enter the blood stream through the lungs and spread through the whole human body, causing problems in many different tissues (Terzano *et al.*, 2010). PM can also be divided in different groups based on origin or composition. Anthropogenic PM is mostly composed of a particle core of carbonaceous material, mainly from combustion processes and vehicular exhaust particles. These products of incomplete combustion are also called black carbon (BC). A variety of compounds can be absorbed on these carbonaceous cores including volatile or semi volatile organic species (e.g. polycyclic aromatic hydrocarbons (PAH's), nitro-PAH's, quinines), transition metals (iron, nickel, vanadium, copper, etc.), ions (sulfate, nitrate), and reactive gases (ozone, peroxides, aldehydes). Other components of PM include materials of biological origin (endotoxins, bacteria, viruses, animal and plant debris) and salts and minerals (ammonium, nitrate, sodium sulfate, silicates, and other crustal elements).

The relation between microbial ecology and PM in the air has not yet been widely studied (Franzetti *et al.*, 2011). Only a few studies have investigated whether, for example, metals in PM have an influence on the bacterial communities. For instance, airborne bacteria isolated

from the polluted area around Karachi (Pakistan) showed a strong inducible tolerance to heavy metals (Nuzhat *et al.*, 2000). Other interesting air pollutants to study in relation to the air microbial ecology are polycyclic aromatic hydrocarbons (PAH's). These are products of incomplete combustion, which typically reach higher levels in urban areas (Chuang *et al.*, 1999) and can be toxic or carcinogenic (Norramit *et al.*, 2005). In soil, microorganisms are already being used for years for bioremediation by conversion of PAH's to harmless molecules (Wilson & Jones, 1993). Although the atmosphere is the most important transportation means of PAH's (Juhasz & Naidu, 2000), bioremediation possibilities with airborne microorganisms, have –to the best of our knowledge- not yet been explored.

3 Materials and Methods

3.1 Sampling

Samples were taken at different sites, at different times during the period September 2012 - March 2013 with the Coriolis®µ air sampler (Bertin Technologies). On sampling days, every location was sampled one after the other. Sets of samples were taken during autumn and winter. For optimization of the sampling and DNA extraction, indoor and outdoor test samples were taken on campus.

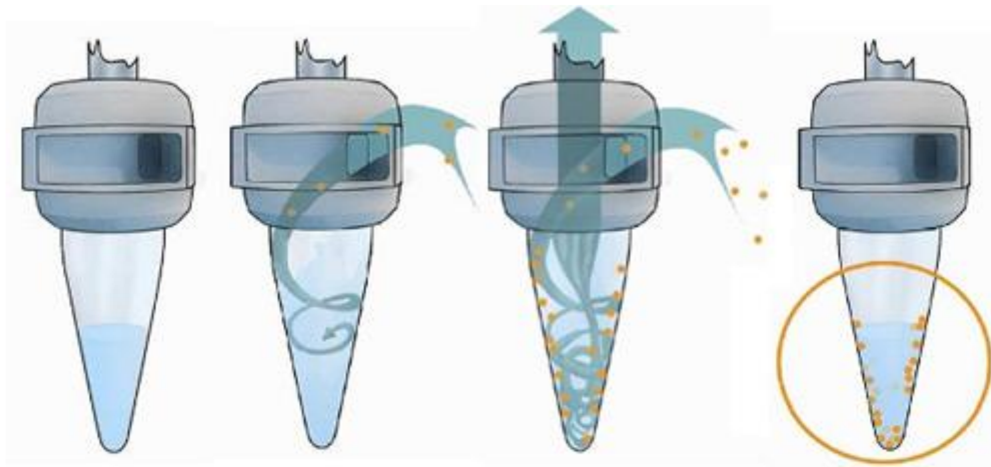

Figure 6: The principle of the Coriolis® technology for air sampling. First, the cone is filled with sterile collection liquid, secondly air is drawn and causes a vortex. Then, due to centrifugal forces, the particles from the air are suspended in the liquid. Finally, the airflow is stopped and the suspended particles are ready for further study (Bertin technologies, "Coriolis® Principle", 2013).

At each sampling site, near-surface-atmosphere bacteria were suspended in sterile collection liquid in the special sampling cones of the sampler (91-CO654-00, Peqlab) as demonstrated in Figure 6. Different collection fluids were used, i.e. the Coriolis collection liquid '15 mL sterile' (Peqlab), PBS (3.8 mM NaH_2PO_4 (Merck), 16.2 mM Na_2HPO_4 (Merck), 150 mM NaCl (VWR), pH 7.0, autoclaved), PBS containing 0,002% Tween®20 (Sigma-Aldrich), or sterile distilled water. For every run, the Coriolis®µ air sampler was set at 10 min and 300 L min^{-1}. The starting volume of the collection liquid was 15 mL. The final volume after sampling was always noted down because of evaporation. All samples were kept on ice after on site sampling. At least 5 mL of each sample was kept with 25% glycerol (Sigma-Aldrich)

after transportation to the lab and all aliquots were then stored at -80°C to preserve bacterial viability.

For some experiments (traits of bacterial culturable fraction), samples of the harbor, green suburb and city were pooled together per location and per season. Samples taken at the harbor on 7 September, 8 October, 16 October, and 5 November (stored with glycerol) were pooled together to a sample called 'Harbor autumn' (HA). The same was done for the samples of these dates taken in the green suburb and in the city (GA and CA respectively). Similarly, samples taken at the harbor on 26 November, 21 December and 8 March were pooled together to a sample called 'Harbor winter' (HW), and samples taken in the green suburb and in the city were pooled in the same way (GW and CW). All the pooled samples were cultured on R2A medium with 100 µg L^{-1} cycloheximide (Carl Roth), added to prevent fungal growth.

3.2 Bacterial cultures

3.2.1 Pure cultures

The following bacteria were used as positive control for molecular methods: *Bacillus subtilis* (LMG accession number 7135) originating from the Belgian Co-ordinated Collections of Micro-organisms (BCCM, Ghent) and *E. coli* Top10F' (Invitrogen). *B. subtilis* and *E. coli* strains were routinely grown overnight at 37°C in shaking conditions (200 rpm). Liquid medium was divided in culture tubes (5 mL) and sterilized at 121°C for 15 minutes. Growth of *E. coli* in liquid Luria Bertani (LB) (BD Biosciences) medium was monitored by measuring the optical densities (OD) of this culture at 600 nm. To grow the bacteria on solid media, LB plates containing 15 g/L agar were prepared. The medium was sterilized and poured in plastic Petri dishes (company) in the laminar flow. After drying, the plates were maintained in sterile condition at 4°C. A standard curve for estimation of the number of colony forming units (CFU) per mL based on the OD_{600nm} was calculated by plating out dilution series of a culture of which the OD_{600nm} was measured.

3.2.2 *Culturing of air samples*

From test samples, 200 µL was spread out and cultivated in duplicate onto plates with R2A medium (for 1 L, 0.5 g yeast extract (Fluka), 0.5 g peptone from casein (Merck), 0.5 g casein hydrolysate (Fluka), 0.5 g D-(+)-glucose, minimum 99.5% (Sigma), 0.5 g soluble starch (VWR), 0.3 g K_2HPO_4 (Fluka), 0.05 g $MgSO_4.7H_2O$ (Fluka)), based on the recipe of Reasoner & Geldreich (1985). This medium was sterilized and prepared as mentioned previously. To avoid fungal growth, 25 µg L^{-1} natamycin was added to the molten agar from a stock of 1 mg natamycin (Sigma) in 1 mL methanol (Merck), as previously described by Pedersen (1992). After samples were plated, the plates were incubated for 48 h at room temperature as previously described by Hyvärinen *et al.*, (1991). Hereafter, the CFU were counted and the concentration of viable culturable bacteria was calculated. The same was done for pooled samples of the locations of interest, but with volumes ranging from 300 µL to 800 µL, incubated up to 8 days. These larger volumes required that the plates were air-dried in the laminar flow before they were covered with the lid and sealed with Parafilm (Pechiney Plastic Packaging).

After 8 days not only CFU were counted, but also the number of pigmented CFU to estimate the fraction of pigmented bacteria of the whole community. To compare pigmentation of airborne bacteria with pigmentation of other communities, samples of other environments were taken. At a grass field on campus, at 1 cm depth, 40 mg of wet soil was collected and suspended in 1 mL of sterile water. After a 1/1000 dilution, 150 µL was spread out on R2A medium (in triplicate). From the tap in the lab, 2 mL tap water was collected in a sterile tube, of which 150 µL was spread on the same medium (in triplicate).

3.2.3 *Isolation of pure cultures*

Random colonies were picked from the culture plates and were brought in pure culture by standard plating. First they were spread out on a new plate to single colony, after which one colony was picked to inoculate liquid R2A medium. The liquid medium was also incubated for 48h at room temperature. Hereafter, two tubes were filled per pure culture: 0.9mL and 0.9mL to which 0.9mL of 50% glycerol was added, followed by storage at -80°C.

To keep the metal resistant bacteria in culture, the medium contained the concentration of metal that the CFU was picked from, since previous studies have shown that metal tolerance genes are often located on plasmids that can be lost during subculturing (Ji & Silver, 1995; Monchy *et al.*, 2007; Smith, 1967).

3.2.4 Metal growth assays

R2A-agar plates were enriched with different metal salts. Either cadmium sulfate, nickel sulfate, or zinc chloride were added. Concentrations of 200 µM and 400 µM, as previously described in a similar study of metal tolerance of saltern (area used for salt making) bacteria (Pereira *et al.*, 2013), while 89 nM was used for Cd. The plates were incubated at room temperature for 7-13 days. Total CFU numbers and pigmented colonies were counted. Colonies were picked, inoculated for pure culture, and stored at -80°C as described above.

3.3 Bacterial cell count by flow cytometry

Test samples were stained: one of them with SYBR Green (10.000x diluted in final volume; Invitrogen) alone for a total cell count or with a mixture of SYBR Green and propidium iodide (Invitrogen) diluted in DMSO for live/dead staining. After adding the stains, samples were vigorously vortexed and incubated in the dark at 37°C for 10 minutes. They were again carefully vortexed and placed a 24-tube rack for flow cytometry in the BD Accuri™ C6 Flow Cytometer (BD Biosciences).

The Flow Cytometer (FCM) was cleaned and tested with a standard bead solution, prepared from CytoCount (1053 beads μL^{-1}; Dako) and filtered Evian water before use. Fluorescent particles or "events" per µL of all samples were then recorded. The FL1-H filter was used to measure green fluorescent light and its threshold was set to 500. Flow speed was set at fast, because the samples were expected to have low concentrations as compared to, for example pure cultures, reaching up to 10^9 CFU mL^{-1}.

3.4 Microbial DNA extraction

After collection, the sample volumes were centrifuged for 20 minutes at 8500 g, at 4°C to obtain pellets, which contained the suspended particles and bacteria. Different protocols and DNA extraction kits were tested on pellets of the test samples to optimize genomic DNA extraction from air samples (Appendix IV).

For optimization of DNA extraction, many protocols and commercial kits were tested, including the DNeasy blood & tissue kit (Qiagen), RTP®Bacteria DNA mini kit (Invitek), PowerSoil® DNA Isolation Kit (MO BIO), a beadbeating DNA-extraction protocol according to Maron and colleagues (2005), and a protocol using cetyl trimethylammonium bromide (CTAB) based on those of Griffiths and colleagues (2000) and Kowalchuk and colleagues (1998). The CTAB-based protocol is described below. After centrifugation for 10 minutes at 5000 g at 4°C of 10 mL of sample, 0.5 g glass beads (0.5 mm, Sigma-Aldrich) were added. Hereto, 0.5mL CTAB buffer (120 mM K_2HPO_4, 0.35 M NaCl, adjusted to pH 8 with NaOH, 5% (w/v) hexadecyltrimethylammonium bromide was added after autoclaving) and 0.5 mL phenol:chloroform:isoamlalcohol (25:24:1) were pipetted and the tubes were vortexed. A beadbeater (type 853022/0, B. Braun Biotechnology International) in a 4°C room mechanically shaked the tubes 3 times 30 seconds, with 10 s break to avoid excessive heating of the contents of the tubes. After centrifugation for 5 minutes at 3000 g, 300 µL supernatant of every tube was brought in a new tube. Again, 0.5 mL of CTAB buffer was added to the original tubes and the beadbeater was used as before. The tubes were centrifuged for 5 minutes at 3000 g and 300 µL of the supernatant of these tubes was added to the tubes that already contained 300 µL supernatant of the previous round. The phenol was removed from the eppendorf tubes by adding an equal volume (600 µL) of chloroform:isoamylaclohol (24:1) to every tube. Tubes were turned to homogenize and centrifuged for 10s. The water phase (top phase) of every tube was transferred to a new tube with 2 volumes of a PEG-6000 precipitation solution (30% (w/v) polyethylene glycol, 1.6 M NaCl, autoclaved). An incubation of 2 hours at room temperature followed to precipitate nucleic acids. Hereafter, the tubes were centrifuged for 10 minutes at 18000 g, the supernatant was discarded and the pellet washed with 1 mL ice-cold ethanol (70%). The tubes were centrifuged again for 10 minutes at 18000 g and the pellets were dried in the

DNA Speed Vac® (Savant). The pellets were resuspended in 100 µL PCR grade water (Sigma) and stored at -20°C.

Due to the postulated high proportion of gram-positive sporulated bacteria in air samples (Lighthart, 1997), some samples underwent a preliminary step of sample incubation before the DNA extractions (Appendix IV) to germinate the spores, to enhance the lysis efficiency, and increase the DNA recovery (Maron *et al.*, 2005). For this step, one milliliter of sample was mixed with 110 µL of TSB 10× and incubated at 37°C for 30 min as previously described (Maron *et al.*, 2005). Subsequently, DNA was extracted from the cells as described above.

3.5 DNA concentration and purity determination

DNA concentrations were measured spectrophotometrically using the NanoDrop™ 1000 Spectrophotometer v3.7 (Thermo Scientific). Absorbance ratios of absorbance at 260 nm and 280 nm, and at 260 nm and 230 nm were an indication for the purity of the DNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm, such as EDTA, carbohydrates and phenol.

3.6 DNA purification

Two different protocols were used for DNA purification of the isolated samples: either by using ethanol precipitation or by using the Wizard® DNA Clean-Up System (Promega) according to the manufacturer's instructions. For the former, twice the sample volume of ice cold 96-100% ethanol was added, followed by incubation at -20°C overnight and centrifugation for 15 minutes at 21460g and 4°C. The pellet was washed with one sample volume of ice cold 70% ethanol, and centrifuged again at full speed for 15 min at 4°C. The main volume of supernatant was pipetted off. Finally, the pellet was dried and resuspendend in a final volume of 30 µL TE buffer (10 mM Tris, 1 mM EDTA).

3.7 DNA gel electrophoresis

To confirm the DNA content and DNA integrity of the samples, 50 mL agarose gels (1% agarose, 1x TBE buffer (Merck) were made, containing 6 µL GelRed™ Nucleic Acid Gel Stain, 10,000X in Water (Biotium)). These gels were loaded in the outermost lanes with GeneRuler™ 1kb Plus DNA Ladder (Fermentas). DNA samples were loaded with 1 µL 6x Loading Dye (Fermentas) and generally 5 µL sample per lane. For 30 minutes, 100V was applied. Finally, the gel was exposed to UV light and photographed.

3.8 Polymerase chain reactions (PCR)

A PCR was executed on several DNA extractions for quality control and further analysis. Hereto, 1 µL of sample was added to the mastermix (High Fidelity PCR Master (Roche), a set of primers, and PCR grade water (Roche) in a final volume of 25 µL. In addition, negative controls were included to detect possible contamination of the mastermix and cross-contamination.

3.8.1 PCR for sequencing of the 16S rRNA gene

To identify the single colonies isolated from air samples, universal bacterial primers for the 16S rRNA gene were used: 8F (5'- AGA GTT TGA TCC TGG CTC AG -3') and 1525R (5'- AAG GAG GTG ATC CAG CCG CA -3') (Weisburg *et al.*, 1991). The end concentrations were 0.5 µM of each primer.

Different templates were used for these PCRs. For metagenomic samples, 1 µL was added to obtain a final reaction volume of 25 µL for every sample. In addition, colony PCR was performed on single colonies. Hereto, ca. 0.5 mm^2 of one colony was collected with a sterile tip and put in a PCR tube containing PCR grade water, followed by incubation in a microwave oven for 2 minutes at full power. Hereafter, the mastermix (without water) was added to obtain the correct concentrations and volume.

The PCR program started with 94°C for 5 min, followed by 30 cycles: denaturing at 94°C for 30 seconds, annealing at 54°C for 45 seconds, and extension at 72°C for 1 min 45 seconds.

PCR products showing good bands were sent for single read Sanger sequencing with both the 8F and 1525R primer to GATC Biotech (via CMPG, KU Leuven). Complete overlapping of complementary sequences, editing and consensus construction was performed using Geneious Pro v5.4.4 (Drummond *et al.*, 2011). Analyzed sequences were compared with known sequences in the GenBank Database using a nucleotide-nucleotide BLAST analysis tool (Altschul *et al.*, 1990).

3.8.2 PCR for DGGE

For DGGE analyses, other universal bacterial primers for the 16S rRNA gene were used: 338F (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG -3'), that has a GC clamp on the 5' side (to use the PCR products for DGGE) and 518R (5'- ATT ACC GCG GCT GCT GG -3') (Muyzer *et al.*, 1993). The final concentrations of the components in the mastermix were: 0.2 µM of each primer, 200 µM of each deoxynucleoside triphsphate (Promega), 1.5 mM $MgCl_2$ (Promega), 1x Taq DNA Polymerase (Promega), 10x Reaction Buffer ($MgCl_2$-free) (Promega), 1.25 U/50 µL of Taq DNA Polymerase (Promega), 400 ng μL^{-1} of bovine albumin (Hoffmann-La Roche), and DNase and RNase free filter sterilized water (Sigma-Aldrich). The PCR program consisted of a 5 minutes hot start of 94°C followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 53°C for 1 minute, and extension at 72°C for 2 minutes, where after the products were incubated at 72°C for 10 minutes and finally at 4°C until removal from the PCR machine.

The products were loaded on an agarose gel as described above with slight modifications, using 2 µL ethidium bromide (Merck) and using MassRuler™ DNA ladder mix (Fermentas). Gels were run at 100V current for 25 minutes, after which a picture of the gel was taken.

3.8.3 PCR for V6 metagenomic sequencing with HiSeq

The hypervariable region V6 of the 16S rRNA gene was amplified with the primers 967F (5'- CAA CGC GAA GAA CCT TAC C -3') and 1046R (5'- CGA CAG CCA TGC ANC ACC T -3') (Sogin *et al.*, 2006). Their concentration was 0.2 µM each and the annealing temperature was optimized using a gradient PCR. The PCR program started with 94°C for 2 minutes, followed

by 30 cycles of denaturing at 94°C for 15s, annealing at 53°C for 30s, extension at 72°C for 20s, and finally one step of 72°C for 5 minutes. Gels (1.5% agarose) were loaded as described above.

3.9 Air pollution data and weather factors

Temperature, wind direction, wind speed, relative humidity, and precipitation at sampling times were added as additional information in the Appendices. They were measured at the weather station Deurne and collected from http://freemeteo.com/.

Data about the levels of certain pollutants at time and location of sampling were obtained from the VMM website: http://luchtkwaliteit.vmm.be/.

The content of heavy metals of the airborne samples was determined by inductively coupled plasma mass spectrometry (ICP-MS) by the department of Prof. K. De Wael (UA), but results were not submitted before the deadline of this thesis.

3.10 Statistical Methods

Results are given as means with standard error (S.E.). The open-source statistical environment R (www.r-project.org) was used. P values smaller than 0.05 were considered to indicate a significant outcome.

4 Results

During this master thesis, various atmospheric samples suitable for microbiological analyses were taken with the Coriolis®µ air sampler at different locations harboring different levels of certain pollutants. In the lab, the bacteria in the samples were analyzed using culture-dependent and molecular techniques. Various functional properties of the culturable fraction, such as pigmentation as a proxy for adaptation to UV radiation, and tolerance to the heavy metals cadmium, nickel, and zinc were assessed. Samples that were taken at (near-) freezing temperatures were used to culture cold-resistant bacteria. Various isolated colonies from these cultures were identified by 16S rRNA gene sequencing. Another aliquot of the samples was used for DNA extraction with the aim of subsequent analysis of the whole community DNA by using the denaturing gradient gel electrophoresis (DGGE) fingerprint technique and metagenomic sequencing. To evaluate the various assays, test samples were taken on campus. Once these techniques were validated, samples taken at the points of interest were processed.

4.1 Study area

Air samples were mainly taken at three different locations in and near Antwerp: in Borgerhout (in the city), in Kapellen (a greener suburb), and at the harbor (industrial area) (Figure 7: **Sampling sites. Pictures were taken in the harbor of Antwerp, where a lot of industry is located (A), the greener suburb, Kapellen (B), and in the city, where, daily, a significant amount of traffic passes through (C).**). Samples taken in the city were within a 150m range of the VMM station “Antwerpen (Borgerhout) - Plantin en Moretuslei (straatkant)”, situated 51°12’34.019”N and 4°25’53.94”E. At the harbor, samples were taken within a 100 m range of the VMM station “Antwerpen –Boudewijnsluis”, situated 51° 16' 51.326"N and 4° 19' 47.863"E. The samples from the greener area were taken in a 100 m range of the VMM station “Kapellen – Fortsteenweg”, situated 51° 19' 13.490"N and 4° 26' 41.186"E. These locations were chosen based on their different air quality index that was calculated with measurement data from the Vlaamse Milieu Maatschappij (VMM), for their different results of microbial endotoxin levels (Brouwers *et al.*, 2012) and based on ongoing measurements of metal concentrations in PM using saturation isothermal remanent magnetization (SIRM) of leaves at the University

of Antwerp (Samson *et al.*, unpublished results). Information of the samples taken at the three different locations can be found in Appendix II and pollution data can be found in Appendix VI.

Figure 7: Sampling sites. Pictures were taken in the harbor of Antwerp, where a lot of industry is located (A), the greener suburb, Kapellen (B), and in the city, where, daily, a significant amount of traffic passes through (C).

Pollutants PM_{10} and BC were estimated for these samples where possible by using VMM data. Table 1 shows that the locations harbor, green suburb, and city were polluted to a significant different degree. Other factors such as temperature, wind direction, wind speed, relative humidity, and precipitation at the time of sampling can be found in Appendix.

Table 1: PM_{10}, black carbon, and total pollution score determined by VMM for every location. Only data measured at time of sampling were used.

	PM_{10} (µg m^{-3})	BC (µg m^{-3})	VMM score
Harbor	28 ± 4[a]	1.9 ± 0.4[a]	3.1 ± 0.3[a]
Green	NA	NA	1 ± 0[b]
City	32 ± 5[a]	4.8 ± 0.8[b]	4.1 ± 0.4[c]

Different letters indicate significant difference in one column.
NA: VMM did not measure this pollutant at this location

4.2 Determination of the CFU per m^3 air of various air samples

4.2.1 CFU per m^3 air of test samples

Various test samples were taken around the Campus Groenenborger area (Antwerp) for the optimization of several methods. Their details can be found in Appendix I. Aliquots were plated out on R2A agar in duplicate, as described in the Materials and Methods (M&M), to estimate the number of culturable CFU per m^3 and to assess the effect of some collection and storage circumstances. Test samples 1 to 4 were used to examine whether freezing bacteria with 25% glycerol, double sampling time, or different collection liquids have an effect on CFU concentrations (Figure 8). This test illustrated that the culturable range varies from approximately 600 to 2000 CFU m3 under the conditions tested.

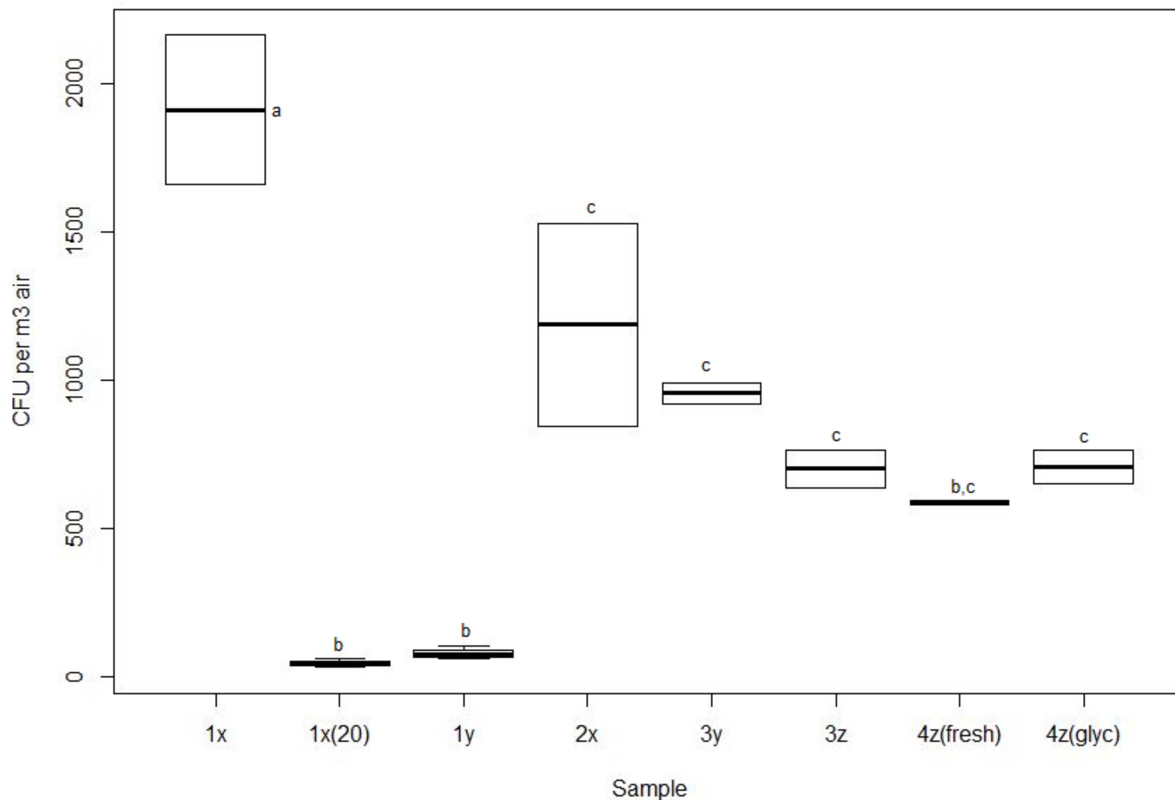

Figure 8: The CFU concentrations per test sample. The numbers of the samples indicate different sampling days and locations: all samples were taken indoors, in the lab, with exception of sample 2x that represents outdoor air. For collection liquid "x" refers to water, "y" to PBS and "z" to coriolis liquid. 4z(fresh) was not stored, but directly spread out on the medium. 4z(glyc) originated from the same sample, but it was frozen to -80°C with glycerol and thawed again before spreading on the medium. Counts of 1x(20) and 1y were too low (<25 CFU) to calculate correct concentrations. Letters "a", "b", and "c" in the graph indicate whether a significant difference exists.

Importantly, CFU m^{-3} air concentrations between the sample frozen with 25% glycerol (4z(glyc)) and of the fresh sample (4z(fresh)) did not show significant differences, while samples frozen without glycerol showed random and unexpected variability (1x, 1x(20), 1y, 2x, 3y, and 3z). This variability can be seen on the plates 1x(20) and 1y in comparison with plates 1x, all stored without glycerol. The CFU of plates 1x(20) and 1y were lower than 25, which is generally taken as the lower limit for plate counts. The CFU m^{-3} air in these samples was therefore lower than 200 CFU m^{-3}. This is significantly lower than that of plates 1x, which was taken at the same time and place. This preliminarily suggests glycerol to be a necessary and suitable cryoprotectant to preserve viability of culturable airborne bacteria.

The cultures of 8y and 8z (taken outdoors, on campus, during November) showed considerable fungal growth and a very low bacterial CFU count per plate (<25) after 7 days. Therefore, in future samples, antifungal compounds were added to the medium as described in M&M.

Figure 9: Culture of test sample 8y showing considerable fungal growth. A volume of 150 µL of collection liquid, used for sampling, was spread out on R2A medium. This picture was taken after 7 days of incubation at room temperature. Fungi had already started to overgrow bacterial colonies.

4.2.2 CFU per m^3 air for the samples from the study area

The means of the CFU concentrations at the harbor, in the city, and in the green suburb were calculated and compared (Figure 10). City air showed a significantly higher mean of 900 CFU per m^3 of air. The harbor air (350 CFU per m^3 of air) and suburb air (360 CFU per m^3 of air) have similar CFU concentrations. In addition, for every location the effect of season (winter or autumn) was assessed, but no significant influence of season could be detected.

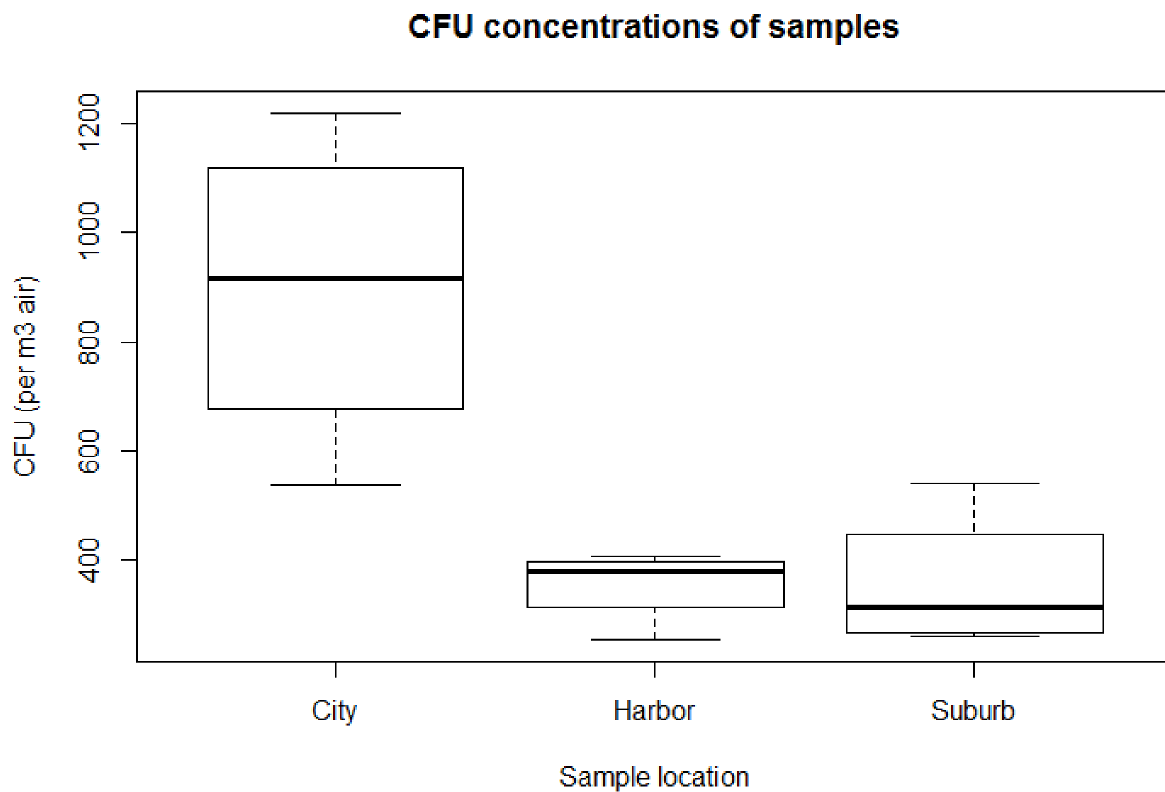

Figure 10: The average CFU concentrations compared between sampling locations. City air showed a significant higher mean of 900 CFU per m^3 of air. The harbor air (350 CFU per m^3 of air) and suburb air (360 CFU per m^3 of air) have similar CFU concentrations.

4.2.3 Determination of the total cell count per m^3 air by FCM

In addition to the determination of the culturable fraction described above, total cell counts of the samples from the three locations of interest were performed by FCM in order to estimate the number of particles containing DNA, i.e. the total biological fraction. This was accomplished by staining the samples with SYBR Green before being run through the FCM.

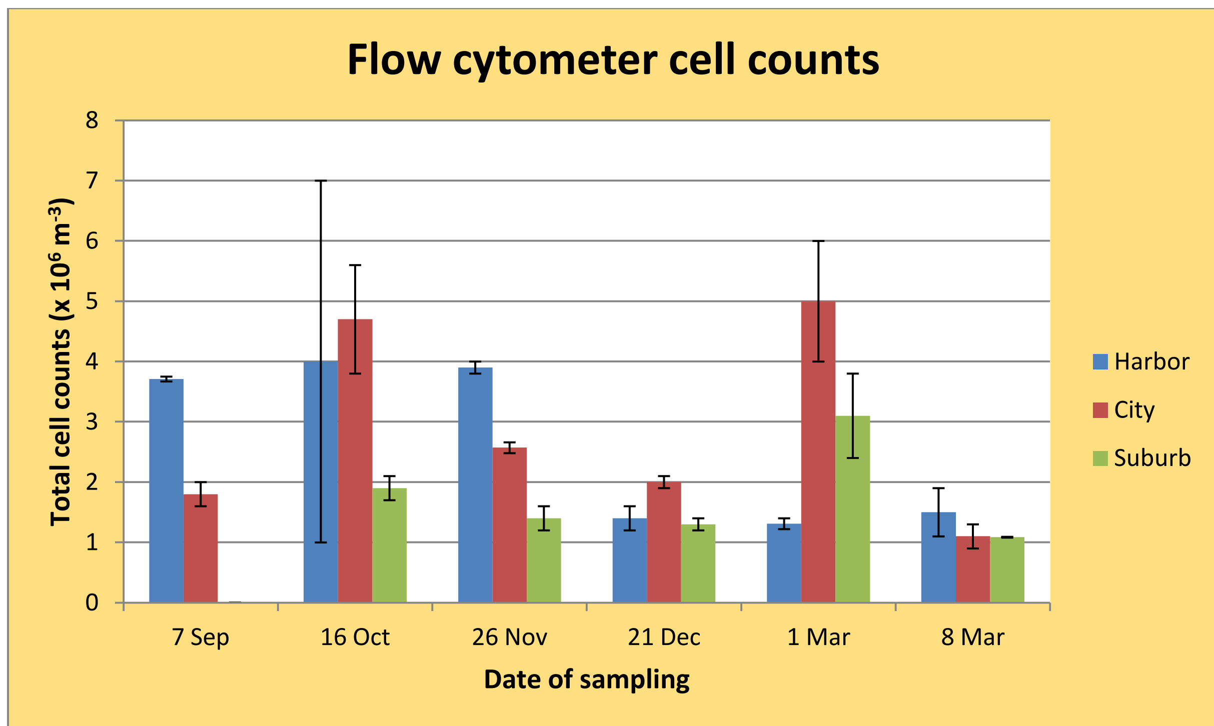

Figure 11: The total cell concentrations of the samples as measured by the flow cytometer. The original data can be found in Appendix III.

As can be seen in Figure 11, the FCM measurements indicate the DNA-containing particles in the air ranged between 9 x 10^5 and 7 x 10^6 per m^3 air. No direct trends are visible on this graph. However, FCM not only includes bacteria, but also other biological particles such as fungi. In order to relate the bacterial culturability with the total cell count of the FCM, plate counts without fungal growth inhibitor were used to estimate bacteria:fungi ratios. Samples in autumn (of suburb and harbor) had a bacterial percentage that was decreased down to 12%. Winter samples showed bacterial percentages of 65 to 100% (of suburb). With these percentages, a crude range of culturablility was calculated (Table 2).

Table 2: The culturable fraction of every pooled sample was determined using plate count and flow cytometry results.

Sample	Particles (x 10^6 per m^3 air)	CFU per m^3 air	Culturablility in %
HA	3.8 ± 0.9	400 ± 10	0.008 - 0.11
HW	2.3 ± 0.5	310 ± 80	0.009 - 0.029
GA	1.5 ± 0.4	267 ± 9	0.013 - 0.187
GW	1.27 ± 0.09	400 ± 100	0.024 - 0.071
CA	3.1 ± 0.7	700 ± 200	0.014 - 0.254
CW	5 ± 3	1100 ± 100	0.047 - 0.109

4.3 Specific functional properties of the culturable airborne bacteria

4.3.1 Slow growth

The CFU counts mentioned above were generally determined after 8 days of growth on R2A agar. However, it was observed that some colonies of the bacterial communities showed continued growth, as shown in Figure 12.

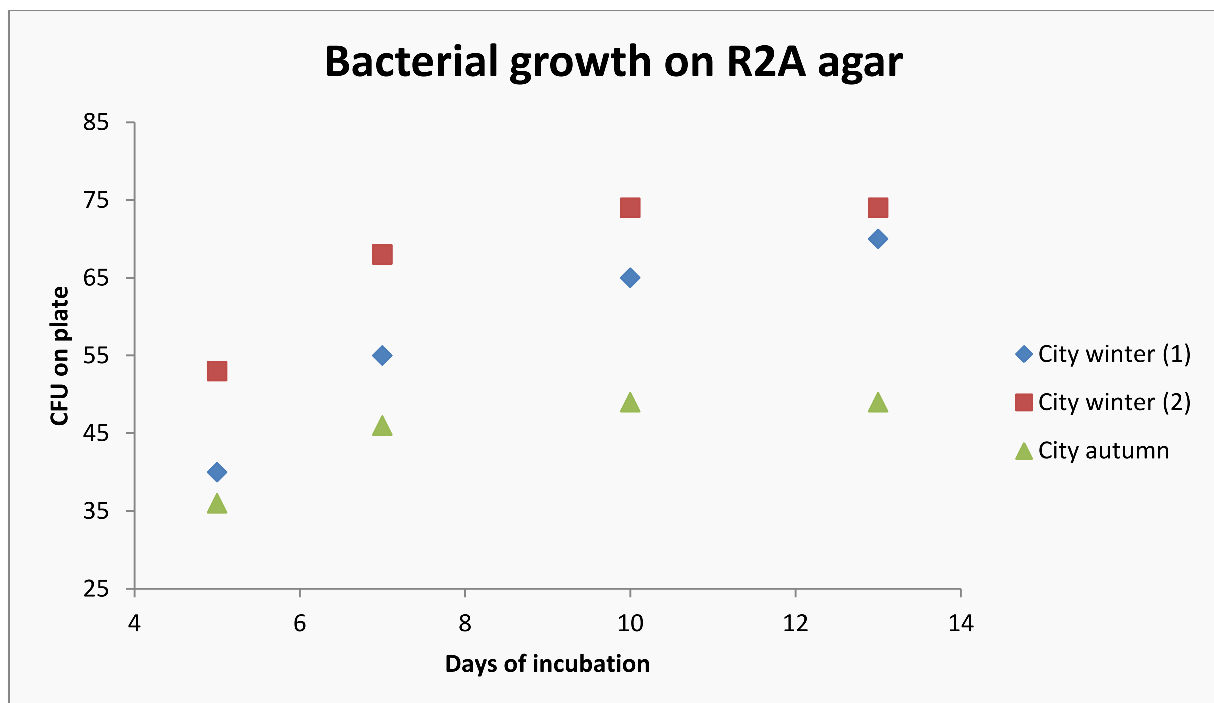

Figure 12: The evolution of CFU counts starting from day 5 of incubation at room temperature. In this graph examples were shown of pooled samples taken in the city. Counts after 8 days of incubation were used for CFU concentration calculation.

4.3.2 Growth at 4°C

During storage of the plated aliquots of the airborne samples in the fridge at 4°C, it was serendipitously noted that a fraction of the bacteria continued to grow (Figure 13). Approximately 60% of the CFU (from cold-air samples, see 'Freeze tolerance') had appeared after longterm storage at 4°C.

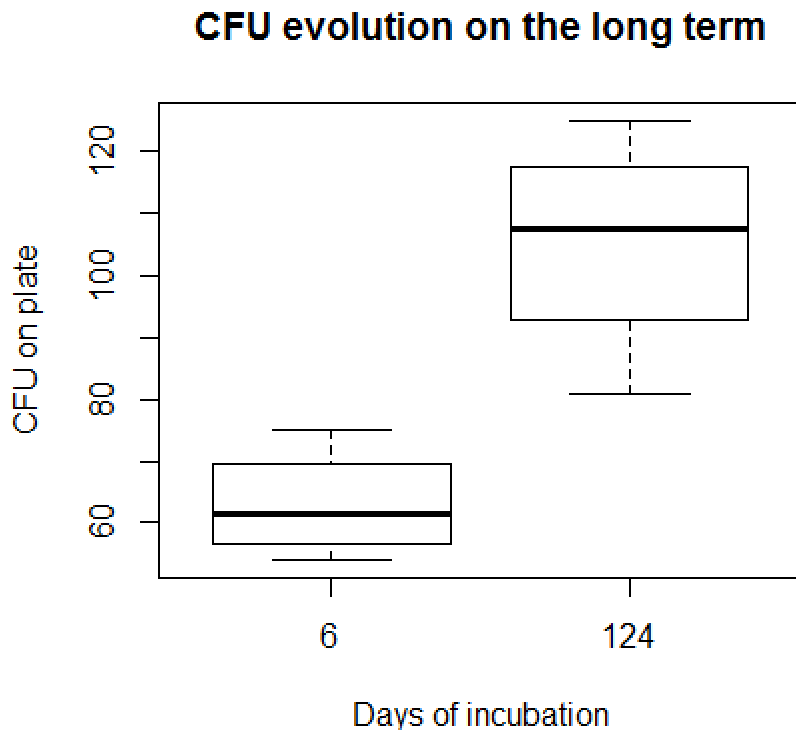

Figure 13: Many of the airborne CFU grew at 4°C. After 6 days of incubation at room temperature and CFU counts, plates were stored in the fridge. CFU were counted again after 118 days of storage in the fridge.

4.3.3 Freeze tolerance

During sampling collection in January, it was noted that the collection liquid froze after two minutes. Despite this, when these samples were plated, a culturable fraction of (1.04 ± 0.08) x 10^2 CFU m^{-3} of air for the green suburb and (7.1 ± 0.3) x 10 CFU m^{-3} for samples from the harbor could still be detected. This suggests that freeze-tolerant bacteria were present in

these samples. Several single colonies were isolated and identified via 16S rRNA gene PCR amplification (Figure 14) and sequencing (Table3).

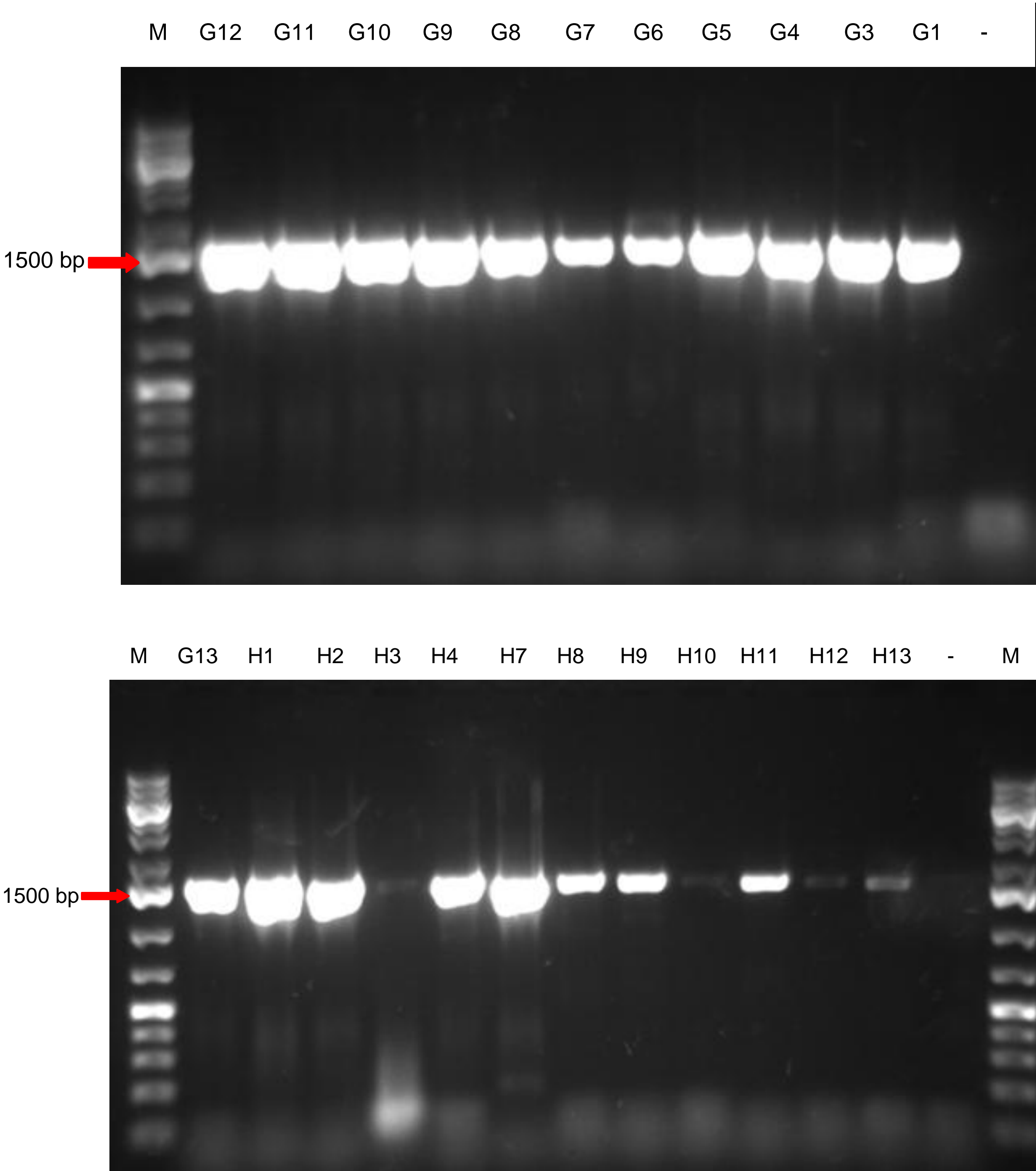

Figure 14: A 1% (w/v) gel showing the PCR products produced using universal bacterial primer pair 8F and 1525R to amplify a ~1500 bp region of the 16S rRNA gene. PCR products of the various colonies from the corresponding air samples from the green suburb (G) and harbour (H) are indicated

Table 3: Sanger sequencing identification of some freezing tolerant bacteria.

Genus	Possible spp.	Phylum	Colony characteristics
Paenibacillus		*Firmicutes*	White
Sphingomonas		*α- Proteobacteria*	Brown
Microbacterium	*phyllospaerae, foliorum*, or *oxydans*	*Actinobacteria*	Fungus like, white centre, green edge

4.3.4 Pigmentation

When counting CFU of the airborne communities, the number of pigmented colonies (yellow, orange, red, pink, and green) was also counted to obtain an estimation of the fraction of pigmented bacteria. For comparison, samples of the soil and of the tap water were taken and cultured on the same medium. These data showed that a significant fraction of the airborne bacteria is pigmented (Figure 15).

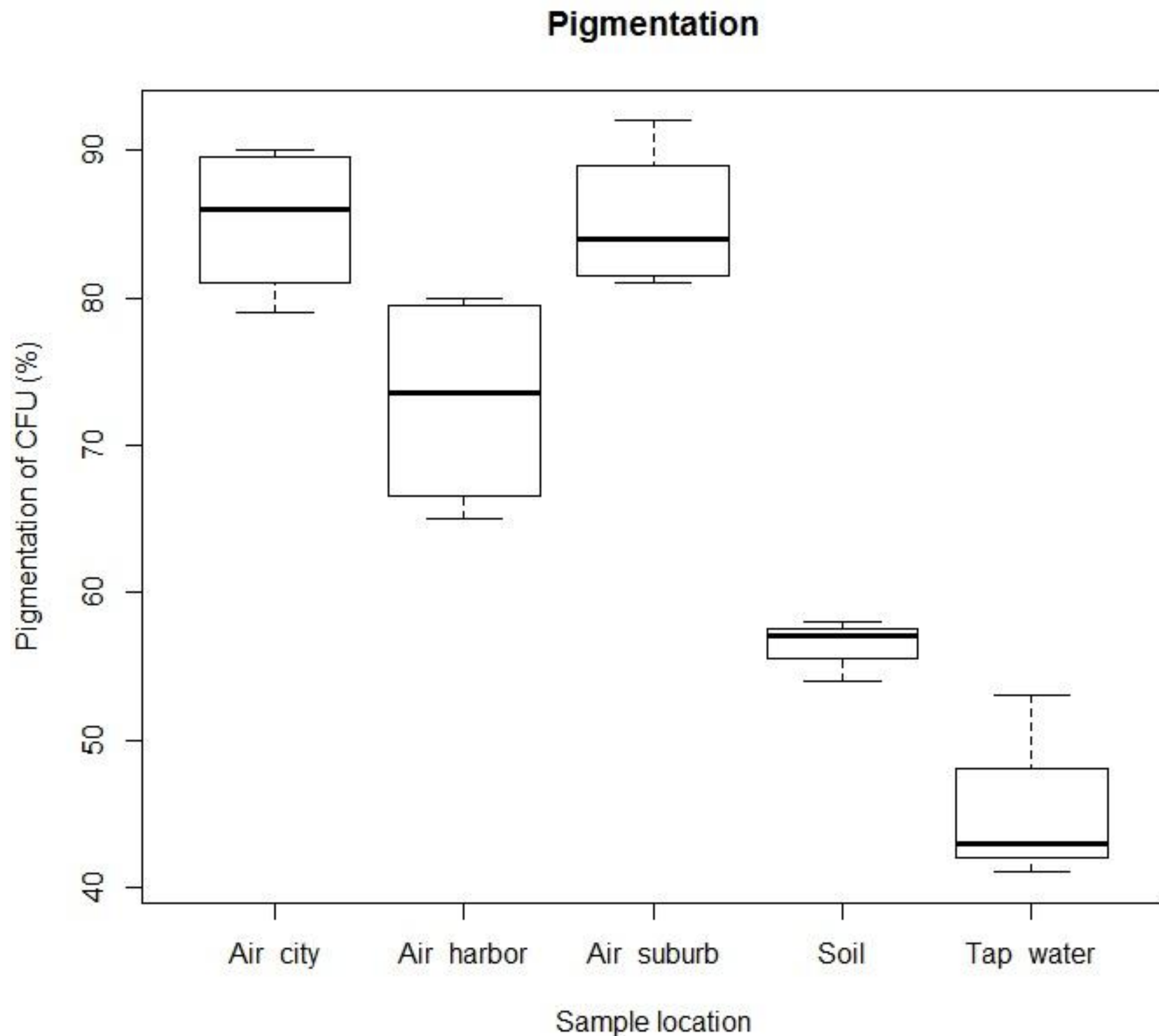

Figure 15: Pigmentation fractions of different bacterial CFU communities. The airborne samples showed a significantly higher pigmented fraction of bacteria compared to the soil and tap water sample.

4.3.5 Metal tolerance

R2A plates with and without metals were prepared for comparative analysis as a proxy for the number of metal tolerant bacteria in the air. The correlation coefficients between concentration (of zinc and nickel separately) and CFU per m^3 air were calculated (results not shown), but no significant differences between locations or seasons were seen. In addition, the resistance of the communities to cadmium, zinc and nickel was assessed. This was done by calculating a survival percentage for every sample. Hereto, the CFU of the metal enriched plates was divided by the average of the CFU on the control plates. The results indicate that

the harbor CFU community is more resistant to cadmium than that of the city (Figure 16). Nickel tolerance appears to be slightly higher in the green suburb than in the harbor, while zinc tolerance appeared to be lower in the harbor.

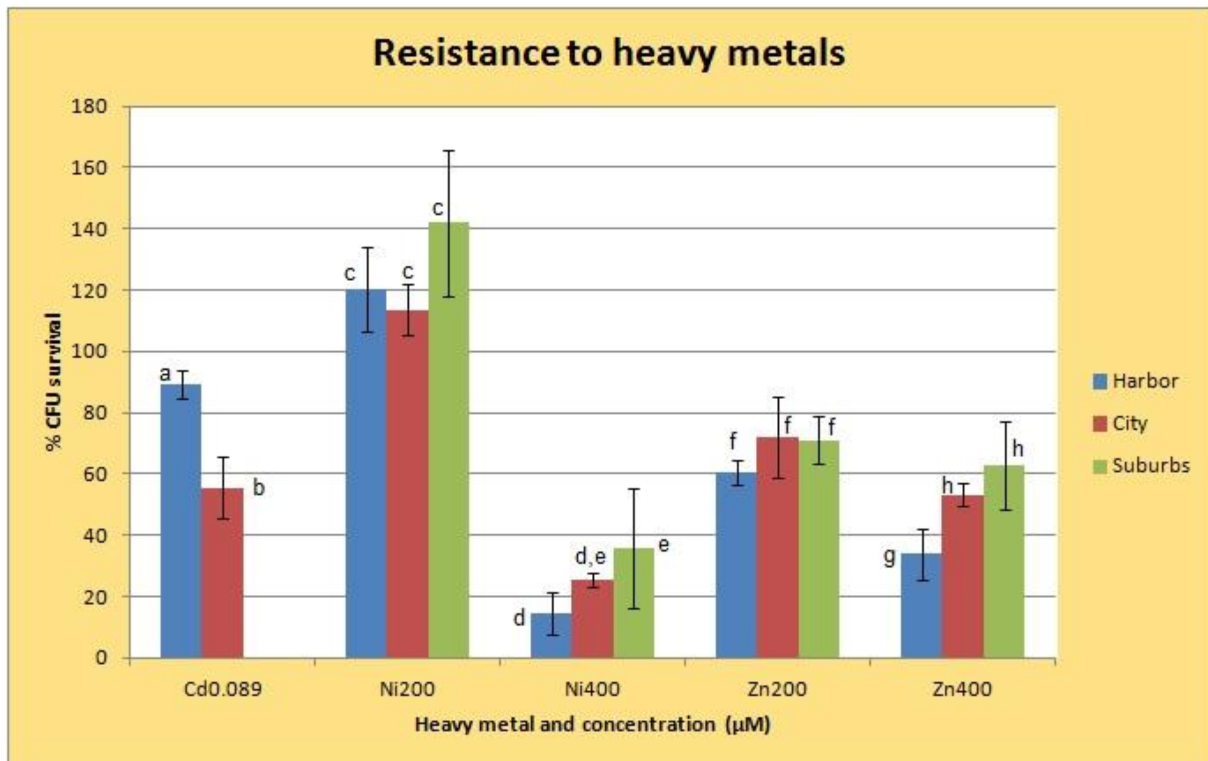

Figure 16: Boxplot of the cadmium, nickel, and zinc resistance of CFU at the harbor, city and green suburb. Letters indicate significant differences within one metal-concentration combination. Survival of more than 100% occurred by counting more CFU on metal enriched plates. The error flags indicate this could be coincidental.

Selected colonies were isolated and identified via 16S rRNA gene sequencing after PCR. Through Sanger sequencing, the genera of selected culturable bacteria resistant to nickel and cadmium were identified (Table 4).

Table 4: Sanger sequencing identification of some heavy metal tolerant bacteria. The column metal indicates on what metal enriched plates they were found.

Genus	Possible spp.	Phylum	Possible source	Metal	Colony characteristics
Arthrobacter		*Actinobacteria*	Common Soil	400 nM Ni	White
Sphingomonas	*aerolata* or *aurantiaca*	*α-Proteobacteria*	Various	400 nM Ni	Orange
Sphingomonas				400 nM Ni	
Microbacterium	*phyllospaerae* or *foliorum*	*Actinobacteria*	Grass	89 nM Cd	Green-yellow, liquidy
Herbaspirillum		*β-Proteobacteria*	Grass roots	89 nM Cd	Light orange

To correlate the metal tolerance capacity of the airborne bacteria with the metals present in the air, the air samples under study were also sent for ICP-MS analysis (Prof. K. De Wael). However, at the time of redaction of this master thesis, no data was available yet.

4.4 DNA-based analysis of airborne samples: optimization with test samples

4.4.1 DNA extraction

To obtain a culture-independent view of the airborne bacterial communities, DNA-based methods were investigated. Hereto, an efficient protocol for total DNA extraction is important. Various protocols for DNA extraction were tested during this master thesis by using test samples (Table 5). With the increased resistance to lysis of Gram-positive cell walls, the use of an extra lysis buffer or extra lysis step was implemented. In addition, low bacterial density and significant levels of pollutants capable of interfering with downstream analyses were important factors to consider.

Table 5: Nanodrop measurements of DNA extractions. A '~**0**' replaces small minus values for concentrations.

DNA extract	DNA extraction	Sample	Origin	Concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
no. 1	DNeasy B&T: normal protocol + purification	2o	CGB outdoors	~0	1.47	0.74
		1y	Lab air	5.5	1.25	0.58
		1x(20)	Lab air	~0	1.6	0.44
		1x	Lab air	~0	1.57	0.61
no. 2	DNeasy B&T: normal protocol + purification	3y	Lab air	3	1.34	0.7
		3z	Lab air	1.4	1.42	~0
		Pos	Tap H_2O	1.2	2.71	0.51
		Neg	Sterile H_2O	0.3	~0	~0
no. 3	CTAB method + purification	1x	Lab air	202	1.39	1.28
		2x	Lab air	280.9	1.31	1.4
		Pos	*B. subtilis*	520.1	1.36	1.56
		Neg	Sterile H_2O	212.1	1.3	1.21
no. 5	DNeasy B&T: for Gram-positive	1x	Lab air	2.6	1.11	1.47
		5i	Lab air	3.2	1.35	0.64
		Pos	*B. subtilis*	2.6	1.57	0.64
no. 5 (purified)	DNeasy B&T: for Gram-positive + purification	1x	Lab air	2.6	1.12	0.71
		5i	Lab air	4.3	1.13	0.5
		Pos	*B. subtilis*	10.4	1.4	0.54
no. 8	PowerSoil: for wet samples	7ig	Lab air	7.1	1.92	0.53
		7o	CGB outdoors	8.6	1.72	0.59
		7og	CGB outdoors	8	2.04	0.11
		Pos	Colony	6.3	2.17	0.39
no. 9	DNeasy B&T: for Gram-positive	9o	CGB outdoors	3.5	1.09	0.98
		Pos	Colony	11	1.62	1.43
no. 10	Beadbeating (Maron *et al.*, 2005) + purification	9o	CGB outdoors	10.3	1.02	1
		Pos	Colony	18.3	1.6	0.36
no. 11	RTP®Bacteria kit: for large volume water sample, Gram-positive	10o	CGB outdoors	66.5	2.97	0.44
		10oS	CGB outdoors	87.9	3.09	0.54
		Pos	Colony	54	3.11	0.75

Location "Lab" refers to the Molecular and Microbial laboratory of the University of Antwerp (department of Bioscience Engineering, room V.535)
"CGB outdoors" refers to the outdoor air at campus Groenenborger of the University of Antwerp.
Sample 10oS was extracted from the same sample as 10o but a spike (same as positive control) was added prior to extraction.

The concentrations of the DNA extractions were often low, ranging from 0 to 10 ng μL^{-1}, except for those of DNA extraction no. 3, ranging from 202 – 520.1 ng μL^{-1} (Table 5). These DNA extracts were however not suitable for nanodrop measurement due to absorbing contaminants left behind by this method (CTAB). In addition, initial experiments showed that

a higher amount of sample volume was needed to obtain results. Therefore in subsequent experiments, DNA was extracted from samples that represented approximately 8 times more air volume (corresponding to approximately 9 m^3 of outdoor air) than for previous DNA extractions. With the CTAB method excluded, DNA extraction using the RTP®Bacteria mini kit yielded the highest concentration of DNA.

The $A_{260/280}$ values, which is the ratio of the absorbance at 260nm and 280 nm, of the DNA extract showed to be lower than 1.8, while the $A_{260/230}$ values were lower than 2.0 for most samples. These absorbance ratios therefore indicate considerable amounts of contaminants, which may also have interfered with concentration measurements. To improve the purity of DNA, ethanol washes were carried out. However, as is shown for sample 5, this had little effect (Table 5). These results therefore suggest that further optimization of DNA purification and extraction is needed.

4.4.2 PCR of DNA extracts

Subsequently, PCR was performed on the DNA extracts, using universal bacterial 16S rRNA gene primers (8F and 1525R). One sample was spiked with the positive control to assess possible PCR inhibition by the components of the air samples. In addition, four colonies of sample 8y and 8z cultures were picked up and used for testing colony PCR (Figure 17).

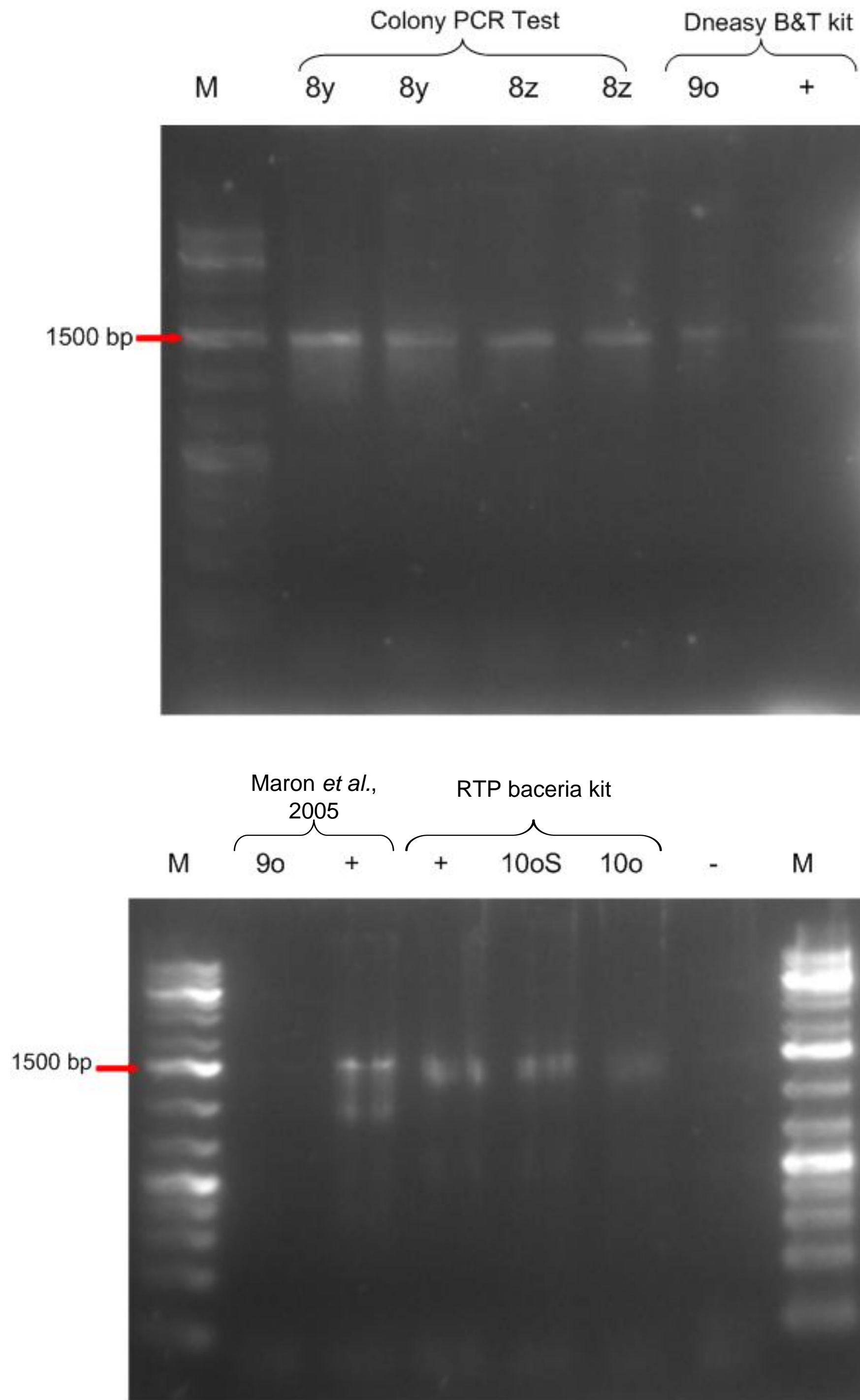

Figure 17: A 1% gel (w/v) of the 16S rRNA gene PCR products. DNA extraction methods and samples are indicated. Lanes M represents the molecular marker (GeneRuler™ 1kb plus), while the positive controls are indicative of corresponding colony PCRs. The negative PCR control (-) consisted of master mix. All bands are at the expected height of approximately 1500 bp.

The results indicate that it was possible to obtain bacterial DNA from outdoor samples since PCR products were obtained with the universal bacterial 16S rRNA gene primers, 8F and

1525R (Figure 17). The RTP®Bacteria mini kit not only yielded the highest DNA concentrations as indicated by the nanodrop readings (Table 5), but it also yielded better PCR results than the DNA extraction of Maron and colleagues (2005) and the DNeasy Blood & Tissue kit (Figure 17). However, since the yield of PCR product was rather low, it was difficult to observe whether PCR inhibitors were present in the DNA extracts.

4.5 DNA based analyses of airborne samples of the three locations of interest

4.5.1 DNA extraction

The RTP® bacteria mini kit (Invitek) was used to extract total DNA from the air samples of the three locations within Antwerp with the varying degrees of air pollution as described previously. In Table 6, the results from the DNA extractions are shown.

Table 6: Nanodrop results of the DNA extractions (RTP® bacteria mini kit, Invitek) of the samples. Date and location of respective samples are given, as is the actual volume of air from which DNA was extracted.

Date	Location	Concentration (ng µL^{-1})	$A_{260/280}$	$A_{260/230}$	Air (m^3)	DNA (ng m^{-3})
05/11/2012	Harbor	69.8	3.02	0.75	4.53	1540
	Suburb	72.2	2.72	1.37	4.59	1570
	City	56.5	2.99	0.25	4.648	1220
26/11/2012	Harbor	83	2.49	1.25	10.73	770
	Suburb	88.5	2.43	1.08	10.44	850
	City	86.4	2.42	1.14	10.73	810
21/12/2012	Harbor	88	2.56	1.36	10.665	830
	Suburb	72.4	2.72	0.7	10.64	680
	City	77.5	2.75	1.67	9.6	810
01/03/2013	Harbor	35.8	3.04	0.76	10.53	340
	Suburb	33.8	2.84	0.39	10.8	310
	City	39.2	2.58	0.8	10.73	370
08/03/2013	Harbor	54.4	3.41	0.58	10.24	530
	Suburb	63.1	3.04	1.05	10.73	590
	City	59.7	3.21	0.35	10.5	570

DNA concentrations ranged from 30 to 90 ng µL^{-1}, in a total volume of 100 µL per sample.

4.5.2 Possible relations between PM10, temperature and DNA yield.

To investigate whether the different airborne characteristics of the sample sites (PM_{10}, BC, temperature, wind speed, wind direction, relative humidity, and precipitation) (Table 1 + Appendix V, VI) have an influence on the DNA concentration after extraction, linear models were tested for significance as described in Materials and Methods (data not shown). PM10 and temperature appeared to be positively correlated with DNA concentrations (p value < 0.01).

4.5.3 PCR for DGGE

To obtain a culture-independent profile of the bacteria present in the different samples, DGGE was explored. Some attempts were done with test sample DNA to obtain PCR products, which would be suited to make a DGGE fingerprint. However, as indicated in Figure 18, the PCR products were not further used, because their bands showed only a weak intensity when viewed on a 1.5% (w/v) agarose gel. It was not possible within the time frame of this thesis to optimize DGGE for airborne analyses.

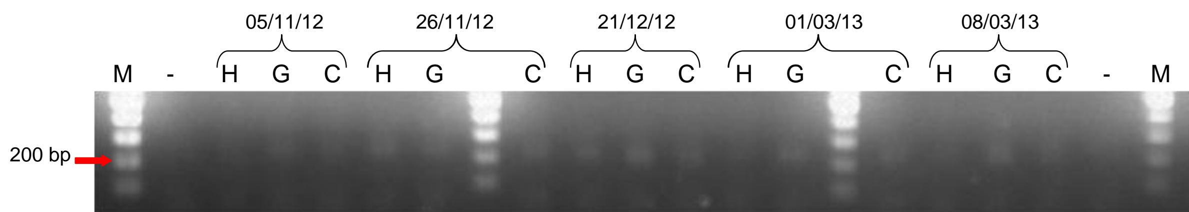

Figure 18: The DNA of the air samples was used to produce partial regions of the 16S rRNA gene with GC-clamps, which would later be used for DGGE. The PCR products were visualized a 1.5% gel (w/v).

4.5.4 PCR for V6 Illumina Hiseq

As an alternative for DGGE, we also aimed for metasequencing (Illumina, Hiseq) at BGI (Beijing Genomics Institute) to obtain a molecularly detailed profile of the bacteria present in the different samples. Hereto, the V6 region was amplified to obtain products of approximately 80 bp long. However, PCR bands obtained for air samples on gel turned out weak, while occassionally also double bands were observed (e.g. lane 3, Figure 19). To

further optimize the PCR protocol, a gradient PCR for these primers was executed, and an annealing temperature of 53°C was concluded to be optimal (though temperatures from 50°C to 57°C seemed to work as well). To decrease PCR inhibition, template dilution (1/10) and BSA addition were tested. They did not improve PCR. Optimization is still ongoing.

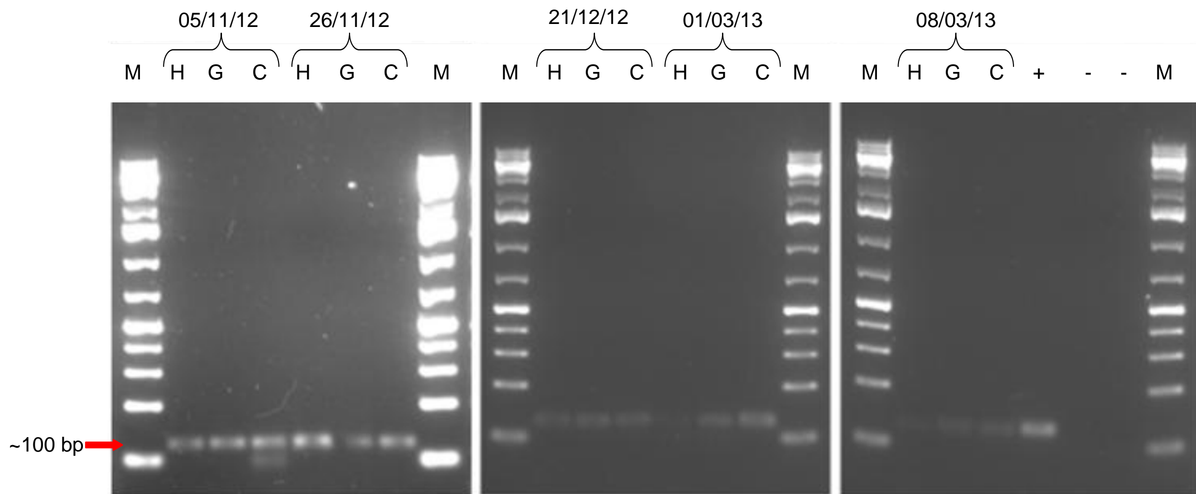

Figure 19: PCR products of the V6 region from the 16S rRNA gene visualized on a 1.5% (w/v) gel. Samples are seen according to sampling date for the locations: harbor (H), green suburb (G), and city (C). The positive control (+) was thePCR product of *E.coil* DNA

4.5.5 Wizard® DNA clean-up system

The problems with the PCRs for DGGE and metasequencing were hypothesized to be partly due to the poor $A_{260/230}$ values of the samples. Therefore, DNA samples were treated with the Wizard® DNA clean-up system in an attempt to improve these values. Three samples and one positive control (purely cultured *E. coli*) each of 50 µL DNA extract, were purified and eluted in 40 µL Milli-Q water in order to concentrate the samples though the $A_{260/280}$ and $A_{260/230}$ values did not improve as can be observed in (Table 7).

Table 7: Nanodrop measurements of samples before and after use of the Wizard® DNA Clean-Up System (Promega).

Sample		Concentration (ng/µL)		$A_{260/280}$		$A_{260/230}$	
		Before	After	Before	After	Before	After
08/03/2013	Harbor	54.4	55.3	3.41	2.9	0.58	0.09
	Suburb	63.1	72.5	3.04	2.48	1.05	0.11
	City	59.7	97.5	3.21	3.32	0.35	0.22
E. coli		85.6	102.2	3.16	2.18	1.97	0.16

Despite the Nanodrop readings showing even lower $A_{260/230}$ readings, the samples treated with the Wizard® DNA clean-up were also re-analyzed by PCR (Figure 20). These results indicate that the Wizard® DNA clean-up improved the PCR yield for the positive control *E.coli*, but not for the samples. In fact, use of the Wizard® DNA clean-up quick seemed to have even decreased the PCR yield of the samples.

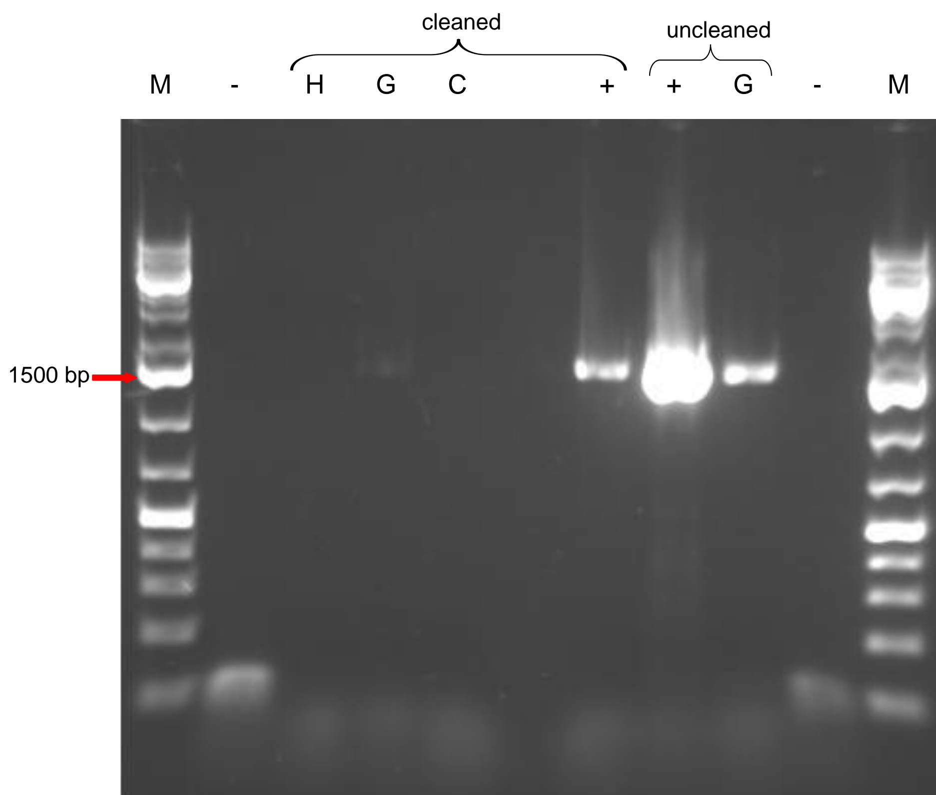

Figure 20: A 1% (w/v) gel of the PCR products using universal bacterial primer pair 8F and 1525R to amplify a ~1500 bp region of the 16S rRNA gene from samples with and without Wizard® clean-up. Samples included cleaned-up DNA of the three locations, sampled the 1st of March (G, H, C) and of the positive control, *E. coli* (+), the same *E. coli* DNA that was not cleaned up (+), and the original sample of the suburb before clean-up.

5 Discussion

5.1 Sampling

In this master thesis, the Coriolis®µ air sampler was used. Different types of Coriolis® samplers have been validated by an independent agency, the Health Protection Agency (HPA) (Bertin technologies, “Coriolis® Validation”, 2013). This validation revealed 100% efficiency for particles 4 µm and bigger, while the size for which the efficiency is 50% is lower than 0.5 µm (Figure 21). Efficiencies were determined using reference methods like filters and impactors. The Coriolis® µ air samplers have been made for sampling a relatively high volume of air in a short period of time, which decreases chances of cell proliferation or loss of cell viability (Langer *et al.*, 2012; Ravva *et al.*, 2012). In addition, Coriolis® δ air sampler has been tested by Carvalho and colleagues (2008) and proved to be at least as efficient as the reference method for collecting pollen grains (10 µm – 120 µm) and spores (1 µm – 10 µm).

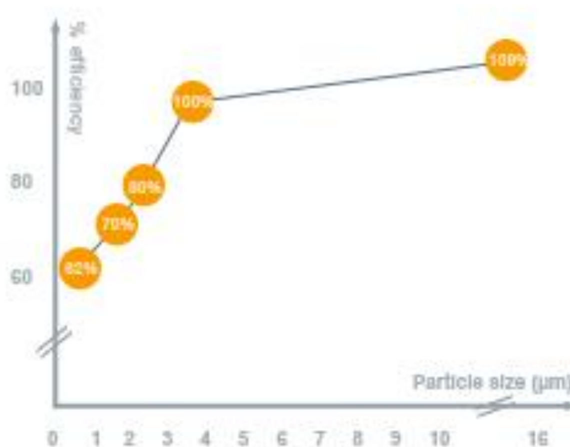

Figure 21: The physical efficiency of the Coriolis® technology. HPA validation proves that efficiency depends on the particle size. Physical efficiency covers the efficiency of all sorts of particles, from inanimate particles to microorganisms (Bertin technologies, “Coriolis® Validation”, 2013).

Peccia and Hernandez (2006) highlighted that all air collection methods show efficiencies varying with particle diameter. Therefore, depending on the sampler, different fractions of the total aerosol are sampled. Combined with the fact that PM size fractions show different associated community compositions (Bertolini *et al.*, 2012), this implies that comparison of results between studies using different types of samplers may not be straightforward.

However, efficiency of the Coriolis® methods shows there is an improvement of previous methods, which suggest it can yield results that are more representative for the real bacterial communities.

During this study, the Coriolis®µ air sampler proved to have a major drawback when samples were taken in temperatures of 2°C or lower. The little amount of sample that was obtained showed significantly lower CFU concentrations than usual, which may be explained by various causes. Bacterial CFU concentrations could have been lower in the air that was sampled, because temperature and season are documented to have an effect on the amount of airborne culturable bacteria (Ravva *et al.*, 2012). However, the difference with the other samples is so high that there might be other effects as well. A fraction of the colony-forming bacteria might have lost their viability due to freezing without cryoprotectant, but the main reason was probably the loss of efficiency of the sampling when the liquid in the cone froze. The bacteria and particles bounce off more frequently on an icy surface than on a liquid one. Also, the impact on ice surface might have proven fatal for many of these organisms. When air temperatures drop near the freezing point, sampling with the Coriolis®µ air sampler becomes unreliable. To compensate for this drawback in the future, it will be important to update the Coriolis®µ air sampler with extra equipment for outdoor monitoring at low temperature, in addition to the option for long term sampling (Bertin technologies, 2013).

Of note, during this master thesis, sampling was mainly performed during autumn and winter. This is outside the growing season of most plants. It can be anticipated that pollen grains (10 µm – 120 µm) are not the main fraction of the biological particles measured (by FCM). This is in agreement with the work of Franzetti and colleagues (2011), who found that chloroplast sequences from plant debris and pollen, were frequently encountered in summer samples, but not in winter samples.

5.2 Culturing and storage of outdoor airborne bacteria

The CFU concentrations for the harbor, city and green suburb during autumn and winter were 900 ± 100, 350 ± 30, and 360 ± 60 CFU per m^3 of air, respectively. Between both seasons, no significant differences could be observed. This is possible since Fang and

colleagues (2007) observed that CFU concentrations sometimes differed over the seasons, depending on the sampled location. Nevertheless, the concentrations of culturable bacteria in this master thesis are similar to CFU/m^3 concentrations at other outdoor locations (Lighthart, 1997). For example, Shaffer & Lighthart (1997), who used another medium (LB agar) but similar growing conditions reported similar CFU concentrations (103 to 609 CFU per m^3 air). Interestingly, they observed higher city concentrations, compared to grass land and forest areas. The average city CFU/m^3 concentration in this study were significantly higher as well. Furthermore, the city concentration is more comparable with the concentrations of the indoor air samples that ranged from 600 – 2000 CFU per m^3 air.

The CFU concentrations and total cell count results were used to calculate culturable fractions of airborne bacteria, which showed to range from 0.008% to 0.25%. This is within the estimated culturability range of 0.001% - 3% for known environments (Amann *et al.*, 1995), but still rather low compared to the average of 1%, estimated by Chi and colleagues (2007). This low culturable fraction may have occurred due to technical limitations or non-optimal culture conditions, as the optimal temperature for culturing of air samples on R2A agar lies between 10°C and 20°C (Hyvärinen *et al.*, 1991) and incubation time may be longer. For example, it was observed that the culturable fraction still increased after 8 days of incubation at room temperature (Figure 12). This is probably related to the fact that many bacteria occur in a dormant state (including as spores) in the air (Dewi Puspita *et al.*, 2012), as mentioned in the literature study. This could lead to an extended lag-phase before airborne bacteria start growing under the tested culture conditions.

The cultures of test samples were useful to deduce a good protocol for storage and culturing. No dilution before culturing was necessary, on the contrary, it was concluded that at least 400 µL sample volume should be spread out on the tested medium and cultured for at least 8 days at room temperature to at least obtain the minimum of 25 CFU per plate. Of note, the CFU concentrations of the test samples taken outdoors on campus, during November, might also have been very low due to excessive fungal growth. To avoid fungi that overgrown bacterial colonies, natamycin or cycloheximide were added to the medium from that point on. Furthermore, presence of 25% glycerol during storage at -80°C proved to maintain the CFU concentrations of the samples better than without this cryoprotectant. This was as expected, because without cryoprotectant, ice crystal formation commonly

causes cryo-injuries and cell death (Prakash *et al.*, 2013). Glycerol has been shown to serve as a useful cryoprotectant before, for other culturable bacterial communities (Guérin-Danan *et al.*, 1999; Ludlam *et al.*, 1989). However, the CFU concentrations might not immediately show how storage with glycerol at -80°C affects the entire bacterial community. It should be noted that glycerol has already been reported to have slight effects on the bacterial communities (Prates *et al.*, 2010). Dimethyl sulfoxide (DMSO) is most likely a better cryoprotectant in general (Hubálek, 2003). However, use of DMSO was avoided due to its serious health risks such as lung irritation and mutagenic effects (ScienceLab, 2013).

5.3 Special functional characteristics of airborne bacteria from Antwerp and surroundings

In this master thesis, several (preliminary) assays on interesting functional properties of the airborne bacteria from the different sample areas were investigated.

Pigmentation is already documented to be a special characteristic for cultured airborne bacteria and is suggested to be related to stress resistance caused by UV irradiation (Fahlgren *et al.*, 2010; Lighthart & Shaffer, 1995). We observed pigmentation percentages ranging from 65% to 92%, which is consistent with the observations of Fahlgren and colleagues (2010). Though pigments in airborne bacteria have not been further studied. Evidently, this requires further investigation.

Survival of freezing can be seen as one part of temperature tolerance, which is expected to occur in airborne bacteria, as explained in the literature study. The identification of colonies from *Paenibacillus* of the *Firmicutes* phylum, and *Microbacterium* of the *Actinobacteria* phylum among the freeze-tolerant bacteria both indicate that survival at freezing temperatures may have occurred due to spore forming.

Of note, another interesting observation was that a significant fraction of the airborne isolates was able to continue growth at 4°C (Figure 13). As mentioned in the literature study, temperature tolerance is considered another important functional property of airborne bacteria. Vaïtilingom and colleagues (2010) and Amato and colleagues (2007a) reported active metabolism and growth by airborne bacteria under cold conditions such as encountered in clouds. They claim this is made possible by the presence of cold-resistant

enzymes. Cold-tolerant enzymes are of interest for various biotechnological applications (Cavicchioli *et al.*, 2011). For example, interesting applications of cold tolerant microorganisms in agriculture are the use of a plant growth promoting bacteria that can be effective at lower temperatures (Mishra & Goel, 1999) or the use of phosphate-solubilizing microorganisms in the soil, which make more effective use of phosphorus possible (Vassilev *et al.*, 2012). Of note, a positive correlation was also found between DNA extraction yield and temperature at time of sampling. This indicates that higher temperatures, in this case still below 20°C, may promote survival of bacteria in the air. Another important facet of airborne microbes is their adhesion on particulates. In this master thesis, a correlation was observed between DNA concentration and levels of PM10, suggesting that particulate matter in the air is an important substrate for airborne bacteria. It has been suggested previously that aggregation of bacteria with other airborne particles is promoting cell survival (Tringe *et al.*, 2008).

5.4 Metal resistance of airborne bacteria as a possible biomonitoring tool

In this master thesis, preliminary assays were performed to investigate whether the prevalence of metal resistant bacteria correlates with locations having different pollution levels. Of importance, since metal resistance genes are often known to be located on mobile elements, such as plasmids (Ji & Silver, 1995; Monchy *et al.*, 2007; Smith, 1967), the metal resistant isolates were cultured and stored in metal enriched medium to ensure they would retain the genes responsible for metal tolerance. When plasmids are no longer coding for necessary proteins, they have been known be lost over some generations (Smith & Bidochka, 1998). Metal-tolerant strains can also be mutants that can be overgrown by revertant stains when the environment is not favoring the metal-tolerant bacteria (Nies, 1999). By ensuring the selection pressure during growth, the metal resistant bacteria were forced to keep the genes coding for this resistance.

The results show that the airborne harbor community is most resistant to cadmium than the (Figure 16). This implies that more cadmium may occur in the harbor aerosol, which would be a logical consequence of the industrial activity at this site. A more thorough investigation of the kind of industry at this location is necessary to really conclude this. However, nickel

tolerance is slightly higher in the green suburb than at the harbor, which is rather unexpected. This may be due to wind transport of pollutants from the harbor to the green suburb, or the hypothesis that nickel tolerance is an indication for heavy metal pollution may not be accurate. Also, zinc tolerance is lower in the harbor, which is rather unexpected as zinc is also used in many industrial processes. Conclusions about the community tolerance as an indicator for heavy metal pollution can be made when we obtain the results of the chemical analysis(ICP-MS) of the airborne samples. If these results indicate that heavy metal resistance is a good indicator for heavy metal pollution, this method could be easily extended to other important stressors and pollutants in the air such as PAHs.

Nevertheless, an expected possible problem with this kind of passive biomonitoring, is that bacteria might be transported by the wind from locations with another environment. However, when the correlation of present species and certain pollutants is strong enough, the airborne bacteria or even the whole community might become an important tool for determining the degree of air pollution.

5.5 Bottlenecks for DNA-based analyses of airborne samples

During this master thesis, most efforts were focused on obtaining good quality DNA from the airborne samples for further downstream analyses. Several bottlenecks were experienced. Firstly, the bacterial content in the air samples is expected to be low, regarding the fact that the culturable fraction ranged between 0.008% and 0.25% and that the DNA content determined by FCM ranged between 9 x 10^5 and 7 x 10^6 per m^3 air. In addition, several bacteria are expected to be in a dormant state (including spores), complicating the extraction of DNA. Nevertheless, we were able to obtain DNA concentrations of 300 - 1500 ng DNA per m^3 air using the RTP® bacteria mini kit (Invitek). The DNA extraction described by Maron and colleagues (2005) and the DNeasy Blood&Tissue kit (Qiagen) showed to be less efficient.

Secondly, various (unknown) contaminants are expected to be present in the DNA samples that could interfere with the downstream PCRs, as was implied by the weak-intensity bands . The $A_{260/280}$ and $A_{260/230}$ values of the DNA extracts indicate that contamination with organic

pollutants seems to be considerable. At present, it is difficult to identify the most important airborne PCR inhibitors. In contrast, PCR inhibition from soil samples is well documented. Inhibitory compounds associated with soil environments comprise primarily of humic acids and fulvic acids collectively referred to as humic substances (Matheson *et al.*, 2010). To counter these contaminants, it is common to use bovine serum albumin (BSA), however this additive showed little effect in the PCRs with the air samples.

Taken together, the quality of the obtained DNA samples showed to be insufficient for further analysis by DGGE and metagenomic sequencing of 16s rRNA genes. This highlights that further optimization will be necessary.

6 Perspectives

The field of air microbiology is still in its infancy compared to detailed community analyses in other environments, such as soil or the human intestinal tract, especially with respect to the use of deep sequencing methods. This, however, also creates the opportunity to study the many facets of air microbiology with tools and techniques already set up for these other fields. In this study, stress factors, such as cold, UV radiation, and presence of excessive heavy metals were assessed for airborne communities of three different locations in Antwerp and its surrounding by using culture-dependent techniques. Attempts were made to complement this data with molecular techniques, however the air has proven to be a very different environment for DNA-extraction with its own inhibitors. Nevertheless, it can be anticipated that these bottlenecks will be shortly overcome and that worldwide efforts such as the Earth Microbiome project for optimization and standardization of methods will start to unravel the most typical airborne bacteria for specific atmospheric environments.

Biomonitoring of air quality with bacteria appears to be a very interesting perspective, resulting from the renewed interest in aerobiology.. For instance, analysis of the local communities are possible (passive biomonitoring), as well as the exposure of certain species to the air to estimate pollution (active biomonitoring). For the latter applications, the advent of synthetic microbiology for the design of specific biosensor bacteria is promising.

Another appealing perspective of aerobiology, is the selection of airborne bacteria, capable of metabolizing or sequestering certain pollutants, for possible applications of atmospheric bioremediation. For example, bioremediation of PAH-contaminated soil is yet widely explored (Wilson & Jones, 1993). As PAHs are also atmospheric pollutants, similar methods for PAH breakdown may be found.

There is still much to be elucidated about the lives of airborne microorganisms themselves. Metabolic activity and dormancy are subjects that were studied by only a few researchers, for a limited fraction of airborne microbes that occur in cloud water (Vaïtilingom *et al.*, 2010). In addition, microbial association with air particles has been observed but not readily studied.. Biodiversity has been observed to be in the same magnitude as other environments such as soil (Maron *et al.*, 2005) and metagenomic analysis of airborne samples shows many

unidentifiable sequences (Bertolini *et al.*, 2012). This can also open new frontiers for biotechnological applications based on the enzymes of these unexplored microbes. Morris and colleagues (2008) suggest that one part of future research of airborne microorganisms could overlap with other fields such as physics and atmospheric chemistry. They highlight that the study of bio-meteorology also needs to elucidate the microbial flux into the environment, and the exchange of microbes between locations. This may eventually give insight in the gene exchange world-wide. This information could also be used to improve the weather forecast (precipitation and cloud formation) and insight in atmospheric chemistry. The atmosphere and its composition are inevitably intertwined with climate change, having serious implications in both ecology and society.

To conclude, the atmosphere still presents a frontier for pioneering microbiologists and it is my sincere hope to become one of them.

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Appendices

Appendix I

Table A.1: Overview of the test samples taken.

Name	Runs (10 min)	Date	Sample location	Collection liquid	Liquid volume(mL)	Sampled air (m^3)	Air (m^3 mL^{-1})
1x	6	27/08/2012	Lab	Water	49.3	18	0.37
1x (20)	4*	27/08/2012	Lab	Water	17.3	12	0.69
1y	2	27/08/2012	Lab	PBS	17.5	6	0.34
2x	6	27/08/2012	CGB	Water	52.8	18	0.34
3y	2*	29/08/2012	Lab	PBS	NA	6	NA
3z	2*	29/08/2012	Lab	Coriolis liquid	NA	6	NA
4i[€]	1	05/09/2012	Lab	Coriolis liquid	NA	3	NA
5i	1	09/10/2012	Lab	Coriolis liquid	NA	3	NA
6i	2	12/10/2012	Lab	PBS	18.5	6	0.32
6o[$]	2	12/10/2012	CGB	PBS	24	6	0.25
7i	2	22/10/2012	Lab	PBS	18	6	0.33
7o	2	22/10/2012	CGB	PBS	20	6	0.3
8y	1	12/11/2012	CGB	PBS + 0.002% Tween	NA	3	NA
8z	1	12/11/2012	CGB	Coriolis liquid	NA	3	NA
9o	6	19/11/2012	Vents of Craeybeeckxtunnel	PBS + 0.002% Tween	65	18	0.28
10o	5	11/12/2012	CGB	PBS + 0.002% Tween	56.5	15	0.27

[€] the ‘i’ indicates that the samples were taken indoor,
[$] ‘o’ indicates that the samples were taken outdoor
For collection liquid “x” refers to water, “y” to PBS and “z” to coriolis liquid
Location “Lab” refers to the Molecular and Microbial laboratory of the University of Antwerp (department of Bioscience Engineering, room V.535)
“CGB” refers to the outdoors of campus Groenenborger of the University of Antwerp.
Air (m^3 mL^{-1}) shows how much volume of air is represented by one mL of collection liquid.
NA: Some of the volumes were not measured.
*After 1 run liquid was added to 15 mL again.

Appendix II

Table A.2: Dates, locations and details about samples taken at the three location of interest.

Date	Location	Time	Runs	Volume (mL)	Sampled air volume (m^3)	Air (m^3)/ml
07/09/2012	City	9:47-10:09	2	17.8	6	0.34
	Harbor	10:58-11:22	2	17.2	6	0.35
	Suburb	13:06-13:27	2	15.2	6	0.39
08/10/2012	Suburb	9:00-9:22	2	20.1	6	0.3
	Harbor	9:54-10.17	2	17.5	6	0.34
	City	11:01-11:23	2	17.6	6	0.34
16/10/2012	City	5:48-6:11	2	21.8	6	0.28
	Suburb	6:38-6:59	2	18.1	6	0.33
	Harbor	7:37-7:59	2	18.6	6	0.32
05/11/2012	City	14:17-14:39	2	21.6	6	0.28
	Suburb	15:12-15:35	2	22	6	0.27
	Harbor	16:07-16:28	2	20.1	6	0.3
26/11/2012	City	19:58-20:56	4	42	12	0.29
	Harbor	21:40-22:30	4	42	12	0.29
	Suburb	23:12-00:04	4	41	12	0.29
21/12/2012	City	6:31-7:19	4	44.5	12	0.27
	Harbor	7:58-8:44	4	43	12	0.28
	Suburb	9:40-10:21	3.66	45	10.98	0.24
12/01/2013	Harbor	11:22-11:32	1	6	3	0.5
	Suburb	10:05-10:50	3	15	9	0.6
01/03/2013	City	12:50-13:31	4	42	12	0.29
	Harbor	11:04-11:57	4	44	12	0.27
	Suburb	9:28-10:21	4	45	12	0.27
08/03/2013	Suburb	10:07-10:56	4	42	12	0.29
	City	11:34-12:22	4	40	12	0.3
	Harbor	13:05-14:01	4	37	12	0.32

Appendix III

Table A.3: Concentrations of SYBR Green stained particles in the air. Every sample except those from the 5th of November were stained and ran through the FCM in duplicate.

Date	Location	Stained particles ($x10^6$ per m^3 air)
07/09/2012	Harbor	3.71 ± 0.04
	Suburb	NA
	City	1.8 ± 0.2
08/10/2012	Harbor	2.804*
	Suburb	0.715*
	City	2.209*
16/10/2012	Harbor	4 ± 3
	Suburb	1.9 ± 0.2
	City	4.7 ± 0.9
26/11/2012	Harbor	3.9 ± 0.1
	Suburb	1.4 ± 0.2
	City	2.57 ± 0.09
21/12/2012	Harbor	1.4 ± 0.2
	Suburb	1.3 ± 0.1
	City	2.0 ± 0.1
01/03/2013	Harbor	1.31 ± 0.09
	Suburb	3.1 ± 0.7
	City	5 ± 1
08/03/2013	Harbor	1.5 ± 0.4
	Suburb	1.086 ± 0.007
	City	1.1 ± 0.2
12/01/2013	Harbor + Suburb	7 ± 2

* An unrealistic amount of beads was measured in the duplicates, so no standard error could be calculated
NA: An unrealistic bead count occurred in both measurements of one sample

Appendix IV

Table A.4: DNA extractions of the test samples. The addition of “g” to the sample means it has a 25% glycerol concentration and TSB step means a preliminary sporulation step was executed by adding 110 µL, 10xTSB to 1mL of sample and incubating this mixture for 30 minutes at 37°C.

No.	Date	Method	Volume & samples	Remark	End product
1	27/08/2012	DNeasy B&T: normal protocol + purification	4mL of 1x,1y,1x (20),2o, pos and neg	No visible pellet after pelleting of bacteria from the sample	Purified DNA in 30 µL TE buffer
2	29/08/2012	DNeasy B&T: normal protocol + purification	4mL of 3y, 3z, pos and neg		Purified DNA in 30 µL TE buffer
3	25/09/2012	CTAB method + purification (@Labmet)	10mL of 1x, 2x, pos* and neg	Visible pellet in positive control and sample 2x	DNA in 100 µL PCR water
4	05/10/2012	RTP®Bacteria kit: protocol for pellet	4mL of 1x, 2x, neg and 0.1mL pos*		DNA in 150 µL elution buffer D
5	09/10/2012	DNeasy B&T: for Gram-positive + purification	6mL of 1x, 5i and 1.2mL pos*	only half of the DNA was purified	Per sample 75 µL buffer AE ar purified DNA in 75 µL TE buffer
6	12/10/2012	RTP®Bacteria kit: protocol for pellet, Gram-positive	8mL of 6i and 6o	only half of the DNA was purified	Per sample 75 µL buffer AE ar purified DNA in 75 µL TE buffer
7	15/10/2012	DNeasy B&T: for Gram-positive	8mL of 6i, 6o, pos**, and neg	only half of the DNA was purified	Per sample 75 µLelution buffer and purified DNA in 75 µL TE buffe
8	23/10/2012	PowerSoil: for wet samples (@Labmet)	4mL of 7o and pos**, 8mL of 7og and of 7ig		DNA in 100 µL Solution C6
9	23/11/2012	DNeasy B&T: for Gram-positive	32mL of 9o and 1mL pos***	Spore germination with TSB step	DNA in 150 µL buffer AE
10	26/11/2012	Beadbeating (Maron *et al.*, 2005)	32mL of 9o and 1mL pos***	Spore germination with TSB step	Purified DNA in 30 µL TE buffer
11	11/12/2012	RTP®Bacteria kit: for large volume water sample, Gram-positive	16mL 10o with spike, 32mL 10o, and 1mL only spike	Spore germination with TSB step, spike was addition of colony 25	DNA in 100 µL buffer D

*The positive control consisted of a liquid culture of Bacillus subtilis (LMG 7135, BCCM), grown at 37°C for five days.

** The positive control consisted of sterile water and a white colony from a plate from sample 3z.

*** The positive control was colony no. 21 added to sterile water from a plate from sample 8o. Colony 25 comes from sample 8o as well.

Appendix V

Table A.5: Table of weather information (Deurne) at the time of collection of the samples.

Date	Location	Temperature (°C)	Wind direction	Wind speed (km/h)	Relative humidity	Precipitation
07/09/2012	City	17	WSW	14	77%	no precipitation
	Harbor	20	WSW	13	68%	no precipitation
	Green	22	WSW	13	53%	no precipitation
08/10/2012	Green	8	ESE	6	93%	no precipitation
	Harbor	9	SE	9	81%	no precipitation
	City	11	SE	9	87%	no precipitation
16/10/2012	City	9	S	19	81%	light precipitation
	Green	10	S	19	81%	light precipitation
	Harbor	10	S	19	87%	light precipitation
05/11/2012	City	8	W	11	93%	light precipitation
	Green	8	W	9	93%	light precipitation
	Harbor	7	N	19	93%	light precipitation
26/11/2012	City	9	SW	26	66%	no precipitation
	Harbor	9	SSW	13	66%	no precipitation
	Green	9	S	11	71%	no precipitation
21/12/2012	City	8	SW	11	93%	light precipitation before
	Harbor	8	SW	9	93%	light precipitation before
	Green	8	SW	9	93%	light precipitation before
01/03/2013	City	5	NNO	11	81%	light precipitation before
	Harbor	4	N	7	93%	light precipitation before
	Green	3	no wind	0	90%	light precipitation before
08/03/2013	Green	11	ESE	9	94%	no precipitation
	City	14	E	12	88%	no precipitation
	Harbor	15	ESE	13	72%	no precipitation

Appendix VI

Table A.6: Table of pollution levels at the time of collection of samples.

Date	Location	PM_{10} (µg m^{-3})	BC (µg m^{-3})	VMM score
07/09/2012	City	41	4.5	5
	Harbor	47	1.67	4
	Suburb	NA	NA	1
08/10/2012	Suburb	NA	NA	1
	Harbor	26	3.78	3
	City	46	5.01	4
16/10/2012	City	14	4.44	3
	Suburb	NA	NA	1
	Harbor	13	0.75	2
05/11/2012	City	22	2.89	4
	Suburb	NA	NA	1
	Harbor	21	1.35	2
26/11/2012	City	24	5.57	3
	Harbor	17	1.26	3
	Suburb	NA	NA	1
21/12/2012	City	21	5.66	4
	Harbor	21	1.76	3
	Suburb	NA	NA	1
01/03/2013	City	32	1.42	4
	Harbor	36	1.17	4
	Suburb	NA	NA	1
08/03/2013	Suburb	NA	NA	1
	City	58	8.75	6
	Harbor	40	3.26	4

NA: VMM did not measure this pollutant at this location