NTNU

Norwegian University of
Science and Technology

Characterization of Airborne Microorganisms at Nationaltheatret Subway Station

Anja Valen

Biotechnology
Submission date: May 2011
Supervisor: Kjetill Østgaard, IBT
Co-supervisor: Janet Martha Blatny, FFI

Norwegian University of Science and Technology
Department of Biotechnology

Acknowledgement

This is a master thesis for the title Biotechnology MSc at the Norwegian University of Science and Technology (NTNU), Department of Biotechnology. The work presented in this thesis has been carried out at The Norwegian Defence Research Establishment (FFI), Division for Protection, from June 2010 to May 2011.

The work has been conducted as a contribution to the project '1203 Biologisk beredskap - Deteksjon og identifikasjon', and was performed in collaboration with the PhD student on the project: Marius Dybwad. The protocol for air sampling and characterization of bacteria was developed by Dybwad, whereas the protocol for detection of virus was developed by the MSc candidate. The experimental work presented in this thesis has been independently performed by the MSc candidate in collaboration with Dybwad.

I would like to express my gratitude to Marius Dybwad. I owe him my thanks for always being gentle, encouraging and taking time whenever details needed attention, and for excellent guidance. I am grateful to my supervisor Dr.ing Janet M. Blatny, for providing the research facilities and for excellent scientific guidance during the studies. Special thanks to my co-supervisor Professor Kjetill Østgaard for encouraging support, and for ensuring good communication between FFI and NTNU.

Abstract

Bioaerosols containing pathogenic microorganisms can have health implications when respired. Of special concern are potential bioterrorism attacks conducted by deliberate aerosolization of hazardous toxins or pathogenic microorganisms. Investigation aiming at understanding the normal state of the bioaerosol environment is essential to facilitate detection of biological threat agents and deviations from the normal background. This MSc thesis presents a pilot study for investigation of the bioaerosol environment at a subway station in Norway.

The aim of this study was to characterize airborne bacteria and Influenza virus at Nationaltheatret subway station in Oslo. A series of studies were conducted to examine the every-day concentrations and diversity of endospores and vegetative bacteria cells. Results showed that 20 times more cultivable bacteria were found during daytime compared to nighttime. An average of 400 CFUs/m^3 was found in daytime samples, of which 3 % were cultivable endospore-forming bacteria. From the cultured bacteria, 92 different bacterial species were observed by tentative 16SrRNA gene identification, and 37 different bacterial genera were identified. The diversity was found to be similar during daytime and nighttime, except for decreased representation of the family taxa *Bacillaceae* during nighttime (6 % compared to 32 % during daytime).

402 cultured bacteria were further characterized based on observed colony morphology, hemolysis activity and antibiotic resistance. Characteristic traits of the ten most represented family taxa were found based on colony morphology. In order to include non-cultivable bacteria for characterization, performance of culture-independent analysis of total bacteria was needed. In order to facilitate such analysis, a bead mill homogenization method for efficient DNA extraction from samples containing both endospores and vegetative bacteria cells was optimized. The concentrations of total bacterial DNA in 15 different air samples were compared, and the observed pattern for daytime and nighttime concentrations resembled the concentrations found for the cultivable bacteria.

Furthermore, a specific PCR assay was developed for detection and quantification of airborne Influenza A virus, and successfully verified by detection of commercial Influenza A virus particles. However, no viral RNA was found in the air samples from Nationaltheatret subway station. Inhibition of the PCR reaction was observed, and hence further investigation regarding inhibition is needed in order to rule out false negative results.

Contents

1 Introduction

1.1 Bioaerosols

Bioaerosols are airborne particles usually defined as particulate matter of plant, soil, animal or human origin, containing microorganisms or organic compounds (Douwes, Thorne et al. 2003). The particles become airborne when suspended in air as a result of wind, turbulence, coughing, traffic etc. When in air, the particles may adhere to tiny droplets of water or dust material, creating bioaerosols that can reside in the air for longer periods of time, depending on the weather conditions and the sizes of the particles (Francoise 2002). Particles below 5 µm remain suspended in the air stream for long periods of time, and they are of primary concern because they penetrate deep into our lungs when respired, potentially causing infections (Thomas, Webber et al. 2008). Bioaerosols can be found in all outdoor and indoor environments, and they often contain bacteria, virus or fungi, which may be pathogenic or non-pathogenic, viable or dead (Douwes, Thorne et al. 2003). The viability of the airborne microorganisms is dependent on measureable factors like relative humidity, solar irradiance and temperature, in addition to special properties of the bacteria themselves, like endospore-forming capability and pigment content (Gilbert and Duchaine 2009).

The interest in bioaerosol exposure has increased over the last few decades, both due to the emerging understanding of its association with a wide range of adverse health effects, and due to the fear of bioterrorism. In hospitals, it could be important to quality test the air because it is a serious and widespread problem that patients acquire infections through the airborne route during hospital stay (L.A. Fletcher, C.J. Noakes et al. 2011) (Killingley, Greatorex et al. 2010). For safety reasons, monitoring the air at public places could help minimizing the proportions of potential bioaerosol attacks. An example of a bioterrorism event propagated by bioaerosols was the anthrax attacks in the United States in 2001 (Centers for Disease Control and Prevention 2001). However, spread of anthrax is self-limiting, as it is not likely to infect other people than those directly exposed. A scenario even more dangerous than a new anthrax attack would be the spread of a contagious agent at a crowded public place, like a subway station, where every infected person will transmit the disease to others after leaving the station (Inglesby, Henderson et al. 1999). Biological agents that are easy to spread and capable of infecting human, causing incapacitation or death, can be considered biological threat agents (Centers for Disease Control and Prevention 2007).

In order to minimize the consequences of a bioterrorism attack, early detection of the dispersed threat agent is necessary. Continual monitoring of the airborne environment for detection of specific agents is possible, but false positive results are likely to occur due to low background levels of naturally-occurring threat

agents (Philip J. Wyatt 2009). Investigation aiming at understanding the normal state of the bioaerosol environment is therefore essential (National Research Council 2005). Airborne fungi are of health concerns, as they are associated with allergy and respiratory diseases (Hope and Simon 2007). However, biological threat agents dispersed in a bioterrorism attack are more likely to be bacteria, toxins or virus, according to the list of critical biological agents obtained from Centers for Disease Control and Prevention (Rotz, Khan et al. 2002).

Relatively many bioaerosol characterization studies have been conducted in hospitals, schools, farm buildings and other industry buildings, but few studies have been conducted in subway stations or train stations (Abdel Hameed and Awad 2002). A study performed in Beijing in 2010 investigated bacteria concentrations in different airborne environments, and found that the cultivable bacteria concentrations were significantly higher in train and metro stations than in hospitals, offices and in outdoor city centre (Dong and Yao 2010). However, among different studies performed in stations, the obtained results are not directly comparable due to use of different air sampling devices and analysis methods (Srikanth, Sudharsanam et al. 2008) (Stellman 1998).

1.2 Bioaerosol sampling

1.2.1 Sampling techniques

Three basic sampling methods exist for collection of airborne microorganisms: filtration, impingement and impaction. Most air sampling devices in use rely on techniques that force surrounding air into the device, where airborne particles are departed from the air stream. These are active air samplers, and the airborne particles can be deposited onto a solid medium (impaction), into a liquid (impingement) or onto a filter (filtration) (John Burke Sullivan and Krieger 2001). Methods for collection can also be passive. An example is use of settling plates, where particles deposit due to gravity. However, gravitational collection is not appropriate for quantitative analyses of airborne microorganisms, as sampling efficiency is highly dependent on motion in the surrounding environment (C. Pasquarella, O.Pitzurra et al. 2000).

1.2.1.1 Impaction

When considering active air sampling, impaction is a sampling method that separates airborne particles from the airflow by leading the airflow into the device, where the particles deviate from the air flow and impacts on a medium. Petri dishes with culture medium are often used as they can be incubated directly for microbiological growth studies after sampling (Cartwright, Horrocks et al. 2009). This makes impaction appropriate for culture-dependent studies, but

insufficient for culturing independent studies (Gilbert and Duchaine 2009). However, there is a high risk of overloading the plates with growing cultures, introducing error when estimating the microorganism concentrations. Therefore, impaction sampling is more suitable for less contaminated bioaerosol environments, or requires shorter sampling time (Cartwright, Horrocks et al. 2009).

Examples of different types of impaction devices are Andersen samplers and Slit samplers (Figure 1.1). These devices are efficient for collection of viable bacteria or virus, dependent on the growth medium or cell culture used. The Anderson sampler is designed for separation of the captured particles into fractions, based on aerodynamic sizes. Interestingly, correlations between particle sizes and types of microorganisms residing on them can be investigated. The Slit sampler can be used for determination of airborne microorganism concentrations as a function of time (Verreault, Moineau et al. 2008). In general, impaction samplers are usually most efficient at capturing large particles (>10 µm), as smaller particles (<10 µm) tend to follow the air stream through the instrument without impacting the medium (Gilbert and Duchaine 2009).

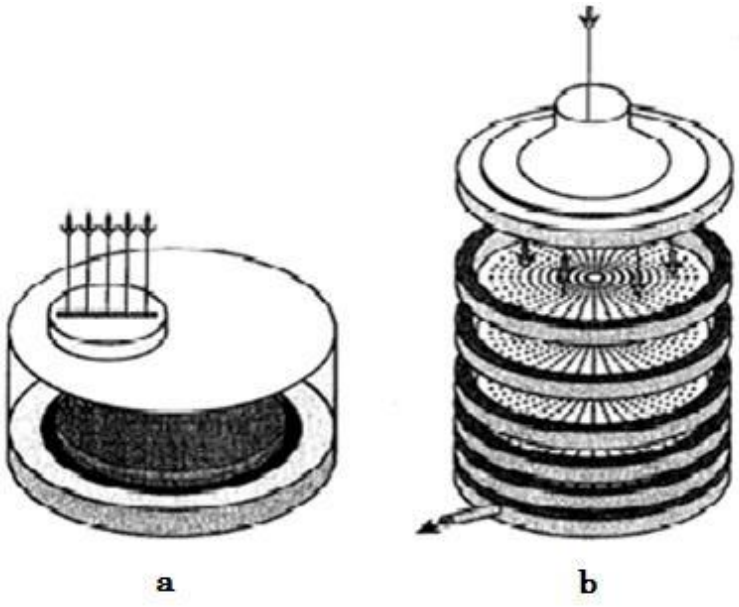

Figure 1.1: Impaction samplers; a) Slit sampler, b) Anderson sampler (Verreault, Moineau et al. 2008)

1.2.1.2 Impingement

The principle of impingement is similar to that of impaction, but here the particles impact a liquid when the air flow abruptly changes its direction, and not a solid medium. The impingement method may induce less physical stress to the collected microorganisms than impaction, making impingement suitable for microbiological studies of viable microorganisms. Impingers also have the

advantage of being able to sample for long time intervals, and the collection in liquid makes multiple analyses possible per sample, including both culturing dependent studies and culture-independent studies by use of molecular biological techniques (Cartwright, Horrocks et al. 2009).

Impingers have been used for collection of airborne bacteria, fungi and virus (John Burke Sullivan and Krieger 2001). However, similar to the impaction method, impingement sampling is most efficient at capturing large particles (>10 μm). Smaller particles (< 10 μm) might also be captured in the liquid, but are likely to re-aerosolize as a consequence of liquid medium evaporation (Verreault, Moineau et al. 2008).

Impingement devices can be exemplified by the Swirling Aerosol collector (manufacture by SKC under the name Biosampler) and the All Glass Impinger (AGI) (Figure 1.2) (Verreault, Moineau et al. 2008). The SKC Biosampler can be considered an improvement of the AGI device in retaining viability of the collected microorganisms, as the swirling motion inside the Biosampler is gentler than the abrupt change in airflow seen in the AGI. Evaporation and re-aerosolization are also minimized by use of the Biosampler compared to the AGI, suggesting better quantitative result reliability (Gilbert and Duchaine 2009). Impingement devices have also been developed where size fractionation of the collected particles is possible. These devices are called multistage liquid impingers (Verreault, Moineau et al. 2008).

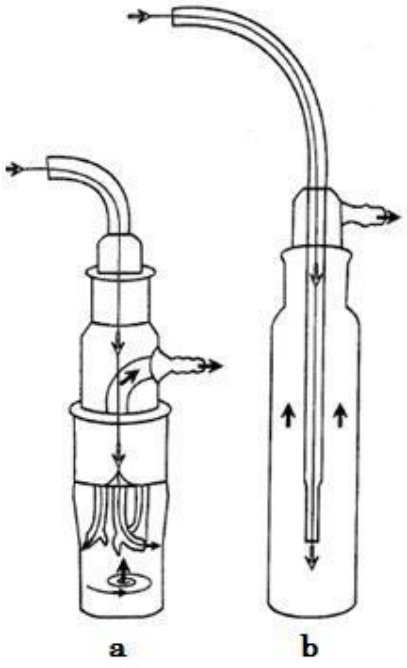

Figure 1.2: Liquid Impingers; a) SKC, b) AGI (Verreault, Moineau et al. 2008)

1.2.1.3 Filtration

Filtration relies on collecting airborne particles by passing air through a porous medium. Depending on the sizes and charges of the particles, in addition to the streamline of the airflow though the filter, the particles will divide from the airflow and impact on the filter surface. This is a relatively simple and effective method for collection of airborne microorganisms (Cartwright, Horrocks et al. 2009) (Verreault, Moineau et al. 2008). An advantage of filtration over impaction and impingement is that filters can be designed for efficient collection of particles with aerodynamic sizes less than 0.5 µm (Verreault, Moineau et al. 2008).

Collection of airborne particles on a filter usually requires extraction of the particles into liquid prior to analysis, although gelatin filters can be placed onto agar plates for direct growth studies. An advantage of the samples extracted in liquid is that they can be tested by multiple assays, and both microbiological culturing analyses and molecular biological analyses are possible (Gilbert and Duchaine 2009). Filter samplers have potential for collection of excessive amounts of airborne microorganisms by long sampling periods or use of high flow rate. However, a drawback of the method is desiccation of microorganisms, resulting in loss of viability. The loss of viability is affected by the sampling time and humidity, appreciating that meteorological conditions should be measured while collecting filter air samples (Cartwright, Horrocks et al. 2009).

Different types of filter samplers differ mainly in composition and pore size of their filters. The filter material affects collection efficiency, but the yields obtained are also affected by the extraction efficiency of the filter in use (Abdel Hameed and Awad 2002). Filter samplers can be exemplified by the Smart Air Sampler System 3100 (SASS 3100) (Figure 1.3), which is a dry filter air sampler developed by Research International (Research International, Inc). The filter used by SASS 3100 consists of micro-fibrous material where each fiber is associated with an electric field, which makes these filters more efficient in capturing particles than conventional glass or cellulosic filters. SASS3100 has a collection efficiency ranging from 50 % of particles of 0.5 µm to 90 % of particles larger than 2.0 µm, which should make it sufficient for collection of virus, bacteria and fungi residing on particles of most sizes. Extraction efficiency from the filters is 100 % when using a 20 ml extraction buffer and a SASS 3010 Manual Particle Extractor (Research International, Inc) (Research International 2011).

Figure 1.3: SASS 3100 filter sampler (Research International 2011)

1.2.2 The choice of sampling equipment

Airborne particle harvesting for analysis purposes have been performed with a wide variety of sampling devices, and new instruments are continually being developed. Choice of sampling device is highly dependent on the aim of the study. For microbiological analyses of viable airborne microorganisms, sampling devices that maintain the viability of the microorganisms throughout the sampling process are required. On the other hand, high-flow sampling devices can be more efficient for molecular biological studies, where viability is not a requirement (Gilbert and Duchaine 2009).

Other criteria that should be considered when choosing sampling device are ease of operation and transportation, cost, sampler reliability and optimum particle size range of the device (Cartwright, Horrocks et al. 2009). For analysis of bioaerosols potentially causing respiratory diseases, it is important to choose a sampling device approved for collection of particles below 10 µm (Thomas, Webber et al. 2008). Size range below 10 µm could also be the goal when aiming at collecting viruses or bacteria, as indicated by the Committee on Materials and Manufacturing Processes for Advanced Sensors, National Research Council (National Research Council 2005), but little is known about the relationship between typical size ranges of airborne particles and the microorganisms they contain. In general, the same kinds of sampling devices have been approved for analysis of airborne fungi and bacteria, whereas modifications have been necessary in order to detect viruses in the air samples (Gilbert and Duchaine 2009). However, there has been a lack of standard protocols for treatment of air samples, making result comparison between different studies difficult (Srikanth, Sudharsanam et al. 2008).

1.3 Microbiological techniques

Traditional microbiological techniques rely on culturing as a means to enumerate and characterize microorganisms from samples (Gilbert and Duchaine 2009). Some great advantages of microbiological culture studies, compared to culture-independent studies, are the possibility for testing the viable bacteria found for pathogenic potential, metabolic requirements and endospore-forming capability.

1.3.1 Culture-dependent quantification

Culture-dependent methods are relatively easy and cheap to perform, and they are applicable to quantification of bacteria, fungi and viruses. For culturing of bacteria and fungi, use of semi-solid growth media is most common. The microorganisms may be collected onto the growth medium directly from air when using impaction-based sampling methods. When sampling is performed by impingement systems, the liquid sample must be spread onto the growth medium prior to culturing. For filter samples, the filter must be extracted in liquid buffer and spread onto the growth medium prior to culturing (Millie P. Schafer and Jensen 1998).

Growth media appropriate for growth of the microorganisms of interest need to be selected. TSA is often used for enumeration of bacteria, while malt extract agar is commonly used for culturing of fungi (Millie P. Schafer and Jensen 1998). However, no single growth medium is suitable for all sorts of bacteria or all sorts of fungi, meaning that only the microorganisms able to grow and multiply on the chosen growth media are found and quantified. These cultivable microorganisms are estimated to represent about 1 percent of the total amount of viable and non-viable microorganisms in the sample (Amann, Ludwig et al. 1995).

Culturing of viruses is performed on cellular growth cultures, consisting of bacteria culture, animal tissue or human tissue. Viruses need to infect living cells in order to multiply, which can be observed as clear spots in the culture. These clear spots are called plaques and each plaque represents one initial viral particle. Counting the plaques gives a measure of the number of initial viruses in the sample poured onto the growth culture, given as plaque forming units (PFU). Bacteria and fungi form colonies when incubated on a growth medium, and each colony represents one single initial microorganism poured onto the medium. Counting the colonies gives a measure of the initial number of microorganisms in the sample, given as colony forming units (CFU). It is common to select for only bacteria or fungi, because the growth of fungi is likely to mask many bacterial colonies present. Growth inhibitors for fungi, like cyclohexamide, can be added to the growth media when bacteria counts are in focus (Cartwright, Horrocks et al. 2009).

Problems related to viable counts are for example quantification error that occurs if large numbers of colonies (above 300) are grown on one culture plate. The colonies are then likely to merge, and one colony might represent several initial microorganisms. If the microorganisms were sampled directly on the culture plate by impaction methods, the sampling time must be reduced in order to reduce the number of colonies. Serial dilutions is needed if impingement or filter sampling resulted in too many colonies. Dilutions do however induce some error and should be avoided if possible (Cartwright, Horrocks et al. 2009).

1.3.2 Traditional taxonomical classification

Characterization of microorganisms by culture-dependent methods is the traditional approach for classification. The science of classification is called taxonomy, where the objective is to classify living organisms based on similarities and differences between the organisms (Millie P. Schafer and Jensen 1998). The classification can permit species-level identification when using appropriate numbers of characteristics tests per microorganism.

Culture-dependent methods for characterization include incubation of microorganisms for studying their growth appearance. Formation of endospores is a characteristic property of some bacteria and fungi, and can be tested for by heat shocking the microorganisms prior to culturing. Bacteria can be classified more closely based on the results from biochemical, physiological and nutritional tests, which evaluate characteristics like temperature optimum, pH tolerance, modes of metabolism etc. (Millie P. Schafer and Jensen 1998). Culture-dependent methods for characterization can also include use of differential media and selective media. Differential media contain indicators that permit the recognition of microorganisms with particular metabolic activities. Growth on blood agar is an example, where growth of bacteria that degrade hemoglobin are detected (Payment, Coffin et al. 1994). Selective media contain compounds that inhibit the growth of particular microorganisms. Growth media with antibiotics are examples of selective media, were only bacteria with antibiotic resistance are able to grow (Michael T. Madigan and John M. Martinko 2006). Hemolytic activity and antibiotic resistance are of health concern, and can be tested for in order to investigate for pathogenic potential in an environment.

For viruses, the need for specific animal or human tissue for reproduction has made large-spectrum characterization of viruses from air samples difficult. For bacteria and fungi, the species not able to grow on the medium provided or under the particular incubation conditions in use, are excluded from the study (L.A. Fletcher, C.J. Noakes et al. 2011). The microbiological techniques give information about types and quantities of cultivable microorganisms in samples,

but no information is to be obtained about the fraction of non-cultivable microorganisms.

1.4 Molecular biological techniques

It has been estimated that only 1 % of microorganisms collected from the environment can be cultivated in laboratories by standard techniques and growth medium (Amann, Ludwig et al. 1995). However, by molecular biological techniques the identity of the 99 % remaining non-cultivable microorganisms can be revealed, by analysis of their nucleotide sequences. Characterization of microorganisms in environmental samples has improved revolutionary as a consequence of the development of molecular biological techniques like Polymerase Chain Reaction (PCR) and DNA sequencing.

1.4.1 Real-time PCR

PCR is a technique that specifically amplifies a selected region of a DNA sequence by use of two short DNA fragments (primers), designed complementary to the ends of the target sequence. This requires sequence information from part of the DNA sequence that is to be amplified (David P. Clark 2005). Sequence information for a huge amount of microorganisms can be found in public databases like GenBank (Benson, Karsch-Mizrachi et al. 2009).

The PCR process relies on cycles of heating and cooling, where the DNA is replicated in three steps: denaturation of the DNA strand, annealing of the primers to the complementary DNA strands, and finally elongation of the primers by DNA polymerase. The double-stranded DNA molecules obtained become targets for replication in the next cycle. The amplification process is exponential, and real-time PCR has been developed for continual measurement of the amount of DNA copies, expressed indirectly by level of fluorescence signal. SYBR Green dye can be added to the PCR for non-specific detection of double stranded DNA (Zipper, Brunner et al. 2004). Other fluorescing probes bind specifically to target sequences, like TaqMan probes (Applied Biosystems, Foster City, CA) (David P. Clark 2005).

Sometimes the sequence of interest consists of RNA and not DNA. In such cases, an additional reverse transcription step is required prior to PCR amplification, where RNA-dependent DNA polymerase synthesizes complimentary DNA (cDNA) from the RNA template. The cDNA can be further replicated by DNA-dependent DNA polymerase (Vellore, Moretz et al. 2004).

1.4.2 DNA sequencing

DNA sequencing is any process used to find the precise sequence of the nucleotides that comprise a strand of DNA. PCR is often required prior to sequencing, because the sequencing techniques require many homologous sequences of appropriate sizes. In general, DNA sequencing methods generate sub-fragments representing all possible lengths of the initial template sequence. This means that each nucleotide base in the template sequence is represented by sequence copies ending at that particular nucleotide base (David P. Clark 2005).

A method often used for sequencing is the chain termination method, where the replication is performed by DNA polymerases in a reaction mixture containing some dideoxynucleotides in addition to the standard nucleotides. Insertion of a dideoxynucleotide by chance leads to termination of replication due to lack of a 3'-OH group required for the formation of a bond between two nucleotides. Each of the resulting replicate fragments end on one of four dideoxynucleotides, which are labeled with four different fluoresce markers. The fragments are separated by length by high-resolution capillary electrophoresis. The shortest fragments reach the laser detector first, where the illuminated colour is recognized and translated into the specific dideoxynucleotide. The whole sequence of the DNA template is revealed when the longest fragment finally has passed the detector (Haqqi, Zhao et al. 2002).

1.4.3 Molecular taxonomical classification

Traditional methods for classification of microorganisms rely on phenotypic analysis, whereas modern classification is heavily dependent on genotypic analysis. The latter is generally fast and highly reproducible, but in order to identify and classify an organism based on its DNA, comparison with a reliable database with known sequences is required. GenBank is a database in daily use around the world, containing a huge amount of DNA sequences from a wide variety of organisms (Benson, Karsch-Mizrachi et al. 2009).

Microorganisms can be classified based on similarities and variations in their DNA sequences, resulting in phylogenetic trees illustrating the evolutionary relationship between them. Ribosomal RNAs (rRNAs) are excellent chronometers, meaning that differences in their nucleotide or amino acid sequences appear to be a function of their evolutionary distance (Michael T. Madigan and John M. Martinko 2006). All cells contain ribosomes, which consist of ribosomal proteins and rRNAs. For bacterial classification, the 16SrRNA gene is often used as a chronometer. 16SrRNA gene sequences are easily obtained from unknown organisms by PCR amplification followed by DNA sequencing (Clarridge 2004). Furthermore, 16SrRNA gene sequences can be classified and given a best match species identity by database search by use of the Ribosomal

Database Project (RDP), which is a database updated monthly with 16SrRNA gene sequences from the International Nucleotide Sequence Database Collaboration (DDBJ, EMBL and GenBank) (Cole, Chai et al. 2007).
When concerning viruses, it is difficult to identify and classify them without already knowing their identity. The diversity of viruses is very large, and there is no known viral chronometer resembling the 16SrRNA gene found in bacteria cells, making viral taxonomical classification based on genotype difficult (Edwards and Rohwer 2005).

1.4.4 MALDI-TOF MS fingerprinting

All bacteria contain a vast amount of ribosomes, consisting of ribosomal RNAs and proteins, making them good candidates for protein fingerprinting. The amino acid sequences of ribosomal proteins are highly conserved, but still there are small differences resulting in spectra that are unique and reproducible for each bacterial species (Sun, Teramoto et al. 2006).

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry has been adapted for identification of bacterial cells directly, with no need for protein extraction prior to analysis (Salaun, Kervarec et al. 2010). The identification is performed by smearing a bacterial colony directly on the sample target. The colony is overlaid with matrix, and thereafter irradiated by a laser pulse. The matrix absorbs most of the laser energy, leaving the proteins ionized but not fragmented. The ionized molecules are accelerated in an electric field and separated in the flight tube according to their mass to charge ratio. The proteins reach the detector at different times, contributing to a spectrum that is unique for each bacterium species (Seng, Drancourt et al. 2009). The mass spectra generated are analysed by dedicated software and compared with stored profiles in order to identify the bacteria (Sun, Teramoto et al. 2006). When developing the databases containing protein spectra of known identity, the MALDI-TOF MS fingerprinting method could hopefully replace the more time-consuming 16SrRNA gene sequencing method for bacterial identification.

1.5 Aims of the study

As part of the FFI project '1203 Biologisk beredskap - Deteksjon og identifikasjon' this work focused on characterization of airborne bacteria and Influenza A virus at Nationaltheatret subway station in Oslo.

The objectives of this work were to:

- investigate the concentration and diversity of total and endospore-forming cultivable bacteria at nighttime and daytime
- characterize the cultured bacteria based on colony morphology, hemolysis activity and antibiotic resistance
- optimize a DNA extraction method for samples containing both endospores and vegetative bacteria cells, in order to facilitate culture-independent analysis of total bacteria
- investigate the presence of Influenza A virus during a typical common flu winter season

Investigating the every-day background of airborne microorganisms is important in order to facilitate continual monitoring for detection of deviations, possibly associated to bioterrorism attacks.

2 Methods and material

Characterization of airborne microorganisms at Nationaltheatret subway station in Oslo was performed during summertime for bacteria and during wintertime for virus. Both bacteria and virus were collected with the same sampling equipment, but processed and analysed differently. The summer and winter investigations were therefore divided into separate sections: 'Characterization of airborne bacteria' and 'Detection of airborne Influenza A virus', respectively.

2.1 Air sampling

2.1.1 Location and times for investigation

Air samples were collected at Nationaltheatret subway station in Oslo. Indoor air sampling was performed at the westbound platform of the subway station, and reference outdoor air sampling was performed outside the station, between the two subway exits (Figure 2.1).

Figure 2.1: Location for outdoor sampling (left), location for indoor sampling (right) (Picture by FFI)

In order to characterize airborne bacteria, 19 air samples were collected during the summer months May - September 2010. From July to September, three samples were collected for each day of investigation: indoor at nighttime, indoor at daytime and outdoor at daytime (reference). The samples were given unique

names including the date of sampling and one of the letters N, D and R, coding short for Night, Day and Reference, respectively (e.g. “160810D”).

In order to test for presence of airborne Influenza A virus, four air samples were collected in February 2011, week 7. The samples were collected indoors at daytime during the morning rush hours, from 7:00 – 9:00 am. These samples were given the unique names D_1, D_2, D_3 and D_4.

2.1.2 Sampling instruments and performance

The instruments used for analysis at Nationaltheatret subway station were a dry filter air sampler with electret filters, SASS3100 (Research International Inc., WA, USA), and an optical particle counter with external temperature and humidity probe, Aerotrak 8220 (TSI Inc., MN, USA) (Figure 2.2). Collection of airborne particles on filter for investigation of their biological content was performed with SASS3100, whereas monitoring of the sizes and concentrations of particles was performed with Aerotrak 8220.

Figure 2.2: Aerotrak 8220 particle counter (left), SASS3100 filter sampler (right) (Picture by FFI)

Each air sampling was performed for two hours. Prior to sampling, the instruments were placed on tripods with their intakes about 1.5 metres above ground level The air flow through the filter sampler SASS3100 was set to 300 litres per minute, corresponding to filter collection of airborne particles from 36 m^3 air after two hour's sampling. The Aerotrak 8220 was programmed to monitor particle concentrations and bin them into the size intervals 0.5-1.0, 1.0-2.0, 2.0-3.0, 3.0-4.0, 4.0-5.0 and >5.0 µm. The particle monitoring and collection were performed in the same time interval for reliable correlation.

Temperature and relative humidity (%RH) was continually monitored by Aerotrak 8220 during sample collection, and a short weather report was noted for each of the samples. For the four samples collected during week 7 in February 2011, only the filter air sampler SASS3100 was used, and the number of people at the station was estimated.

All the collected filter samples were processed and analysed at the Norwegian Defence Research Establishment (FFI), located at Kjeller. Transportation time from Nationaltheatret subway station to the laboratory was approximately 1 hour. The airborne particles collected on filter were extracted by use of a manual particle extractor, SASS3010 (Research International Inc., WA, USA) (Figure 2.3). Each filter was extracted with 20 ml extraction buffer (Phosphate buffered saline + 0.05 % Triton X-100) (Sigma-Aldrich, Inc.). The particle concentrations and their size distribution profiles were obtained from Aerotrak 8220, and expressed as particles per m^3.

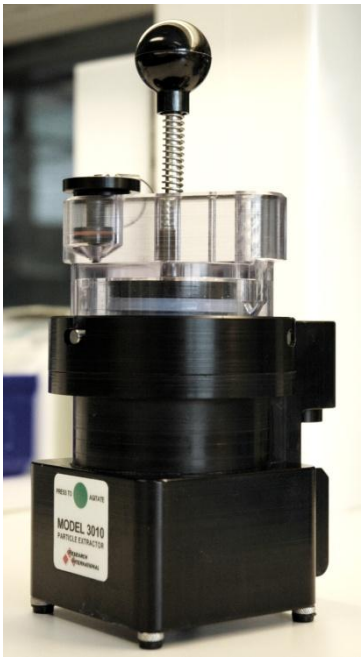

Figure 2.3: Manual Particle Extractor (SASS3010) (Picture by FFI)

2.2 Characterization of airborne bacteria

Culture-dependent bacterial analysis was performed for all the 19 air samples collected during the months May – September 2010 (section 2.2.1), including determination of concentrations and investigation of diversity. Culture-independent estimation of total bacterial concentrations was performed for 15 samples collected in the months July to September 2010 (section 2.2.2).

2.2.1 Culture-dependent bacterial analysis

2.2.1.1 Culturing

Culture plates with two different growth media were prepared: Reasoner's 2A (R2A) with cycloheximide (100 µg/ml) and Trypto-Casein Soya Agar (TSA) with cycloheximide (100 µg/ml). For the TSA growth medium, 40 grams of TSA powder (Bio-Rad Laboratories, Inc.) was added deionized water to a final volume of 1000 ml. It was autoclaved in a closed bottle (121 ˚C, 15 min), and then left to cool down to approximately 55 ˚C prior to adding cycloheximide stock solution (4 ml) (Sigma-Aldrich, Inc.). The growth medium was poured into sterile culture plates and left to solidify (30 minutes). The procedure was equal for the R2A growth medium except that 18 grams of R2A powder (Oxoid Ltd., UK) was weighed out.

For enumeration of total cultivable bacteria, each extracted sample was plated in triplicates on TSA and R2A culture plates (100 µl per plate). For enumeration of endospore-forming bacteria, each extracted sample was heat-shocked in water bath (75 °C for 20 min) prior to being plated (100 µl per plate). For the heat-shocked samples, triplicate TSA and R2A plates were made for both aerobic and anaerobic incubation. For anaerobic incubation, the culture plates were placed in an anaerobic incubation chamber (Oxoid Ltd., UK), set up in accordance with the manufacturer's instruction. All the culture plates were marked with sample name and incubated at 30 °C in the dark for 48 hours.

After incubation, the number of colony forming units (CFUs) on each culture plate was counted. For each sample, the average CFU was calculated from the triplicate plates, and the standard deviation was found. This was performed both for the TSA and R2A triplicates. The average CFU values and the standard deviations found were converted to concentrations per m^3 air, by multiplying with the dilution factor and dividing by the amount of collected air (Formula 2.2).

$$CFU\ (m^3\ air)^{-1} = (average\ CFU \times 200) / (120 \times 0.3\ m^3\ air) \qquad (Formula\ 2.2)$$

According to the formula, the limit of detection (LOD) was 5.5 CFU per m^3, corresponding to an average CFU value equal one.

2.2.1.2 Microbiological characterization

For each sample incubated for enumeration of total cultivable bacteria (section 2.2.1.1), all the morphologically distinct colonies observed were selected for further analysis. This selection was performed independently from TSA and R2A culture plates. For the samples incubated for enumeration of endospore-forming bacteria, all colonies were selected for further analysis, independent of their morphologies. Each selected colony was given a unique identifier including the sample name and a colony number (e.g. "160810D-02").

Clonal isolates were made by streaking each selected colony onto a new TSA culture plate. The plates were marked with the unique identifiers and incubated until appropriate size colonies were visible (~48 hours). After incubation, the morphologies were studied. Size, colour, shape and surface appearance of single colonies were noted, and interesting phenomenon and representative plates were photographed. Further downstream analyses of the clonal isolates were performed, as described in section 2.2.1.3 - 6.

From each plate of pure colonies derived, multiple bacterial colonies were selected for freeze storage. Freeze storage medium was made by weighing out Brain Heart Infusion powder (18.5 g) (Oxoid Ltd, UK) into an autoclavable bottle, and adding deionized water (385 ml) and 85% Glycerol (115 ml) (Merck KGaA, Darmstadt, Germany). The bottle was heated on water bath to dissolve the powder prior to autoclaving (121 °C, 15 min). The cooled medium was pipetted (1 ml) into Cryo tubes (2.0 ml, Thermo Fisher Scientific Inc.). Each selected colony was transferred to a tube marked with its unique identifier, and stored at -80 °C.

2.2.1.3 Examination of hemolytic activity

All the clonal isolates (section 2.2.1.2) were tested for hemolytic activity. Culture plates with Colombia blood agar (5 % sheep blood) was obtained from Oxoid (Oxoid Ltd, UK). From each clonal isolate, a colony was transferred to blood agar and incubated at 30 °C in the dark for 48 hours. After incubation, each colony was classified as α-hemolytic (green halo around the colony), ß-hemolytic (transparent halo around the colony) or γ-hemolytic (no hemolysis).

2.2.1.4 Determination of antibiotic resistance

All the clonal isolates (section 2.2.1.2) were tested for antibiotic resistance on five different antibiotics media. Five bottles with 1000 ml TSA growth medium were prepared (section 2.2.1.1) to make five different media by adding ampicillin, tetracycline, nalidixic acid, streptomycin and chloramphenicol (Sigma-Aldrich, Inc.) to the final concentrations of 50, 10, 20, 50 and 25 µg/ml, respectively. The antibiotics growth media were poured into sterile culture plates and left to solidify (30 minutes). A colony from each clonal isolate (section 2.2.1.2) was transferred to each of the five different antibiotic culture plates, and incubated at

30°C in the dark for 48 hours. After incubation, each plate was inspected and antibiotic resistance noted for each colony as positive or negative.

2.2.1.5 16SrRNA gene sequencing

From each plate of pure colonies derived, a bacteria colony was transferred to an Eppendorf tube (1.5 ml, Axygene Inc.) with nuclease-free water (100 µl) (Ambion, Life Technologies), marked with the colony's unique identifier. Lysis of the bacteria cells was performed with four freeze/thaw cycles with liquid nitrogen bath for freezing (1 minute) and heat block for thawing (94 ˚C, 1 min). The tubes were vortexed between the cycles. After lysis, the tubes were centrifuged (10,000 *g*, 3 min) to pellet bacterial cell content. The supernatants were used as template for real-time PCR amplification using SYBR Green detection (Zipper, Brunner et al. 2004).

96-well PCR plates were prepared by adding 27 µl reaction mixture to each well and then 3 µl of template. Per reaction, the mixture contained SYBR Green master mix (15 µl) (Roche Diagnostics, USA), nuclease-free water (6 µl) and 10 µM forward and reverse primer (3 µl each). Universal bacterial 16SrRNA gene primers were obtained from Invitrogen (Invitrogen Ltd, UK), with the following sequences: forward 27F (5'-GAGTTTGATCMTGGCTCAG-3') (Lane 1991) and reverse 1492R (5'-ACGGYTACCTTGTTACGACTT-3') (Weisburg, Barns et al. 1991)., where M codes for the nucleotide bases A or C, and Y codes for C or T.

The PCR was performed in a LightCycler 480 instrument (Roche Diagnostics Corp., IN, USA) (Figure 2.4) under the following conditions: initial denaturation (95 °C, 5 minutes), 35 cycles of denaturation (95 °C, 20 seconds), annealing (55 °C, 20 seconds) and extension (72 °C, 90 seconds), and finally a terminal extension (72 °C, 10 minutes).

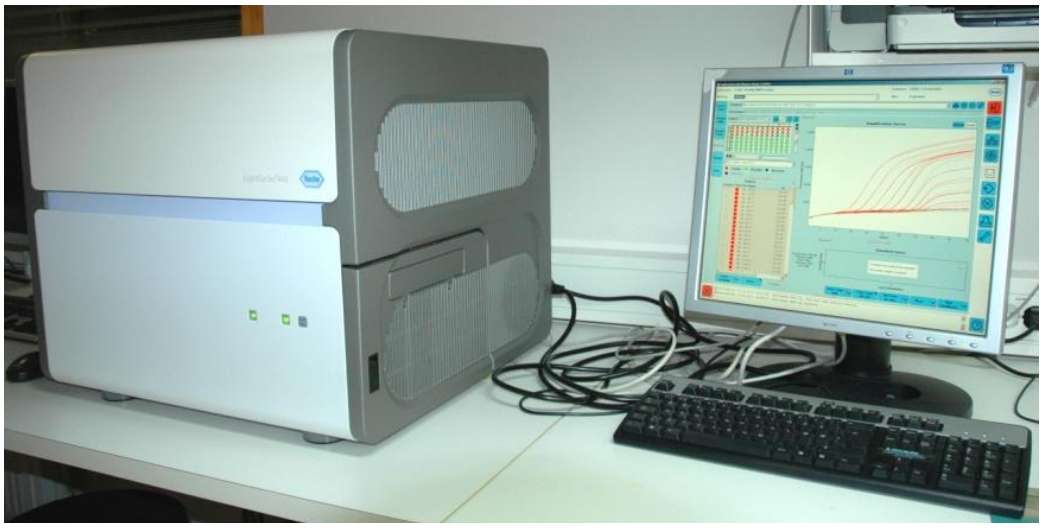

Figure 2.4: LightCycler 480 PCR machine (left) and LightCycler Computer Program (right) (Roche Diagnostics Corp., IN, USA) (Picture by FFI)

The 16SrRNA gene PCR amplified samples were sent to Eurofins MWG Operon in Germany for purification and 16SrRNA gene sequencing (Eurofins MWG Operon, Germany). Both forward and reverse sequencing were ordered for better result reliability. The result sequences were obtained from Eurofins MWG webpage and imported into the standardized Biological Background Study BioNumerics database (BioNumerics 6.0, Applied Maths, Belgium). In BioNumerics, the two sequences (forward and reverse) from each clonal isolate were combined to one sequence that was given the unique identifier name of the original clonal isolate (e.g. "160810D-02"). The sequences were further trimmed, manually inspected and corrected for obvious base calling errors (performed by Marius Dybwad at FFI). Only combined sequences with <1% ambiguous bases and more than 400 base pairs were used for further analysis.

All the approved sequences were taxonomically classified down to genus level by the Classifier tool at the Ribosomal Database Project (RDP), release 10.18 (Cole, Chai et al. 2007). The classification results were compared to the corresponding colony morphology observations (section 2.2.1.2), in order to look for interesting patterns for microbiological identification. Furthermore, by RDP_SeqMatch the sequenced isolates were given the best species scores available for identification (Cole, Chai et al. 2007).

2.2.1.6 MALDI-TOF MS fingerprinting

The reliability of MALDI-TOF MS fingerprinting for identification of bacterial isolates was compared to the best match species identities given by RDP_SeqMatch, as MALDI-TOF MS fingerprinting is quicker and less expensive than the sequencing process required prior to PDR_SeqMatch analysis. In order to reduce replicate isolates for this analysis, hierarchical clustering was performed for the sequenced isolates. The sequences were binned into operational taxonomic units (OTUs) of 97 % sequence similarity, and within each group, the sequence that most accurately represented all the group members was chosen as the OTU representative isolate.

Freeze stored isolates representing the 84 resulting OTUs (section 2.2.1.5) were thawed and streak-plated onto new TSA plates marked with the unique colony identifiers. After incubation (30 °C, 48 hours) the colonies were given a best-score identification by MALDI-TOF MS fingerprinting technology.

Matrix solution was prepared prior to analysis as following: stock solution was made in an Eppendorf tube by mixing ultra pure water (475 µl), acetonitrile (ACN) (500 µl) and 100% trifluoro acetic acid (TFA) (25 µl) (Sigma-Aldrich, Inc.). From this stock solution, 250 µl was transferred to a tube with portioned dry α-Cyano-4-hydroxycinnamic acid (HCCA), obtained from Daltonics (Bruker Daltonics Inc). The matrix was dissolved by vortexing at room temperature until

clear solution, to obtain a final concentration of 10 mg HCCA/ml. Analysis of the 84 OTU isolates was performed by a direct transfer method, by smearing a single colony as a thin film directly on a MALDI target. Metal plates with 96 spots were used, where each OTU isolate was transferred in three parallels onto the plate. An *Escherichia coli* standard was included on each plate (Bruker Daltonics Inc). Both the standard and all the samples were overlaid with HCCA matrix solution (1 µl) and left to air dry prior to analysis.

When dry, the bacterial isolates were analysed by Biotyper 2.0, which is a fully automated system coupled to a Microflex MALDI-TOF MS instrument (Figure 2.5) (Bruker Daltonics, Inc.). The best-match identification results obtained were compared to the results obtained from database search with RDP_SeqMatch (section 2.2.1.5).

Figure 2.5: MicroFlex MALDI-TOF MS instrument (Bruker Daltonics Inc., Germany) (picture by FFI)

2.2.2 Culture-independent bacterial analysis

2.2.2.1 Bead mill homogenization optimization

Analysis of total microbial DNA in environmental samples required an extraction method efficient in lysis of both endospores and vegetative cells. Cell lysis and DNA extraction have often been performed by freeze-thawing, bead mill

homogenization, sonication and chemical lysis. However, a study by K.L. Anderson and S. Lebepe-Mazur compared all these methods for bacterial lysis, and found that bead mill homogenization gave the greatest quantity of extracted DNA (Anderson and Lebepe-Mazur 2003). Unfortunately, no common bead mill homogenization protocol for combined samples with bacterial endospores and vegetative cells was found during a literature study, so investigation was needed to find the optimal homogenization conditions for this experiment.

Optimization was performed separately for bacterial endospores (*Bacillus atrophaeus)* and vegetative cells (*E. coli)*, in order to compare the effect of the different homogenization conditions on endospores and cells. DNA extraction was performed by MoBio UltraClean Soil DNA Isolation kit (MoBio Laboratories, Solana Beach, CA), with some modifications. The bead tubes supplied with the kit were replaced with autoclaved (121 °C, 45 minutes) bead mill homogenization tubes (2 ml, Sarstedt AG & Co, Germany) filled with Zirconia/Silica beads (0.1 mm, BioSpec Products Inc., USA). A variety of different bead mill homogenization conditions were tested for bacterial samples (100 µl, $1.0*10^6$ endospores or cells /µl):

- the optimal amount of the DNA extraction reagents: MoBio Bead Solution, Inhibitor Removal Solution and S1 Lysis Solution (MoBio Laboratories, Solana Beach, CA)
- the effect of antifoam A (Sigma-Aldrich, Inc.)
- the optimal amount of Zirconia/Silica beads
- the optimal duration of bead beating for release of maximum amount of DNA with minimum fragmentation
- final DNA isolation by silica column (supplied with the kit) or ethanol precipitation

For each test, the amount and quality of the resulting extracted DNA was tested by real-time PCR (section 2.2.1.5).

2.2.2.2 Isolation of total DNA

For 15 samples collected at Nationaltheatret subway station, the remaining sample extract (~18 ml) from culture-dependent analysis (section 2.2.1.1) was used for total DNA extraction, by use of MoBio UltraClean Soil DNA Isolation kit.

15 bead mill homogenization tubes were prepared with MoBio Bead Solution (440 µl), Inhibitor Removal Solution (200 µl) and S1 Lysis Solution (60 µl), as found to be optimal (section 2.2.2.1). The 15 sample extracts were centrifuged (5 °C, 6,000 *g*, 45 min), and the supernatants discarded prior to adding the pellets to the bead tubes marked with the unique sample names. Bead mill homogenization was performed in a Mini Beadbeater-8 (BioSpec Products Inc., USA) for 2 minutes at maximum speed.

After centrifugation (10,000 *g*, 3 min), the supernatants were transferred to 15 new Eppendorf tubes (2.0 ml) and added S2 Protein Precipitation Solution (500 µl). The tubes were briefly vortexed and chilled for 10 minutes at 4 °C, and finally centrifuged for 3 minutes at 10,000 *g*. The supernatant (~1400 µl) was combined with MoBio S3 High Salt DNA-binding Solution (2800 µl), vortexed briefly and run on silica column in 650 µl aliquots. The column was washed with MoBio S4 High Salt Ethanol Wash Solution (300 µl) and the nucleic acids were eluted by running MoBio S5 Elution Buffer (60 µl) through the column three consecutive times to maximize the elution efficiency. The 15 DNA isolates were stored at – 20 °C until further analyses.

2.2.2.3 Analysis by gel electrophoresis

2 µl from each of the 15 DNA isolates (section 2.2.2.2) were amplified by real-time PCR (section 2.2.1.5) prior to analysis by gel electrophoresis. The remaining DNA isolates were stored for future analyses.

The PCR conditions were as described in section 2.2.1.5, except use of 55 ºC during the annealing steps. The reaction mixture was prepared with 2X SYBR Green master mix (10 µl), nuclease-free water (3 µl), 13.4 µg/µl Bovine Serum Albumin (BSA) (1 µl) (Applied Biosystems, USA) and 10 µM forward and reverse primer (2 µl each), per reaction. A 96-well PCR plate was prepared by adding 18 µl reaction mixture to each well and then 2 µl of template (DNA isolate).

Nitrile glows were used for safety during gel electrophoresis set up. Agarose gel was prepared by adding 1 gram agarose (LE agarose, Seakem) to 100 ml Trizma Borsyre EDTA (TBE) buffer (11 g/L Trizma, 6 g/L Borsyre, 4 ml/L 0.5 M EDTA) and heating this in a microwave oven at maximum effect until a homogene solution was formed (2 minutes). The agar solution was left to cool down to about 50 °C before SYBR safe (10 µl) (Invitrogen Ltd, UK) was added and mixed in by gentle swirling. A gel container was prepared with a 30-wells comb placed 2 mm from the bottom. The agar solution was poured into the container and left to solidify (approximately 40 minutes). When solid, the comb was removed and the gel placed in the electrophoresis container, filled with TBE buffer.

Each of the 15 PCR amplified DNA isolates were prepared by mixing 4 µl PCR product with 4 µl Loading Buffer (0.05 % xylene cyanol, 0.25 % bromfenol, 60 % glyserol) and applied in separate wells on the gel. A negative PCR control was included, and 1 kb plus DNA ladder (5 µl) (Invitrogen Ltd, UK) was loaded on the gel in the first and the last well. The gel electrophoresis was performed at 88 Volt for ~1 hour. The gel was then photographed under UV-radiation for visualization of the PCR products.

2.3 Detection of airborne Influenza A virus

The four samples collected during February 2011, week 7 (section 2.1.1) were investigated for presence of Influenza A virus RNA.

2.3.1 RNA isolation

2.3.1.1 Method verification

A trizol-chloroform based method was used for RNA extraction as recommended for samples contaminated with particulate matter (Fabian, McDevitt et al. 2009). The protocol for trizol-chloroform RNA extraction published by Gern et al was slightly modified for this experiment and tested on commercial Influenza A virus particles in order to verify the method (Gern, Martin et al. 2002). Virus particles were obtained from Helvetica Health Care (HHC, Switzerland) and were of type H1N1 Influenza A (strain Singapore/63/04). The concentration was determined to be $7*10^8$ virus particles per ml based on the manufacturer in house standard.

Influenza A virus particles (100 µl, $7*10^7$ particles/µl) were mixed with 1 ml TRIzol LS Reagent (Invitrogen Ltd, UK) in a 2 ml Eppendorf tube by vortexing (25 °C, 10 min). The resulting mixture was supplied with chloroform (270 µl), vortexed (25 °C, 5 min), and then centrifuged (4 °C, 12,000 *g*, 15 min). The aqueous phase (~800 µl) was transferred to a new tube and mixed with 5 µl RNase-free glycogen (20 µg/µl) (Applied Biosystems, USA) and 670 µl isopropanol (Sigma-Aldrich, Inc.) and incubated at −20°C for 1 hour to precipitate RNA. The RNA precipitant was pelleted by centrifugation (4 °C, 12,000 *g*; 10 min). Further, the RNA pellet was washed once with 75 % ethanol (1400 µl) (Arcus, Norway), centrifuged (4 °C, 12,000 *g*, 5 min), and air dried for 10 minutes prior to being resuspended in nuclease-free water (20 µl). Qualitative detection of isolated RNA was performed by one-step reverse transcriptase real-time PCR, as described in section 2.3.2.

2.3.1.2 Isolation from samples

The four filter air samples collected at successive days at Nationaltheatret subway station (D_1, D_2, D_3 and D_4) were each extracted with 20 ml extraction buffer (section 2.1.2). The sample extract was centrifuged in two 15 ml tubes (5 °C, 6000 *g*; 45 min), and the supernatant transferred to 8 ml ultracentrifugation tubes while the pellets were kept on ice prior to RNA extraction. Ultracentrifugation was performed for 90 minutes (4 °C, 136,000 *g*) (Krammer, Nakowitsch et al. 2010). Thereafter the supernatant was discarded and the pellets combined with the pellets kept on ice, giving approximately 100 µl concentrated sample. RNA extraction was performed (section 2.3.1.1), and the samples were resuspended in nuclease-free water (12 µl).

RNA isolates from the two first samples (D_1 and D_2) were frozen at -80 ˚C until the third day, when they were analysed together with sample D_3, as described in section 2.3.2. Sample D_4 was analysed as described in section 2.3.3.

2.3.2 Reverse transcriptase real-time PCR

2.3.2.1 Primer and probe design

The highly conserved matrix protein gene of the viral influenza A genome was selected as the amplification target (Fouchier, Bestebroer et al. 2000). Two pairs of primers known to be specific for the matrix gene were tested, in order to choose the most sensitive and specific pair for this study. The first primer pair with a corresponding hybridization probe has been used in a number of projects for Influenza A detection, and was designed by van Elden et al (van Elden, Nijhuis et al. 2001). For this study, the van Elden primers and probe were obtained from TIB Molbiol (GmbH, Germany).

The second primer pair, with corresponding hybridization probe, was designed by Ward et al (Ward, Dempsey et al. 2004) and obtained from TIB Molbiol (GmbH, Germany). The hybridization probes were designed with 5´reporter dye (6FAM) and 3´quencher dye (BBQ) for specific real-time fluorescence monitoring of gene copies made during PCR. The expected lengths of the PCR products were calculated from information about primer hybridization locations in the target gene (Table 2.1).

Table 2.1: Van Elden and Ward primer and probe sequences. The Y-nucleotide base codes for nucleotide C or T.

van Elden assay	Sequence	Nucleotide location
Reverse primer	5´-GGACTGCAGCGTAGACGCTT-3´	217–236
Forward primer	5´-CATYCTGTTGTATATGAGGCCCAT-3´	382–405
Probe	5´-TCAGTTATTCTGCTGGTGCACTTGCCA-3´	349–376
PCR product	Length of PCR product = 405–217+1 = 188 bp	
Ward assay	Sequence	Nucleotide location
Forward primer	5´-AAGACCAATCCTGTCACCTCTGA-3´	169-191
Reverse primer	5´-CAAAGCGTCTACGCTGCAGTCC-3´	242-263
Probe	5´-TTTGTGTTCACGCTCACCGT-3´	209-228
PCR product	Length of PCR product = 263–169+1 = 95 bp	

The specificity of the primers and probes from both assays were tested by *in silico* BLAST-search among consensus sequences in GenBank, belonging to all sequenced viral, fungal and bacterial strains (Benson, Karsch-Mizrachi et al. 2009). 100 % specificity for the Influenza A genome was required in this study due to low expected virus concentration in the air samples.

The sensitivity of the assays (van Elden and Ward) were tested by looking for base-pair hybridization errors between the primer and probe sequences and the matrix gene sequence from different Influenza A strains, obtained from the Influenza Research Database (Squires, Chang et al. 2008). The specificity and sensitivity were also tested by molecular biological techniques, as described in section 2.3.2.2.

2.3.2.2 Method optimization

Influenza A virus genome detection required a reverse transcriptase step for conversion of the viral RNA genome to cDNA prior to DNA-dependent DNA amplification (Fouchier, Bestebroer et al. 2000). Both reverse transcription and PCR amplification were performed in the same PCR well by use of a reaction mixture for one-step reverse transcriptase real-time PCR; 'RealTime Ready RNA Virus Master' (Roche Applied Science, USA).

LightCycler 480 real-time PCR System with 96-well reaction plates was used for analysis (Roche Applied Science, USA). The PCR assay was performed with a final volume of 20 µl per well, containing RNA sample, probe, primers, reaction mixture (buffer and enzyme blend) and water, according to the manufacturer's instructions. Two assays corresponding to the two different primer pairs were compared, and they required slightly different amounts of PCR reagents (Table 2.2). Slightly different PCR conditions were also recommended for the two assays, for optimal amplification of target sequence (Table 2.3).

Table 2.2: PCR reaction mixtures

Amount per PCR well (µl)		Reagent
van Elden	Ward	
4.4	4.4	2X master mix
1	1	10µM Probe
2	2	10µM Forward primer
1	3	10µM Reverse primer
6.6	5.6	Water (nuclease-free)
5	5	RNA sample

Table 2.3: Amplification programmes. 45 cycles of amplification were recommended for van Elden assay, and 50 cycles for Ward assay

	Van Elden assay		Ward assay	
Program	Temperature (°C)	Duration	Temperature (°C)	Duration (min)
Reverse transcription (1 cycle)	50	8 minutes	50	8 minutes
Initial denaturation (1 cycle)	95	30 seconds	95	30 seconds
Amplification	95	1 second	95	15 seconds
	60	20 seconds	58	45 seconds
	72	1 second	58	45 seconds
Cooling (1 cycle)	40	30 seconds	40	30 seconds

Amount of RNA isolated from commercial virus particles (section 2.3.1.1) were tested by both assays (van Elden and Ward). Genomic RNA from Influenza A virus (A/H1N1/Virginia/ATCC/2009) was ordered as control RNA for the PCR amplification process (LCG Standards, England). For each PCR plate, negative controls (water) and positive RNA controls (ATCC RNA) were included.

After PCR, the products were analysed on agarosis gel (section 2.2.2.3) to compare the specificity and sensitivity of the two assays (van Elden and Ward).

2.3.2.3 Standard curve construction

Two alternative methods for standard curve construction were tested. The first method constructed a curve based on theoretical concentrations of RNA, calculated from known initial virus particle concentration prior to RNA extraction. Loss of RNA during RNA extraction was expected, both when extracting RNA from commercial viral particles for standard curve preparation and when extracting RNA from unknown air samples. The percentage-wise loss of RNA was expected to stay constant in both cases, and thereby work as an internal calibration, making it possible to find the true number of initial virus particles collected, independent of the RNA loss caused during extraction.

The second method for standard curve construction did not calculate for the loss of RNA during extraction, but gave exactly correct concentrations of the standards used. A DNA sample of known concentration was used directly for standard curve construction, and therefore no extraction procedure was needed prior to measurement, ensuring no unexpected loss of DNA.

RNA standard curve

1 ml solution containing 7*10^8 virus particles were obtained from Helvetica Health Care (HHC, Switzerland). Viral RNA was extracted with TRIzol (section 2.2.1.2), and the RNA pellet was resuspended in 14 µl nuclease-free water to obtain a theoretical concentration of $5.0*10^7$ RNA copies /µl. This concentrated solution was used as the highest standard for standard curve construction. 11 lower concentrated standards were prepared by five-fold serial dilution from the highest concentrated solution (5 µl standard + 20 µl water).

Reverse transcriptase real-time PCR was performed with 4 parallels of each standard, where 2 µl standard solution was added to each PCR well containing 18 µl reaction mixture. The Ward PCR assay was used for amplification (Table 2.2 and Table 2.3), with 7.6 µl water per reaction. After PCR, a standard curve was constructed by plotting the crossing point value (Cp value) obtained for each standard against the log quantity of the corresponding standard RNA copy number. In this case the standard RNA copy numbers were the theoretical start concentrations of RNA in the PCR wells (Table 2.4). The standard curve was saved to be included in later analyses of samples for quantification of their viral content.

DNA standard curve

Plasmids containing the matrix protein gene (DNA) of Influenza A virus were obtained from TIB Molbiol (GmbH, Germany), verified by OD measurement to contain 10^{10} DNA copies. The DNA was dissolved in 100 µl water to obtain a concentration of $1.0*10^8$ copies /µl. This concentrated solution was used as the highest standard for standard curve construction. 5 lower concentrated standards were prepared by ten-fold serial dilution from the highest concentrated solution (10 µl standard + 90 µl water), and then 5 lower standards were made by five-fold dilution (5 µl standard + 20 µl water).

Reverse transcriptase real-time PCR was performed with 3 parallels of each standard, where 5 µl standard solution was added to each PCR well containing 15 µl reaction mixture. The Ward PCR assay was used (Table 2.2 and Table 2.3). A standard curve for viral RNA quantification was constructed by plotting the Cp value obtained for each standard against the log quantity of the corresponding standard copy number. In this case, the standard copy numbers were the start concentrations of DNA in the PCR wells (Table 2.4). The standard curve was saved to be included in later analyses of samples for quantification of their viral content.

Table 2.4: Standard concentrations in PCR

Standard number	Copy number in PCR well (/µl)	
	RNA standard	DNA standard
1	$5{,}0*10^{6}$	$2{,}5*10^{7}$
2	$1{,}0*10^{6}$	$2{,}5*10^{6}$
3	$2{,}0*10^{5}$	$2{,}5*10^{5}$
4	$4{,}0*10^{4}$	$2{,}5*10^{4}$
5	$8{,}0*10^{3}$	$2{,}5*10^{3}$
6	$1{,}6*10^{3}$	$2{,}5*10^{2}$
7	$3{,}2*10^{2}$	$5.0*10^{1}$
8	$6{,}4*10^{1}$	$1.0*10^{1}$
9	$1{,}28*10^{1}$	$2.0*10^{0}$
10	$2{,}56*10^{0}$	$4.0*10^{-1}$
11	$5{,}12*10^{-1}$	$8.0*10^{-2}$
12	$1{,}024*10^{-1}$	

2.3.2.4 Quantitative analysis of virus content in samples

The RNA isolated samples D_1, D_2 and D_3 (section 2.3.1.2) were tested for Influenza A viral RNA by Ward reverse transcriptase real-time PCR assay (section 2.3.2.2). The PCR was prepared with two parallels of each sample, positive controls (ATCC RNA) and negative controls (water). Included in the PCR were also two parallels of the third highest concentrated RNA- and DNA standards (Table 2.4), for correlation with the corresponding saved standard curves.

Finally, after amplification, the Cp values obtained from the amplification curves of the unknown samples were plotted on the standard curves for quantification of influenza A virus RNA copies.

2.3.3 Inhibition test

A fourth air sample (D_4) was collected at Nationaltheatret subway station in order to test for inhibition of the PCR amplification process. The RNA in the sample was extracted with trizol (section 2.3.1.1), and re-suspended in 20 µl nuclease-free water. Ward reaction mixture was prepared, both with and without BSA (13.4 µg/µl). A pre-study performed by Marius Dybwad revealed samples from Nationaltheatret subway station inhibited 16SrRNA gene PCR, and that addition of BSA counteracted the observed inhibition (Marius Dybwad, FFI, unpublished results). Those air samples were not extracted with trizol, but the effect of reducing inhibition with BSA was described also for trizol-extracted samples in an article published by Silvy et al (Silvy, Pic et al. 2004).

A PCR plate was prepared with five wells containing reaction mixture (RM) with BSA (13 µl per well) and seven wells containing RM without BSA (13 µl per well). A RNA spike was prepared from aliquots saved during construction of the RNA standard curve. RNA spike (2 µl) and isolated RNA from air sample D_4 (5 µl) were added to the PCR wells (Table 2.5). Included in the PCR were also a RNA standard and a DNA standard (Table 2.4) for correlation with the corresponding saved standard curves. The PCR was run with Ward reverse transcriptase real-time PCR conditions (section 2.3.2.2).

Table 2.5: Schematic PCR assay

	1	2	3	4	5	6
A	RM+BSA Spike Sample	RM+BSA Spike water	RM+BSA water Sample	RM+BSA negative	RM+BSA negative	RM DNA Standard
B	RM Spike Sample	RM Spike water	RM water Sample	RM negative	RM negative	RM RNA standard

An agarosis gel was prepared for analysis of the PCR products from inhibition test (section 2.1.4.3). The PCR products amplified in the PCR wells A1-4 and B1-4 (Table 2.5) were applied on the gel in separate wells, and included was also previously amplified positive control (ATCC) RNA.

3 Results

3.1 Air sampling

3.1.1 Samples and sampling conditions

In order to characterize the airborne bacteria at Nationaltheatret subway station, 19 air samples were harvested in the period May - September 2011 (Table 3.1). The weather conditions for the nighttime, daytime and reference samples were stable throughout the sampling period (Table 3.1 and Figure 3.1). The nighttime and reference samples showed greater variations in humidity than the daytime samples, but this observation was not found to influence on the bacterial concentrations found (Table 3.3).

Table 3.1: Weather conditions measured by Aerotrak 8220, expressed as average values for the time interval of measurement +/- the standard deviations

Sample name	Time interval	Weather report	Temperature (°C)	Humidity (%RH)
180510D	11:00 – 13:00	Indoors conditions	17.5 +/-0.4	69.1 +/-3.8
140610D	11:00 – 13:00	Indoors conditions	18.3 +/-0.3	62.9 +/-6.4
280610D	11:00 – 13:00	Indoors conditions	19.6 +/-0.4	69.8 +/-3.3
280610N	02:00 – 04:00	Indoors conditions	18.9 +/-0.5	63.3 +/-2.0
260710D	10:30 – 12:30	Indoors conditions	19.4 +/-0.5	75.6 +/-0.5
260710N	02:30 – 04:30	Indoors conditions	20.4 +/-0.3	71.6 +/-1.5
260710R	09:00 – 11:00	Overcast, no wind	20.2 +/-1.2	65.2 +/-4.4
160810D	11:00 – 13:00	Indoors conditions	21.7 +/-0.5	69.5 +/-5.9
160810N	01:30 – 03:30	Indoors conditions	19.9 +/-0.2	74.7 +/-3.4
160810R	09:00 – 11:00	Overcast, light wind	19.0 +/-0.6	56.2 +/-3.3
300810D	10:56 – 12:56	Indoors conditions	19.3 +/-0.3	66.3 +/-6.9
300810N	01:20 – 03:20	Indoors conditions	18.8 +/-0.3	61.1 +/-1.4
300810R	08:48 – 10:48	Sunshine, no wind	17.7 +/-1.1	46.0 +/-3.7
130910D	10:54 – 12:54	Indoors conditions	19.3 +/-0.5	69.8 +/-5.0
130910N	01:20 – 03:20	Indoors conditions	18.3 +/-0.5	67.1 +/-1.1
130910R	08:45 – 10:45	Sunshine, no wind	15.9 +/-1.7	67.4 +/-8.3
270910D	09:47 – 11:47	Indoors conditions	16.6 +/-0.3	62.4 +/-7.4
270910N	01:30 – 03:30	Indoors conditions	14.9 +/-0.4	47.6 +/-0.8
270910R	11:50 – 13:50	Sunshine, light wind	12.6 +/-1.0	47.6 +/-3.2

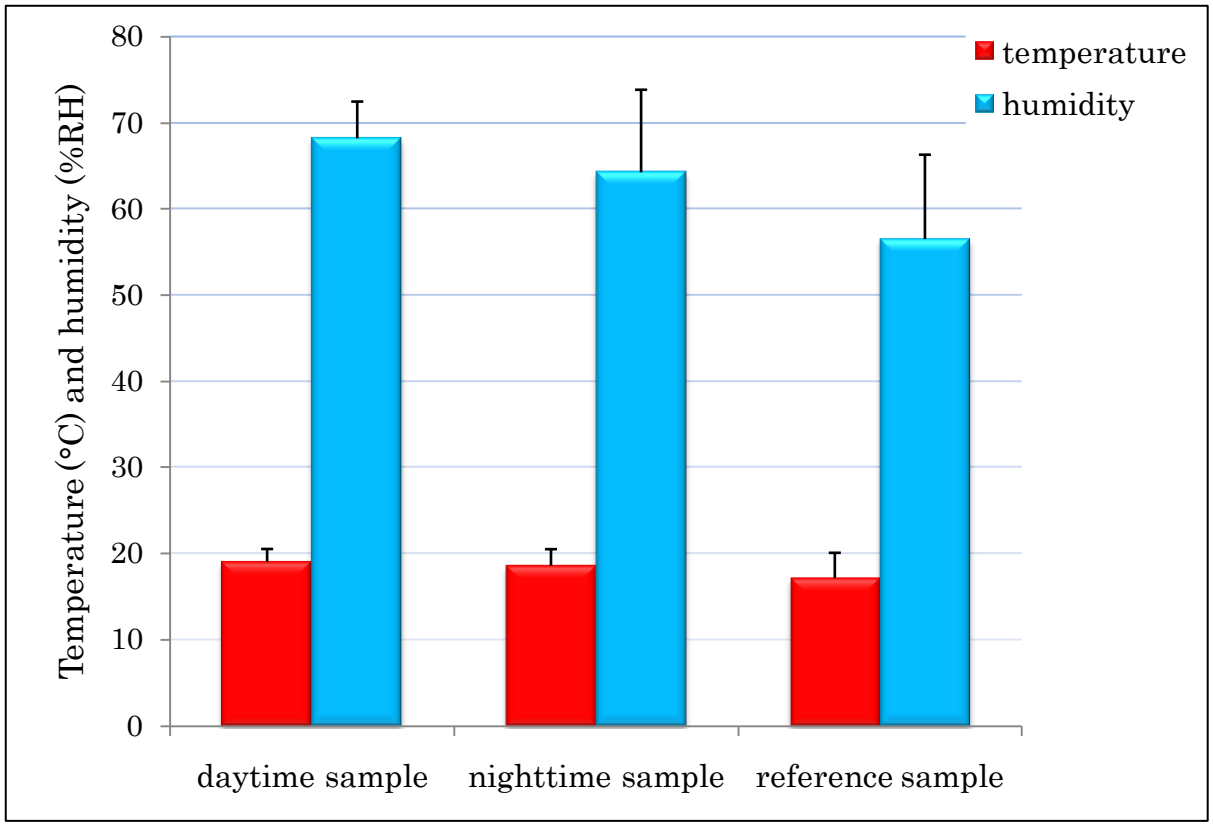

Figure 3.1: Average temperature and humidity values obtained for the daytime, nighttime and reference samples, with error bars illustrating the standard deviations

In order to investigate the presence of Influenza A virus during the common flu winter season, four air samples were collected in week 7, February 2011 (Table 3.2). The number of people at the station stayed constant for the four days of sampling.

Table 3.2: Number of people waiting for a train or passing by at the westbound platform during the morning rush hours in week 7, 2011

Sample name	Time interval	Average number of people
D_1	07:00 – 09:00	Approximately 100
D_2	07:00 – 09:00	Approximately 100
D_3	07:00 – 09:00	Approximately 100
D_4	07:00 – 09:00	Approximately 100

3.1.2 Airborne particle concentrations

In order to investigate aerosol size distribution profiles for samples collected indoor at day, indoor at night and outdoor, aerosol sizes and concentrations were monitored during sample collection in May – September 2011 (Figure 3.2). The aerosol concentrations and size distribution profiles were stable throughout the study for the daytime, nighttime and reference samples. No apparent correlation was observed between the aerosol concentrations and the daily weather conditions wind, temperature and %RH (Table 3.1 and Table A.1 in Appendix A).

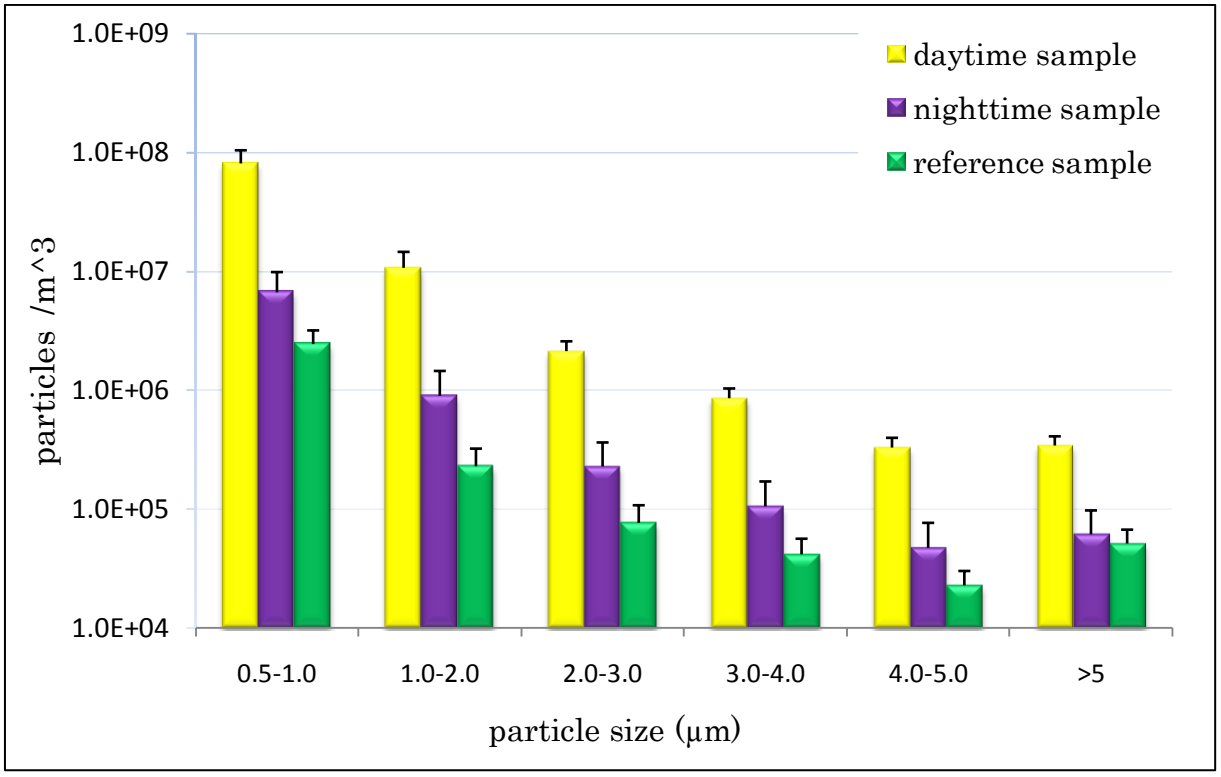

Figure 3.2: Airborne particle concentrations and size distribution profiles for the average daytime, nighttime and reference sample, with error bars illustrating the standard deviations

For all size intervals, the particle concentrations were highest for indoor daytime samples and lowest for outdoor reference samples. 90 % of the total numbers of airborne particles for indoor, outdoor and reference samples where represented by indoor daytime samples (Figure 3.2). For the particle size distribution profiles it was observed that the smallest size-interval (0.5 - 1.0 µm) represented 85 % of the total particle count.

3.2 Characterization of airborne bacteria

3.2.1 Cultured bacteria

3.2.1.1 Sample concentrations

In order to characterize as many viable bacteria as possible, total culturing and selective endospore culturing were performed on both TSA and R2A growth media (Table 3.3). However, neither TSA nor R2A was found to provide better conditions for growth, so average colony count numbers from TSA and R2A media were used for further result analysis. No correlation was observed when comparing the cultivable bacterial concentrations found and the weather conditions (Tables 3.1 and 3.3).

Table 3.3: Average bacterial concentrations calculated from colony growth on triplicate plates, expressed as CFU m^{-3} of air +/- the standard deviation

Sample	CFU m^{-3} *					
	Total culturing		Endospore culturing			
	Aerobe incubation		Aerobe incubation		Anaerobe incubation	
	TSA	R2A	TSA	R2A	TSA	R2A
180510D	480 +/-26	517 +/-22	<LOD	<LOD	<LOD	<LOD
140610D	461 +/-82	454 +/-74	11 +/-0	17 +/-10	<LOD	<LOD
280610D	352 +/-69	344 +/-20	15 +/-8	7 +/-8	<LOD	<LOD
280610N	13 +/-18	30 +/-13	<LOD	<LOD	<LOD	<LOD
260710D	222 +/-29	289 +/-67	<LOD	<LOD	<LOD	<LOD
260710N	17 +/-6	30 +/-14	<LOD	<LOD	<LOD	<LOD
260710R	35 +/-17	76 +/-55	22 +/-24	76 +/-63	<LOD	30 +/-14
160810D	461 +/-212	276 +/-12	<LOD	<LOD	<LOD	<LOD
160810N	31 +/-3	30 +/-3	<LOD	<LOD	<LOD	<LOD
160810R	181 +/-25	104 +/-33	20 +/-12	11 +/-10	6 +/-0	<LOD
300810D	441 +/-50	444 +/-112	11 +/-10	6 +/-6	<LOD	<LOD
300810N	43 +/-20	28 +/-17	<LOD	<LOD	<LOD	<LOD
300810R	161 +/-19	228 +/-124	13 +/-3	6 +/-6	<LOD	<LOD
130910D	469 +/-143	493 +/-43	65 +/-98	11 +/-6	<LOD	<LOD
130910N	6 +/-6	7 +/-3	<LOD	<LOD	<LOD	<LOD
130910R	57 +/-26	137 +/-18	7 +/-8	11 +/-11	<LOD	<LOD
270910D	341 +/-46	350 +/-47	26 +/-18	7 +/-3	22 +/-11	9 +/-6
270910N	7 +/-8	<LOD	<LOD	<LOD	<LOD	<LOD
270910R	70 +/-35	94 +/-15	7 +/-3	9 +/-12	9 +/-8	<LOD

*The LOD was set to 5.5 CFU per m^3 air, as found to correspond to average CFU from triplicate plates equal one (Formula 2.2 section 2.2.1.1)

The concentrations of airborne bacteria found from total culturing were stable for the daytime, nighttime and reference samples (Figure 3.3). However, the concentration of the average daytime sample was higher than the average nighttime and reference sample, by a factor of approximately 20 and 4, respectively.

The daytime samples contained 75 % of all the cultivable bacteria found (Table 3.3). Further, 90 % of the total average particle concentrations were measured by Aerotrak 8220 for the daytime samples (Figure 3.2), giving that the nighttime and reference samples together were 2.5 times higher concentrated with cultivable bacteria per collected particle than the daytime samples.

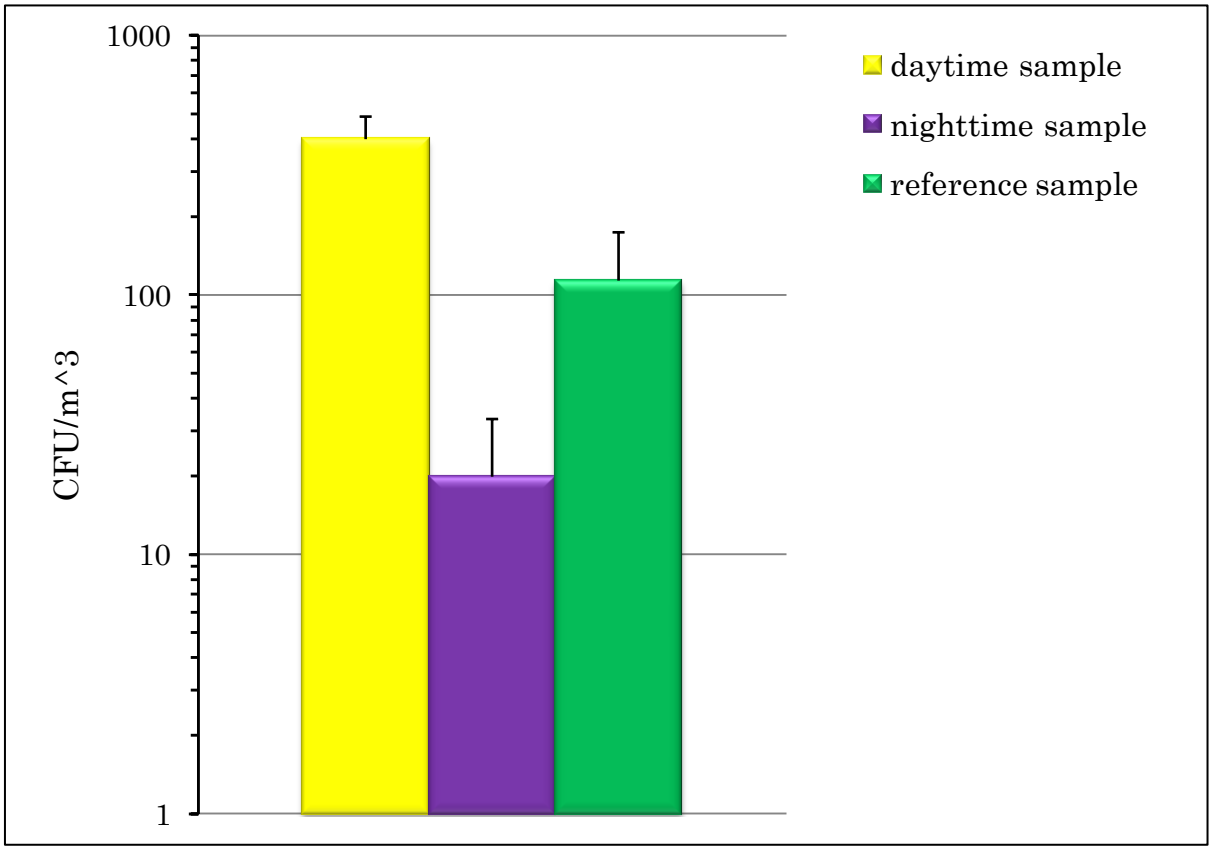

Figure 3.3: Average bacterial concentrations calculated from total culturing on TSA and R2A, with error bars illustrating the standard deviations

From selective endospore culturing, growth was observed for 10 of in total 19 samples, where aerobe incubation resulted in 82 % of the observed endospore-forming bacteria (Table 3.3). The aerobic endospore fraction found in the average outdoor reference sample was higher than the fractions for the average indoor daytime and nighttime samples, by a factor of approximately 5 and 4, respectively (Figure 3.4).

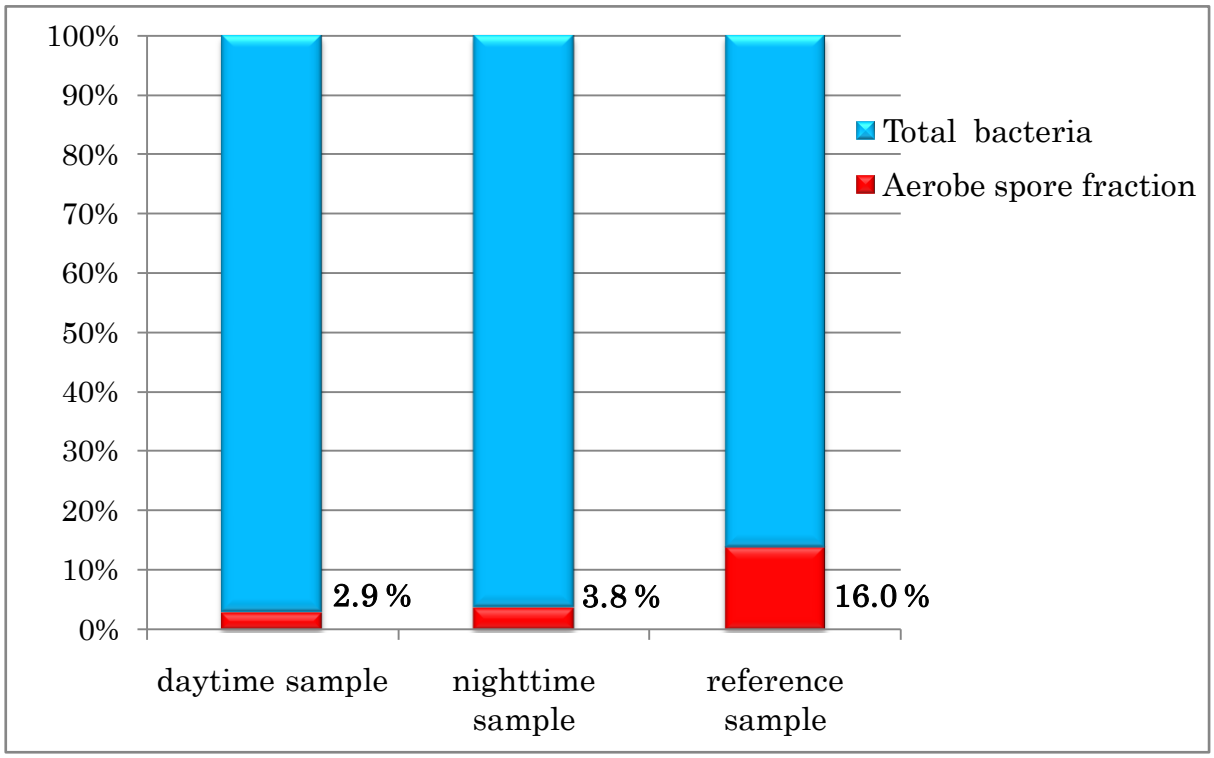

Figure 3.4: Aerobe endospore fractions found in the average daytime, nighttime and reference samples

3.2.1.2 Taxonomic classification results

In order to investigate the diversity of the cultivable bacteria found, isolates were taxonomically classified. From total culturing, all colonies with unique morphology appearance were selected for classification, and from endospore culturing, all colonies found were selected (section 2.2.1.2). 291 isolates from total culturing and 111 from endospore culturing were successfully 16SrRNA gene sequenced and classified down to genus level (section 2.2.1.5). The classified isolates were distributed between only three phyla: *Actinobacteria* (35 %), *Firmicutes* (58 %) and *Proteobacteria* (7 %). However, 22 distinct bacterial families were represented, under which in total 37 different bacterial genera were observed (Table 3.4).

The majority of the classified isolates belonged to the genera *Bacillus* (31.3 %), *Micrococcus* (23.4 %) and *Staphylococcus* (18.7 %), implying that species of these genera show high diversity and varying colony morphologies. However, it should be noted that the major genera also could be due to replicate isolates, in addition to high species diversity. Distinct morphologies were selected from total culturing independently for each sample, and all colonies from endospore culturing were selected, resulting in possibility for replicates (section 2.2.1.2).

Table 3.4: Taxonomical classification results for 402 bacterial isolates, including the numbers of isolates representing each family and genus

Isolates		Family		Isolates		Genus
133	33.1 %	*Bacillaceae*		126	31.3 %	*Bacillus*
107	26.6 %	*Micrococcaceae*		94	23.4 %	*Micrococcus*
75	18.7 %	*Staphylococcaceae*		75	18.7 %	*Staphylococcus*
18	4.5 %	*Paenibacillaceae*		17	4.2 %	*Paenibacillus*
13	3.2 %	*Pseudomonadaceae*		13	3.2 %	*Pseudomonas*
11	2.7 %	*Microbacteriaceae*		9	2.2 %	*Microbacterium*
8	2.0 %	*Streptomycetaceae*		8	2.0 %	*Streptomyces*
6	1.5 %	*Dermacoccaceae*		6	1.5 %	*Dermacoccus*
6	1.5 %	*Nocardiaceae*		5	1.2 %	*Arthrobacter*
5	1.2 %	*Planococcaceae*		5	1.2 %	*Kocuria*
4	1.0 %	*Enterobacteriaceae*		5	1.2 %	*Rhodococcus*
3	<1 %	*Rhodobacteraceae*		3	<1 %	*Lysinibacillus*
2	<1 %	*Acetobacteraceae*		3	<1 %	*Paracoccus*
2	<1 %	*Caulobacteraceae*		3	<1 %	*Rothia*
2	<1 %	*Corynebacteriaceae*		2	<1 %	*Brevundimonas*
1	<1 %	*Comamonadaceae*		2	<1 %	*Corynebacterium*
1	<1 %	*Dietziaceae*		2	<1 %	*Paenisporosarcina*
1	<1 %	*Intrasporangiaceae*		2	<1 %	*Pantoea*
1	<1 %	*Leuconostocaceae*		2	<1 %	*Planococcus*
1	<1 %	*Moraxellaceae*		2	<1 %	*Roseomonas*
1	<1 %	*Promicromonosporaceae*		2	<1 %	*Viridibacillus*
1	<1 %	*Sphingomonadaceae*		1	<1 %	*Brevibacillus*
				1	<1 %	*Cellulosimicrobium*
				1	<1 %	*Comamonas*
				1	<1 %	*Curtobacterium*
				1	<1 %	*Dietzia*
				1	<1 %	*Enhydrobacter*
				1	<1 %	*Erwinia*
				1	<1 %	*Exiguobacterium*
				1	<1 %	*Gordonia*
				1	<1 %	*Janibacter*
				1	<1 %	*Plantibacter*
				1	<1 %	*Serratia*
				1	<1 %	*Sphingomonas*
				1	<1 %	*Sporosarcina*
				1	<1 %	*Tumebacillus*
				1	<1 %	*Weissella*

21 of the 22 bacterial families found were represented in the indoor samples, compared to only 11 of 22 families represented in the outdoor samples (Table 3.5). However, this observation could not be considered a proof of richer bacterial diversity indoors compared to outdoors. For the indoor samples, colonies with unique morphologies were selected from in total 3708 bacterial colonies, whereas for the outdoor samples, colonies with unique morphologies were selected from only 742 bacterial colonies (Table 3.3). This implied that the families only observed indoors could have been found outdoors if higher numbers of outdoor bacteria were obtained prior to morphology-based selection.

Table 3.5: Family taxa observed in indoor and outdoor samples

Indoor samples (day and night)				**Outdoor samples (day)**		
Family taxa	**Isolates**			**Family taxa**	**Isolates**	
Acetobacteraceae	1	<1 %		*Acetobacteraceae*	1	<1 %
Bacillaceae	84	28 %		*Bacillaceae*	49	46 %
Caulobacteraceae	2	<1 %				
Comamonadaceae	1	<1 %				
Corynebacteriaceae	2	<1 %				
Dermacoccaceae	5	2 %		*Dermacoccaceae*	1	<1 %
Dietziaceae	1	<1 %				
Enterobacteriaceae	4	1 %				
Intrasporangiaceae	1	<1 %				
Leuconostocaceae	1	<1 %				
Microbacteriaceae	8	3 %		*Microbacteriaceae*	3	3 %
Micrococcaceae	91	31 %		*Micrococcaceae*	16	15 %
Moraxellaceae	1	<1 %				
Nocardiaceae	5	2 %		*Nocardiaceae*	1	<1 %
Paenibacillaceae	8	3 %		*Paenibacillaceae*	10	9 %
Planococcaceae	4	1 %		*Planococcaceae*	1	<1 %
				Promicromonosporaceae	1	<1 %
Pseudomonadaceae	10	3 %		*Pseudomonadaceae*	3	3 %
Rhodobacteraceae	3	1 %				
Sphingomonadaceae	1	<1 %				
Staphylococcaceae	55	19 %		*Staphylococcaceae*	20	19 %
Streptomycetaceae	8	3 %				
Total	**296**	**100 %**		**Total**	**106**	**100 %**

The only relatively high deviations observed between the sampling locations were a higher percent representation in the outdoor samples of *Bacillaceae* (46 % to 28 %) and *Paenibacillaceae* (9 % to 3 %), and a lower representation of *Micrococcaceae* (15 % and 31 %) (Table 3.5).

Among the isolates originating from endospore culturing, 80 % were classified as the bacterial genera *Bacillus, Paenibacillus, Viridibacillus, Tumebacillus* and *Brevibacillus* and 15 % as the genus *Staphylococcus.* These observations correlated well with the observation of a higher endospore fraction in the outdoor samples (Figure 3.4) and a higher percent representation of the family taxa *Bacillaceae* and *Paenibcillaceae* in the outdoor samples (Table 3.5).

In order to investigate the effect of human activity and train traffic on the bioaerosol environment, bacterial diversity was compared for indoor daytime samples and nighttime samples (Table 3.6).

Table 3.6: Family taxa observed in indoor daytime and nighttime samples

Daytime indoor samples				**Nighttime indoor samples**		
Family taxa	**Isolates**			**Family taxa**	**Isolates**	
Acetobacteraceae	1	<1 %				
Bacillaceae	81	32 %		*Bacillaceae*	3	6 %
Caulobacteraceae	2	<1 %				
Comamonadaceae	1	<1 %				
				Corynebacteriaceae	2	4 %
Dermacoccaceae	5	2 %				
Dietziaceae	1	<1 %				
Enterobacteriaceae	1	<1 %		*Enterobacteriaceae*	3	6 %
Intrasporangiaceae	1	<1 %				
Leuconostocaceae	1	<1 %				
Microbacteriaceae	6	2 %		*Microbacteriaceae*	2	4 %
Micrococcaceae	75	30 %		*Micrococcaceae*	16	35 %
Moraxellaceae	1	<1 %				
Nocardiaceae	1	<1 %		*Nocardiaceae*	4	9 %
Paenibacillaceae	7	3 %		*Paenibacillaceae*	1	2 %
Planococcaceae	4	2 %				
Pseudomonadaceae	9	4 %		*Pseudomonadaceae*	1	2 %
Rhodobacteraceae	3	1 %				
Sphingomonadaceae	1	<1 %				
Staphylococcaceae	43	17 %		*Staphylococcaceae*	12	26 %
Streptomycetaceae	6	2 %		*Streptomycetaceae*	2	4 %
Total	**250**	**100 %**		**Total**	**46**	**100 %**

Fewer family taxa were observed in the nighttime samples than for the daytime samples. However, as also smaller bacterial concentrations were obtained for the nighttime samples (Figure 3.3), there is a possibility that more taxa could have been observed if more nighttime samples were collected. Interestingly, the largest

deviation in percent representation between the daytime and nighttime samples was observed for *Bacillaceae* (Table 3.6), which also was found to represent the largest deviation between the indoor and outdoor samples (Table 3.5).

3.2.1.3 Characteristic morphologies

In order to further characterize the 402 classified bacterial isolates, colony morphology (Table B.1 in Appendix B) was linked to the taxonomical classification results (Table 3.4). Dominant traits observed for the largest family taxa were suggested as morphologies characteristic for these families. The characteristic patterns found included colony colour, size, shape and surface appearance, noted after 48 hours incubation (Table 3.7 and Figure 3.5a-f).

Table 3.7: Characteristic morphologies for the ten largest families, where percentage abundance of the trait among the family isolates are given

Taxonomical family	Isolates	Colour	Size	Shape	Surface
Bacillaceae	133	Beige 94 %	5 mm 88 %	Irregular 78 %	Textured 62 %
Micrococcaceae	107	Yellow 78 %	1 mm 90 %	Round 78 %	Smooth 77 %
Staphylococcaceae	75	Beige 71 %	1 mm 79 %	Round 89 %	Smooth 89 %
Paenibacillaceae	18	Beige 67 %	5 mm 50 %	Irregular 61 %	Smooth 89 %
Pseudomonadaceae	13	Yellow 54 %	1 mm 69 %	Irregular 77 %	Ruffled 54 %
Microbacteriaceae	11	Yellow 91 %	1 mm 100 %	Round 90 %	Smooth 100 %
Streptomycetaceae	8	White 88 %	1 mm 100 %	Round 75 %	Ruffled 88 %
Dermacoccaceae	6	Orange 50 %	1 mm 100 %	Round 100 %	Smooth 100 %
Nocardiaceae	6	Pink 33 %	1 mm 100 %	Round 83 %	Smooth 100 %
Planococcaceae	5	Orange 60 %	1 mm 100 %	Round 80 %	Smooth 100 %

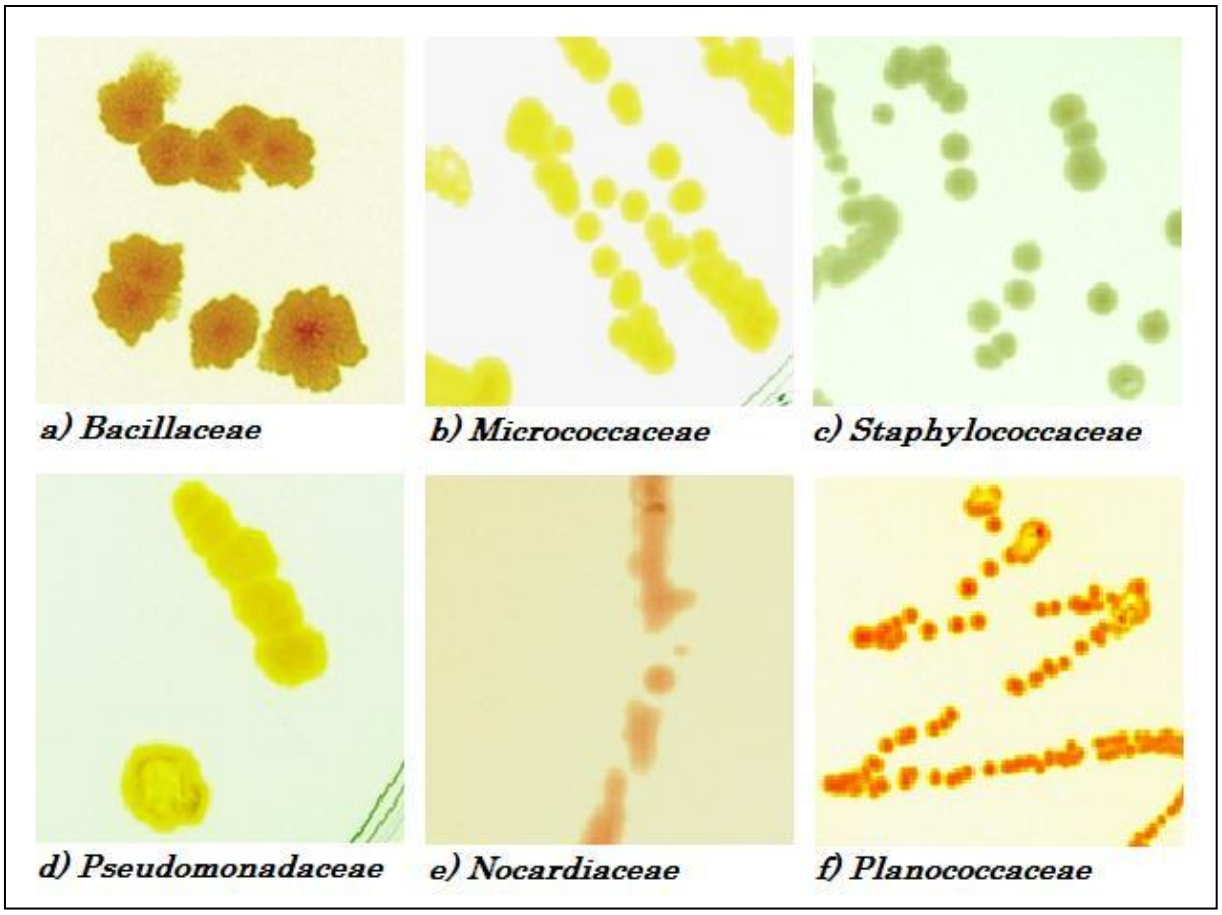

Figure 3.5 a-f: Examples of colonies with morphologies found to be characteristic for the family taxa a)*Bacillaceae, b)Micrococcaceae, c)Staphylococcaceae, d)Pseudomonadaceae, e)Nocardiaceae* and *f)Planococcaceae.* Colours appear in picture not exactly as observed in laboratory (Picture by FFI)

3.2.1.4 Hemolytic activity

The 402 classified isolates were successfully assayed for hemolytic activity. 22 % of the isolates showed hemolytic activity, in which all isolates were classified as inducing beta-hemolysis, except two that were classified as inducing alpha-hemolysis. 87 % of the isolates showing hemolytic activity were classified as the genus *Bacillus*. The other genera were *Staphylococcus, Streptomyces, Erwinia* and *Pseudomonas.*

3.2.1.5 Antibiotic resistance

Among the 402 classified isolates, 32 %, 12 %, 65 %, 3 % and 8 % showed resistance against the antibiotics ampicillin, streptomycin, nalidixic acid, tetracycline and chloramphenicol, respectively. The distribution of antibiotic resistance in the dominant genera (Table 3.4) illustrated the trend (Table 3.8). Among the dominant genera, only *Paenibacillus* were frequently resistant against streptomycin. Very few isolates among the dominant genera displayed resistance against tetracyclin, except some isolates of *Pseudomonas*.

Table 3.8: Distribution of antibiotic resistance in the dominant genera

Genus	AMP	STR	NAL	TET	CHL		Colour codes
Bacillus	48 %	14 %	17 %	2 %	13 %		Very high >90%
Micrococcus	4 %	6 %	98 %	1 %	2 %		High > 30%
Staphylococcus	53 %	9 %	95 %	1 %	4 %		Moderate >10%
Paenibacillus	12 %	76 %	12 %	0 %	12 %		Low <10%
Pseudomonas	31 %	8 %	54 %	15 %	23 %		

3.2.1.6 Species identity

In order to tentatively identify the bacterial isolates on species level, they were all compared to sequences of known identity by RDP_SeqMatch (2.2.1.5), giving a best-match identity score. From the 291 isolates selected based on different colony appearance (total culturing), 92 unique bacterial species were found. It should be noted that the 291 colonies were selected independently for each of the 19 samples (section 2.2.1.2). Furthermore, colonies were selected independently from TSA and R2A culture plates per sample, giving that 38 replicates were expected per species. However, most of the species replicates found were selected multiple times from one sample, and only 17 of the 92 different species were observed in more than two different samples. The only specie observed in more than 9 samples was *Micrococcus luteus*, which was found in 17 of the 19 samples. The lack of replicates indicated high bacterial diversity in the airborne environment at Nationaltheatret subway station.

Furthermore, it was found that 89 of the 291 isolates were replicates of another isolate, collected at the same time and location and grown on the same medium. This observation suggested that selection of different bacteria species based on observation of different colony appearance was an insufficient method. However, it was found that 69 of the 89 unnecessary replicates were due to *M. luteus.* When excluding *M. luteus,* only 7 % of the isolates were incorrectly selected as unique bacteria species based on colony appearance.

From endospore culturing, all colonies found were selected for classification, and therefore replicate isolates were expected. Among the 111 classified endospore-forming isolates, 37 unique bacterial species were found by RDP_SeqMatch database search (2.2.1.5).

In order to investigate the identification reliability of MALDI-TOF MS fingerprinting compared to RDP_SeqMatch, representative isolates from each OTU (n=84) were analysed by MALDI technology, and the results compared to the RDP_SeqMatch results (Table C.1 in Appendix C). 33 of 84 bacterial isolates were identified with corresponding species names, 24 of 84 bacterial isolates were

identified with corresponding genus names (wrong species), and 26 of 84 bacterial isolates were identified with non-corresponding names. However, 50 % of the isolates identified with non-corresponding species names were found to be lacking in the MALDI database (Table C.2 in Appendix C). When subtracting for the identifications that could never have been equal the RDP_SeqMatch results because of lack of their identification spectra in MALDI-database, the identification of bacterial isolates by MALDI-TOF MS fingerprinting gave corresponding genus identification for 74 % of the OTU isolates. One isolate failed to give MALDI-spectra for unknown reasons.

3.2.2 Culture-independent bacterial analysis

3.2.2.1 Optimal DNA extraction method

In order to optimize a method for extraction of microbial DNA from samples containing endospores and vegetative bacteria, various bead mill homogenization conditions were tested (section 2.2.2.1). The optimization study resulted in the optimal conditions, as verified by PCR:

- use of MoBio Bead Solution (440 µl), Inhibitor Removal Solution (200 µl) and S1 Lysis Solution (60 µl)
- no use of antifoam A
- final DNA isolation by silica column

These conditions were found to be optimal both for endospores (*B. atrophaeus)* and vegetative cells (*E. coli)*. However, the optimal amount of beads and duration of bead beating varied for the endospores and the vegetative bacteria cells (Table D.1 in Appencix D). A compromise that seemed to disfavor the cells and the endospores equally was chosen as the standard conditions for the unknown air samples; 1.5 gram beads and bead beating for two minutes.

3.2.2.2 16SrRNA gene quality and quantity

In order to analyse the total microbial DNA extracted from 15 air samples (section 2.2.2.2), their 16SrRNA gene PCR products were run on agarosis gel (Figure 3.6). All of the bands in the gel corresponding to the daytime samples (D) appeared strong, whereas the bands corresponding to the reference samples (R) appeared strong only for two of five samples. All the five nighttime samples (N) showed weak bands in the gel, implying that there were less DNA in these samples.

These results correlated well with the results from the culture-dependent analysis, where higher numbers of cultivable bacteria were found in the daytime samples than in the nighttime and reference samples (section 3.2.1). This trend

seemed to be true also for total bacteria, represented by the amount of amplified 16SrRNA gene visible on the gel (Figure 3.6).

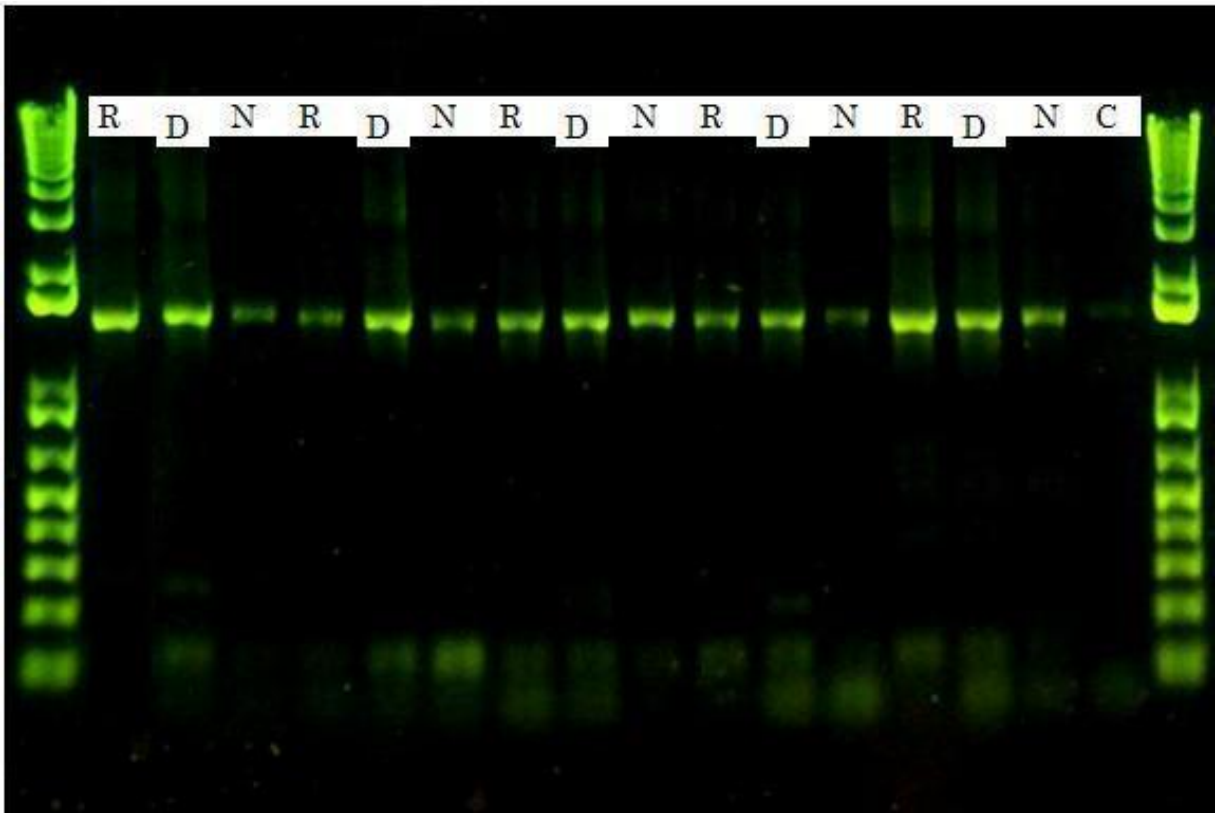

Figure 3.6: 16SrRNA gene amplified total DNA, representing five reference, daytime and nighttime samples, and a negative control

A very weak band could be seen in the lane of the negative PCR control (C) (Figure 3.6). The band appeared in the same row as the 16SrRNA gene products of the samples, and was most likely due to cross-contamination.

The remaining total DNA extracts (section 2.2.2.3) will be further analysed in a culture-independent diversity study using DGGE and 16SrRNA gene based microarrays, by Marius Dybwad at FFI.

3.3 Detection of airborne Influenza A virus

3.3.1 RNA isolation

In order to verify the RNA extraction method prior to testing air samples for viral RNA, extraction was performed from commercial whole virus particles (section 2.3.1.1). Successful PCR detection of viral RNA extracted from commercial virus particles confirmed the RNA extraction method, although the losses from the isolation process remained unknown. Successful detection of target RNA also confirmed the one-step reverse transcriptase real-time PCR assay (section 2.3.2).

3.3.2 Reverse transcriptase real-time PCR

Testing and comparison of the two competing PCR assays designed by van Elden and Ward (sections 2.3.2.1 and 2.3.2.2) revealed better results for the Ward assay. All the primers and probes were found to be specific for the Influenza A genome by *in silico* BLAST specificity testing in GenBank (Benson, Karsch-Mizrachi et al. 2009). However, sensitivity testing by sequence alignment revealed that the Ward probe bound best to all the target sequences tested, whereas the van Elden probe had from two to four mismatches in some target sequences. No mismatches were found for the primer sequences.

In order to test the specificity and sensitivity of the two assays in laboratory, their PCR products were compared on agarosis gel (Figure 3.7). Both assays were found to be equally specific, as only bands corresponding to PCR products of the expected lengths (Table 2.1, section 2.3.2.1) were visible on the gel, except for the primer dimers which also appeared in the negative samples. However, a weak band in well number six indicated that the van Elden assay was less sensitive in amplification of the trizol-extracted RNA than the Ward assay. This observation, together with the *in silico* test results, made us choose the Ward assay for further studies.

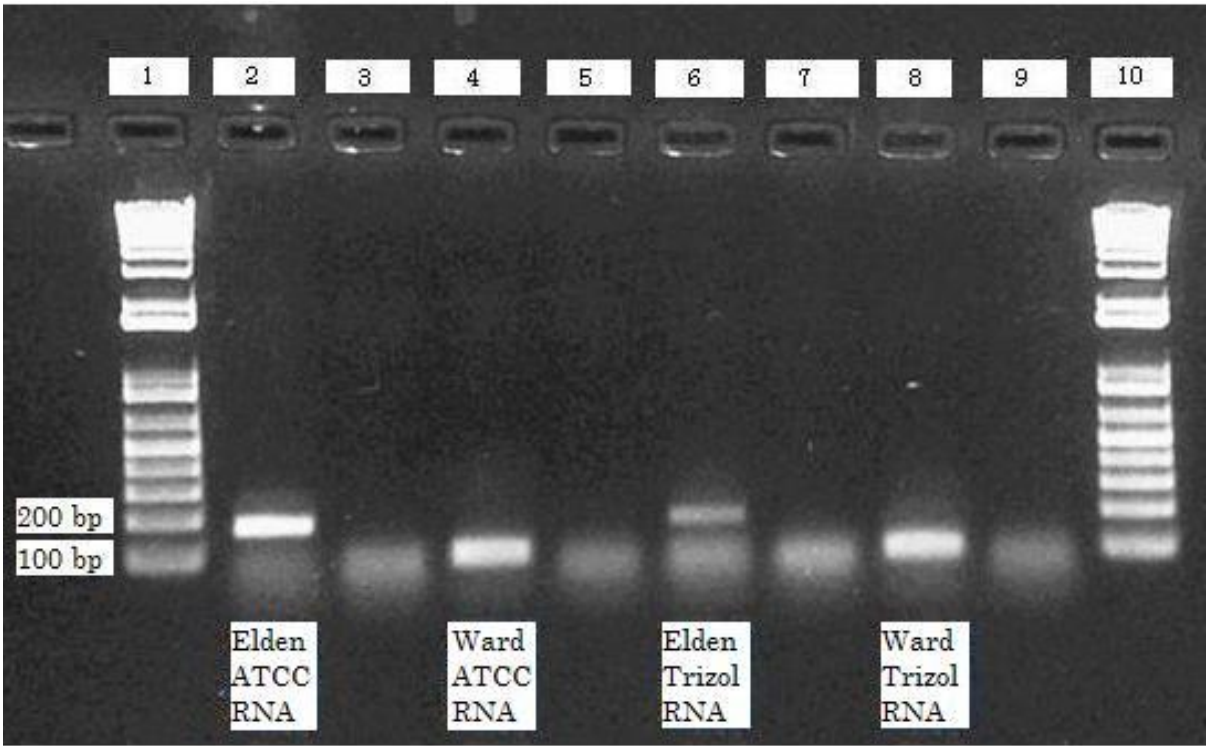

Figure 3.7: PCR products from amplification of positive control RNA (ATCC RNA) and trizol-extracted RNA (Trizol RNA), by van Elden and Ward assays. Negative PCR controls are shown in well number 3, 5, 7, and 9

In order to quantify Influenza A viruses, potentially present in the air samples, two standard curves were prepared (section 2.3.2.3). For the RNA standard curve, the lowest concentrated standard that appeared in the linear area of the curve represented a theoretical initial concentration of $1.6*10^3$ copies per µl in PCR (Figure 3.8). This limit of quantification for the RNA standard curve ($1.6*10^3$ copies per µl in PCR) was higher than expected, indicating huge losses of RNA during the RNA extraction process (section 2.3.1). Internal calibration (section 2.3.2.3) corrects for the RNA loss, but still an initial concentration of virus particles corresponding to minimum $1.6*10^3$ RNA copies per µl in PCR need to be collected, in order to quantify the virus particles in the sample correctly. However, qualitative detection is possible for concentrations below the limit of quantification. The LOD was estimated to be 10 RNA copies per µl in PCR from the standard concentrations (Table 2.4, section 2.3.2.3) and standard curve picture (Figure 3.8).

For the DNA standard curve, perfect linearity was seen for the six highest concentrated standards, but also the seventh standard was included as part of the linear area (Figure 3.9). This was considered reliable as no deviation was observed for the three standard parallels. The error value obtained for the curve

was 0.00548; far below the required <0.2, and the efficiency of the curve was found to be 1.951 (Lightcycler Computer Program). The lowest concentrated standard in the linear area of the curve represented a theoretical initial concentration of 50 copies per µl in PCR. This limit of quantification was considered suitable for the study, and still qualitative detection was possible below the limit of quantification. However, it should be noted that the DNA standard curve only gives information about RNA concentrations after RNA extraction, and not information about the number of initial viral particles collected.

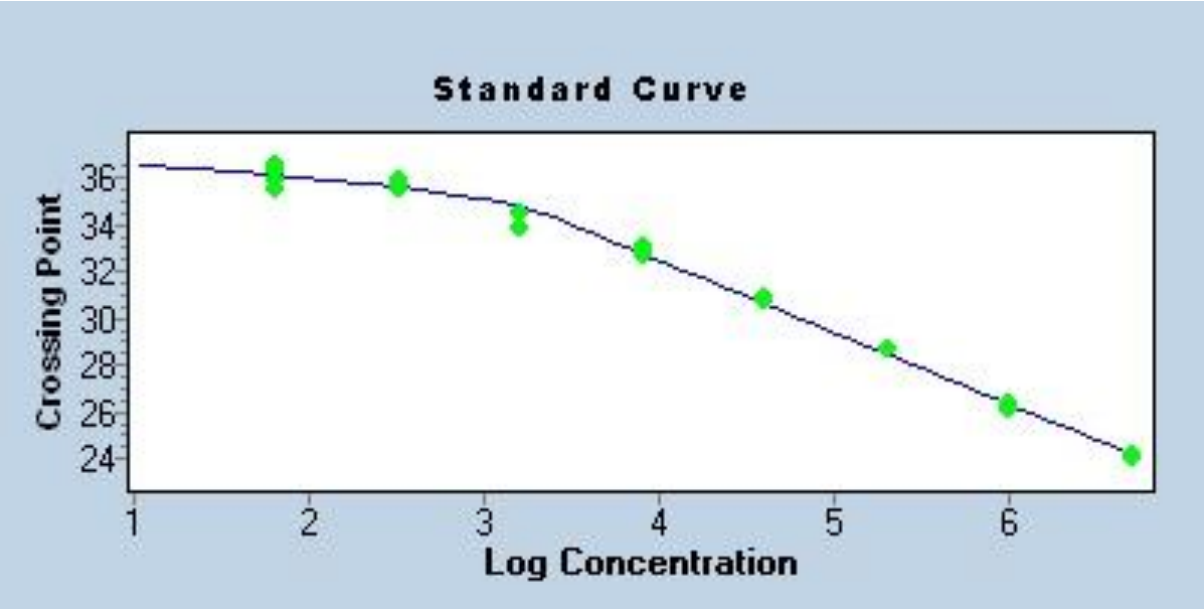

Figure 3.8: RNA standard curve

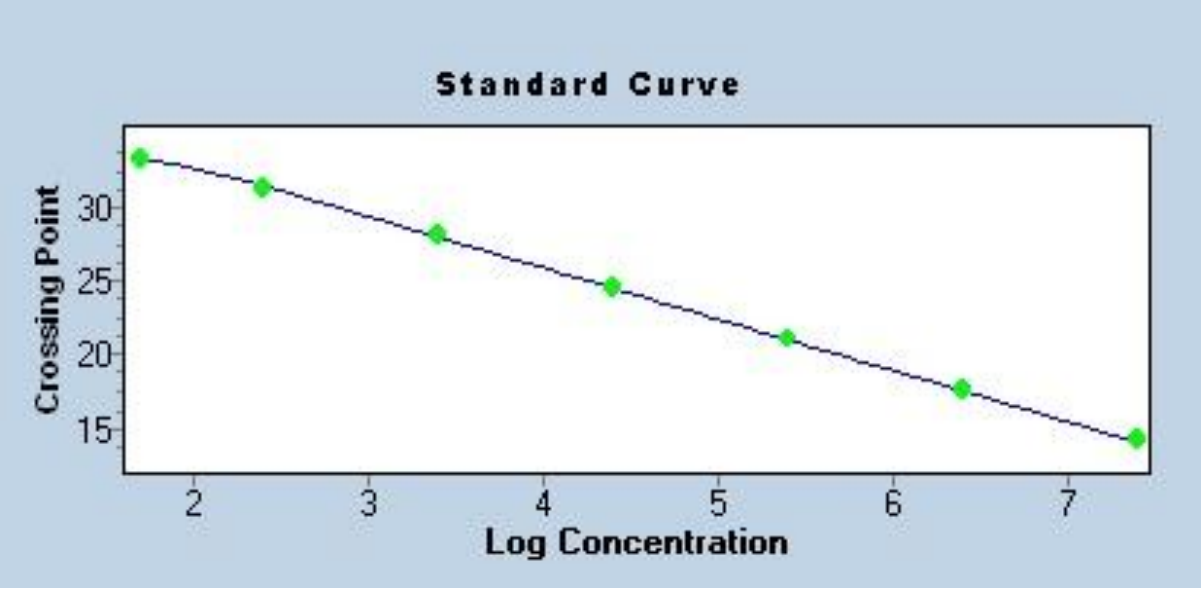

Figure 3.9: DNA standard curve

3.3.3 Detection and quantification of airborne Influenza A virus

In order to test for presence of Influenza A virus RNA in the air samples, a specific reverse transcriptase PCR assay was performed on the RNA isolated air samples (section 2.3.2). The PCR assay worked correctly because the positive controls were amplified and the negative controls were not. The standards included in the assay for correlation with the saved standard curves obtained equal crossing points with their corresponding saved standards, implying good reproducibility. However, no amplification was seen for the RNA isolated air samples (Figure 3.10).

The RNA isolated air samples were not given crossing points by the PCR analysis program, and could therefore not be related to the standard curves for quantification. Logically, no crossing point was given when no amplification occurred; indicating lack of target sequences in the RNA isolated air samples.

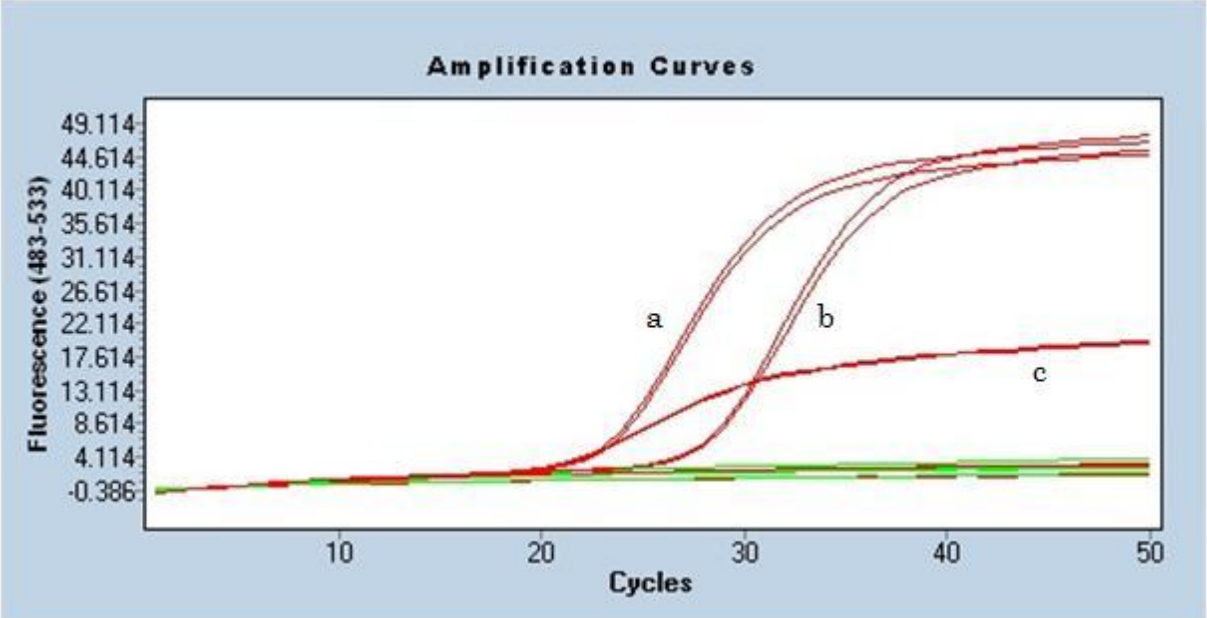

Figure 3.10: PCR showing amplification of a) positive RNA control, b) RNA standard and c) DNA standard, but no amplification of negative controls or air samples

In order to test the RNA isolated air samples for inhibition, reverse transcriptase PCR assay was performed with and without BSA (section 2.3.3) (Figure 3.11). The assay worked correctly because again the positive controls (in this case the standards and spike) were amplified, and the negatives were not amplified. However, no amplification was observed in the PCR wells containing RNA isolated air sample from Nationaltheatret subway station. This was true also for the air samples spiked with high concentrations of RNA, confirming inhibitors in the air samples. Unfortunately, the inhibited samples containing BSA showed no recovery from inhibition compared to the samples without BSA.

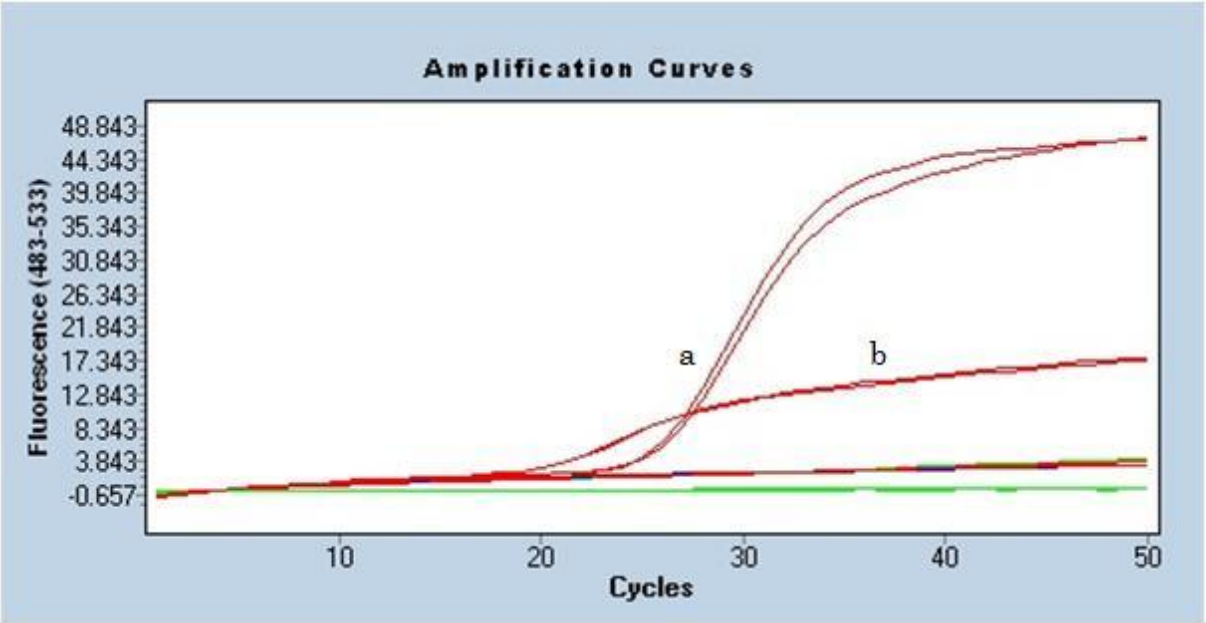

Figure 3.11: PCR showing amplification of a) RNA spike and standard, b) DNA standard, but no amplification of negative controls or air samples

Gel electrophoresis of the inhibition-tested PCR products (section 2.3.3) showed that no PCR products were visible in the wells containing sample (well 3, 5, 7, 9), not even primer dimers (Figure 3.12). For the negative controls (well 6 and 10), weak bands corresponding to primer dimers were visible. The RNA spikes without inhibiting RNA isolated air sample and the control RNA showed clear bands corresponding to fragments of the same lengths. The observations showed that the inhibition from the air samples was total. No effect, positive or negative, was observed from use of BSA, which was added in well 3-6.

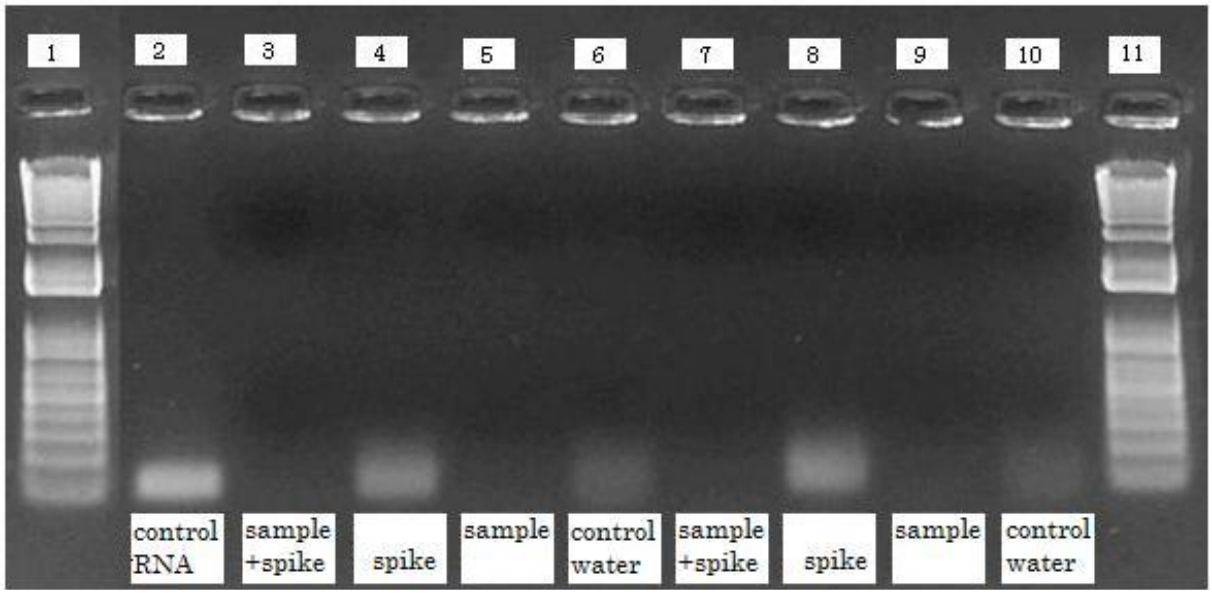

Figure 3.12: Gel picture showing total inhibition of PCR products containing RNA isolated air sample from Nationaltheatret subway station

4 Discussion

This study can be considered the first one that deals with investigation of the bioaerosol environment at a subway station in Norway. Experiments were conducted in order to characterize the airborne microorganisms at Nationaltheatret subway station. According to the aims of the study:

- the concentration and diversity of total and endospore-forming cultivable bacteria were investigated for daytime and nighttime samples
- the cultured bacteria were further characterized based on colony morphology, hemolysis activity and antibiotic resistance
- a DNA extraction method for samples containing both endospores and vegetative cells was optimized
- a specific quantitative PCR detection assay was developed for investigation of the presence of airborne Influenza A virus

4.1 Air sampling

In this study, all air samples were harvested with SASS3100 filter sampler (Research International, Inc), which was chosen because of its collection efficiency, wide particle size range, ease of handling and transportation, and ease of filter extraction (Research International 2011). As a high-flow air sampler with potential for long-time sampling, a drawback with SASS3100 is desiccation of microorganisms during sampling, possibly reducing their viability (Cartwright, Horrocks et al. 2009) (section 1.2.1.3). In this study, it was observed that the nighttime and reference samples together contained 2.5 times more cultivable bacteria per airborne particle than the daytime samples (section 3.2.1.1). The number should be considered approximate, as it relies on 100 % collection efficiency by SASS3100 filter sampler (section 1.2.1.3). However, the results implied that reduced viability was observed for the daytime samples. As the daytime samples contained 75 % of the total number of cultivable bacteria found (section 3.2.1.1), reduced viability could be a consequence of higher bacterial concentrations on the daytime filters, as previously suggested by Hirvonen et al. (Hirvonen, Huttunen et al. 2005).

Whether loss of viability during filter collection occurred in a randomly fashion, or dominated for certain types of bacteria, could not be interpreted from the data obtained in this study. If the last case was true, the observed diversity for the daytime samples could not be trusted to give a correct characteristic pattern of bacterial genera residing in air during daytime (Table 3.6, section 3.2.1.2). For further investigation, it would be interesting to test whether certain bacterial genera dominate in losing their viability during filter sampling. SKC Biosampler

(section 1.2.1.2) is an example of a gentle air sampler, which could be used in parallel with SASS3100 to test the reproducibility of the bacterial diversity found.

Meteorological data can influence the airborne particle concentrations and the viability of microorganisms residing on them (section 1.1). In this study, the air sampling conditions stayed constant for all samples, and relative humidity was measured to be within the range of highest survival for microorganisms in the airborne environment (40 - 80 %RH) (Gilbert and Duchaine 2009) (Table 3.1, section 3.1.1). Constant weather conditions reduced the possibility for observing the effect of these conditions on viability. However, the concentration and diversity comparison performed in this study, between the daytime, nighttime and reference samples (sections 3.2.1.1 and 3.2.1.2), could be considered reliable because of the constant weather conditions.

The results from the particle counter Aerotrak 8220 showed that 85 % of the total number of detected particles were of sizes <1.0 µm (section 3.1.2). This observation is of health concerns, because airborne particles below 5 µm penetrate deep into our lungs when respired, potentially causing infections (Thomas, Webber et al. 2008). For further investigation, it would be interesting to investigate if there exist some correlation between types of microorganisms and airborne particle sizes, but that will require use of other air samplers, like the Anderson sampler (Verreault, Moineau et al. 2008) (section 1.2.1.1).

4.2 Cultivable bacteria

4.2.1 Concentrations

From total culturing, the indoor daytime samples were found to be 20 times more concentrated with cultivable bacteria than the indoor nighttime samples (section 3.2.1.1). This observation suggested that human activity and traffic was the main cause of indoor bioaerosols, as few bacteria were found during nighttime when no trains or people were at the station.

An average value of 400 bacterial CFU/m^3 was found during daytime at Nationaltheatret subway station in Oslo. For comparison, 12,639 bacterial CFU/m^3 was found at a subway station in the highly populated and traffic crowded Beijing, measured by Biosampler instrument and gelatin filters (Dong and Yao 2010) (section 1.1). The CFU numbers were not directly comparable due to use of different collection methods (Srikanth, Sudharsanam et al. 2008). However, observation of 31 times more cultivable airborne bacteria in a Beijing subway station suggested that indoor bioaerosol concentrations in general could be related to the level of people and traffic.

Compared to the CFU numbers from total culturing, the aerobic endospore fractions were 2.9 % and 3.8 % at Nationaltheatret subway station during daytime and nighttime, respectively (section 3.2.1.1). However, the aerobic endospore fractions found in the outdoor reference samples were 16.0 %. These results were similar to those previously reported by Hameed et al, where slightly higher levels of endospore-forming bacteria were found in Cairo at an outdoor station compared to an indoor station (Abdel Hameed and Awad 2002).

4.2.2 Diversity

In order to investigate the bacterial diversity at Nationaltheatret subway station, all cultivable bacteria with different colony morphologies were classified down to genus level. Among the selected isolates, 37 different genera were observed, in which the major genera were *Bacillus* (31.3 %), *Micrococcus* (23.4 %) and *Staphylococcus* (18.7 %) (Table 3.4, section 3.2.1.2). These findings correlated well with other studies performed in indoor environments, where for example the same three genera was found to dominate in 100 different indoor locations studied in Poland (Rafal L. Gorny and Jacek Dutkiewicz 2002).

Database search by RDP_SeqMatch tentatively identified the selected isolates on species level, resulting in observation of 92 different bacterial species (Cole, Chai et al. 2007) (section 3.2.1.6). Only 17 of the 92 different species were observed in more than two different samples, suggesting that more than 19 air samples were needed in order to find characteristic patterns of bacteria diversity on species level. However, only 85 different bacterial species were observed in together 100 different indoor locations in Poland (Rafal L. Gorny and Jacek Dutkiewicz 2002), implying that most species residing in the airborne environment possibly may have been found in this study, although few replicates were observed.

Bacterial diversity deviations observed between daytime and nighttime samples were mainly due to higher concentration of the family taxa *Bacillaceae* at daytime (32 %) compared to at nighttime (6 %) (Table 3.6 section 3.2.1.2). Observations of 46 % *Bacillaceae* in outdoor samples implied that the airborne particles containing *Bacillaceae* taxa originate outdoors, and come into the station through open doors during daytime. Furthermore, as the fraction of *Bacillaceae* was the most reduced taxa in the nighttime samples compared to the daytime samples, the results also implied that *Bacillaceae* reside on larger particles than other family taxa, resulting in settling to the ground in shorter time. However, this relationship between family taxa and particle sizes is only a theory, possibly explaining the observations seen in this study. Investigation is needed in order to learn more about whether such relationships between family taxa and particle sizes occur and are reproducible.

For the endospore-forming bacteria found during selective cultivation, comparison between daytime and nighttime diversity was not performed because growth was below LOD for all nighttime samples (Table 3.3, section 3.2.1.1).

For tentative species identification, MALDI-TOF MS fingerprinting was compared to RDP_SeqMatch results, and was found to give corresponding genus identity for 74 % of the isolates investigated (section 3.2.1.6). However, this result required subtraction of false identifications due to lack of species-spectra in MALDI-TOF MS database, which implied that still, MALDI-TOF MS is only appropriate for indications of genus identity and not for indications of species identity, and additional methods are required for reliable identifications. However, the score values given by the Biotyper 2.0 program seemed to be good indicators of the reliability of the results given (Table C.1 in Appendix C).

In this study, the MALDI-TOF MS results were compared to RDP_SeqMatch results, but the 16SrRNA gene databases used by RDP_SeqMatch do not yet contain correct unique sequences for all bacterial species, suggesting that also identifications based on sequences need additional validation by other methods in order to be confirmed (Song, Liu et al. 2003) (section 1.4.3).

4.2.3 Characteristics

Downstream analysis of the selected cultivable bacteria revealed that it was possible to find characteristic morphology traits for the bacterial family taxa (Table 3.7, section 3.2.1.3). However, even though most of the characteristic colours, colony sizes, shapes etc. were observed for more than 70 percent of the family members for each family, these observations might not be reproducible as most of the families were represented by less than 18 isolates. The three largest families, *Bacillaceae, Micrococcaceae* and *Staphylococcaceae* (Table 3.4, section 3.2.1.2), represented 75 % of the 291 isolates selected from total culturing based on different morphologies. This implied that those three families showed more morphology variations for their members. In order to investigate the distribution of each family in the airborne environment, all colonies found should be analysed in further studies.

One species, *M. luteus*, was selected based on different morphologies 69 times more than predicted. This implied that *M. luteus* appeared with many different colony morphologies, or perhaps that some of the different *M. luteus* species actually were other species for which there were no correct best match identity in the 16SrRNA gene databases (Cole, Chai et al. 2007) (section 1.4.3). However, except for *M. luteus*, this study showed that most bacterial colonies showing different morphologies actually were different species (section 3.2.1.6).

22 % of 402 classified isolates showed hemolytic activity, meaning that they produced exotoxins which act on red blood cells to lyse or break them down (section 3.2.1.4). Hemolysis is generally regarded as a bacterial virulence factor, meaning that the presence of hemolytic bacteria in the airborne environment can be regarded as an indicator for pathogenic potential (Payment, Coffin et al. 1994) (section 1.3.2). More investigation is needed in order to describe the potential health threat from respiration of airborne hemolytic bacteria. However, some bacteria, like *Staphylococcus aureus*, is known to cause infections through airborne transmission in hospitals (Shiomori, Miyamoto et al. 2001). *S. aureus* was found in two of the air samples in this study, and was classified as beta-hemolytic (section 3.2.1.4). This implied that the air at Nationaltheatret subway station potentially can cause infection if respired by immunodeficient people.

Also of health concern, the 75 characterized isolates of *Staphylococcus* showed high resistance against the antibiotics ampicillin (53 %) and nalidixic acid (95 %) (Table 3.8, section 3.2.1.5). Potential for emerging antibiotics resistance among bacteria as a consequence of overuse in hospitals is a cause for concern, as this makes bacterial infections difficult to treat (Hawkey 2008) (Stevens, Bisno et al. 2005).

4.3 Culture-independent total bacteria

For culture-independent analysis, the samples from Nationaltheatret subway station needed to be bead beaten in order to release DNA from both bacterial cells and endospores prior to DNA isolation. The culture-dependent study showed that there were approximately 3 %, 4 % and 16 % aerobe endospore-forming bacteria in the daytime, nighttime and reference samples, respectively (Figure 3.4, section 3.2.1.1). However, the number of endospore-forming bacteria that were collected as endopores is not known, appreciating the importance of an optimal DNA extraction method for combined samples of endospores and cells.

A problem observed in this study was that the endospores released more DNA when bead beaten for minimum three minutes, whereas the vegetative cells released their DNA instantly, leading to lower yields after only one minute bead beating, probably due to DNA fragmentation (section 3.2.2.1). Two minutes bead beating was chosen because it resulted in reproducible PCR detection of endospore DNA *(B. atrophaeus)* in the optimization tests, and still the DNA from the vegetative cells *(E. coli)* had good quality (Table D.1 in Appencix D). However, it is not unlikely that two minutes bead beating was too short time for breaking all types of endospores in the mixed environmental samples, where the concentration of each endospore probably was low. The loss of the DNA from vegetative cells would on the other hand have been high if the bead beating interval was increased further.

Ideally, the DNA from all species of endospores and vegetative bacteria should be extracted, in order to facilitate correct characterization based on molecular biological techniques (section 1.4). In order to achieve this goal, dividing each environmental sample into two parts prior to bead beating should be considered if repeating these experiments. When dividing the samples, optimal bead beating conditions could be used for both the endospores and the vegetative bacteria. In order to maintain maximum number of bacteria per sample, two filter air samples could be collected in parallel.

4.4 Detection of airborne Influenza A virus

RNA isolated from commercial Influenza A virus particles was successfully detected by one-step reverse transcriptase PCR (section 3.3.2). When investigating RNA isolated from air samples for presence of Influenza A virus, the PCR results were negative, indicating too low viral concentrations in air for PCR detection. Quantification of virus content by the constructed standard curves (section 3.3.2) was therefore not possible. However, during construction of the RNA standard curve, it was found that the LOD was 10 RNA copies per µl in PCR. The LOD for the DNA standard curve was even lower, but not realistic as the losses of RNA during extraction was found to be high. For the experiments performed, a LOD equal 10 copies /µl in PCR corresponded to collection of 480 virus particles per filter sample, because the extracted RNA was resuspended in 12 µl water to obtain two parallels in PCR. Testing for viral RNA without PCR parallel should have been considered, as only 200 virus particles would have been needed per filter sample when resuspending in 5 µl water.

480 viral particles per filter sample corresponded to collection of four viral particles per minute. Lower concentrations could have been obtained during sample collection, but other aspects should be considered prior to drawing any conclusions.

Prognosis from the public health institute showed that people still got infections from Influenza A virus during the week of sample collection (Nasjonalt folkehelseinstitutt 2011). Further, the samples were collected at a high traffic location where the number of people stayed constant (Table 3.2, section 3.1.1), indicating high potential for creation of bioaerosols containing Influenza A viruses by coughing, talking and sneezing (Killingley, Greatorex et al. 2010). Each air sample obtained represented 36 m^3 air, filtered through a filter capable of collecting particles of sizes in the range 0.3-5.0 µm (Research International 2011), and viability was not a requirement for detection of the collected viruses. These considerations suggested that other factors than viral concentrations and sampling method might have influenced the negative PCR results.

Environmental samples are known to contain inhibitors of different sorts which can lower the sensitivity of the PCR assay in use (Maher, Dillon et al. 2001). In this case, the environmental samples were treated with trizol, chloroform, isopropanol and ethanol in order to isolate pure RNA (section 2.3.1). Still inhibition from the RNA isolated air sample D_4 was found to be total, as no PCR detection was observed when addition of high-concentrated RNA spike to the sample (section 3.3.3). The nature of this inhibition is not known, but environmental samples often contain humic acids, which inhibit PCR (Kreader 1996). It has been reported that BSA can reverse the inhibition from humic acids when added to PCR (Kreader 1996). In this study, BSA was added in PCR for air sample D_4, but without observable effect. It is not known whether the inhibition still was total because inhibitors not affected by BSA were present, or because the concentrations of humic acid were so extreme that more BSA would be needed in order to counteract the inhibition.

The inhibition test showed that detection of airborne viruses at Nationaltheatret subway station is difficult, if not impossible. More investigation is needed in order to solve the PCR inhibition problem for viral RNA amplification.

4.5 Biological threat agents

Investigation of the every-day background of airborne microorganisms is essential in order to facilitate continual monitoring for detection of deviations from the normal background, possibly associated to bioterrorism attacks. In this study, none of the bacterial species found were among those listed as critical biological agents by Centers for Disease Control and Prevention (Rotz, Khan et al. 2002) (section 1.1). However, many more biological agents than those listed as critical can be considered threat agents, being relatively easy to spread and capable of infecting humans (Centers for Disease Control and Prevention 2007). The number of isolates selected for identification in this study was too limited for characterization of the every-day background of microorganisms on species level, implying that this study should be repeated in larger scale in order to characterize the every-day background of airborne microorganisms on species level.

5 Conclusions

The aim of this study was to characterize the airborne bacteria and Influenza A virus at Nationaltheatret subway station.

By culture-dependent methods, airborne cultivable bacteria were found to be 20 times more concentrated in air during daytime compared to nighttime. This pattern was found to be similar for total bacteria, estimated by analysis of total extracted DNA. A bead mill homogenization method for DNA extraction from both endospores and vegetative bacterial cells was optimized, in order to facilitate culture-independent analysis of total bacteria.

From the cultured bacteria, 92 different bacterial species were observed by tentative 16SrRNA gene identification, and 37 different bacterial genera were identified. The diversity was found to be similar during daytime and nighttime, except for decreased representation of the family taxa *Bacillaceae* during nighttime (6 % compared to 32 % during daytime). The results obtained can be used as indicators for the numbers of replicate samples needed in order to perform in-depth studies regarding bacterial every-day diversity. Tentatively species identification indicated that 19 air samples was a limited number for such comprehensive investigations, as few species were observed in more than two air samples. Use of supplementary air samplers should also be considered when optimizing the results.

In this study, 402 bacterial isolates were closely characterized based on colony morphology, hemolysis activity and antibiotic resistance, and characteristic traits of the ten most represented family taxa were found based on colony morphology. However, in order to verify these results, repeated studies need to be done. For future investigations, use of more than 402 isolates is advised as most families in this study were represented by less than 18 isolates, limiting the reproducibility of the results.

A specific PCR assay was successfully developed for detection and quantification of commercial Influenza A virus. However, no viral RNA was found in the air samples from Nationaltheatret subway station. Inhibition of the PCR reaction was observed, and hence further investigation regarding inhibition is needed in order to rule out false negative results. Furthermore, longer sampling times should be tested in order to ensure collection of detectable concentrations of virus. The efficiency of the SASS3100 air sampler in collecting virus could be tested by use of other air samplers in parallel to SASS3100 in hospitals or other indoor locations known to contain airborne Influenza virus.

References

Abdel Hameed and A. Awad (2002). "Environmental Study in Subway Metro Stations in Cairo, Egypt." Journal of Occupational Health **44**: 112-118.

Amann, R. I., W. Ludwig, et al. (1995). "Phylogenetic identification and in situ detection of individual microbial cells without cultivation." Microbiol Rev **59**(1): 143-169.

Anderson, K. L. and S. Lebepe-Mazur (2003). "Comparison of rapid methods for the extraction of bacterial DNA from colonic and caecal lumen contents of the pig." J Appl Microbiol **94**(6): 988-993.

Benson, D. A., I. Karsch-Mizrachi, et al. (2009). "GenBank." Nucleic Acids Res **37**(Database issue): D26-31.

C. Pasquarella, O.Pitzurra, et al. (2000). "The index of microbial air contamination." Journal of Hospital Infection **46**: 241-256.

Cartwright, C., S. Horrocks, et al. (2009) "Review of Methods to Measure Bioaerosols from Composting Sites."

Centers for Disease Control and Prevention (2001). "From the Centers for Disease Control and Prevention. Update: Investigation of bioterrorism-related anthrax and interim guidelines for exposure management and antimicrobial therapy, October 2001." JAMA **286**(18): 2226-2232.

Centers for Disease Control and Prevention. (2007). "Emergency Preparedness and Response, Bioterrorism Overview." from http://emergency.cdc.gov/bioterrorism/overview.asp.

Clarridge, J. E., 3rd (2004). "Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases." Clin Microbiol Rev **17**(4): 840-862, table of contents.

Cole, J. R., B. Chai, et al. (2007). "The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data." Nucleic Acids Res **35**(Database issue): D169-172.

David P. Clark (2005). Molecular Biology, Understanding the Genetic Revolution, Elsevier Academic press.

Dong, S. and M. Yao (2010). "Exposure assessment in Beijing, China: biological agents, ultrafine particles, and lead." Environ Monit Assess **170**(1-4): 331-343.

Douwes, J., P. Thorne, et al. (2003). "Bioaerosol health effects and exposure assessment: progress and prospects." Ann Occup Hyg **47**(3): 187-200.

Edwards, R. A. and F. Rohwer (2005). "Viral metagenomics." Nat Rev Microbiol **3**(6): 504-510.

Fabian, P., J. J. McDevitt, et al. (2009). "An optimized method to detect influenza virus and human rhinovirus from exhaled breath and the airborne environment." Journal of Environmental Monitoring **11**(2): 314.

Fouchier, R. A., T. M. Bestebroer, et al. (2000). "Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene." J Clin Microbiol **38**(11): 4096-4101.

Francoise, F. (2002). "Biosolids and Bioaerosols: The Current Situation." Report for the Quebec Ministry of Environment.

Gern, J. E., M. S. Martin, et al. (2002). "Relationships among specific viral pathogens, virus-induced interleukin-8, and respiratory symptoms in infancy." Pediatr Allergy Immunol **13**(6): 386-393.

Gilbert and Duchaine (2009). Bioaerosols in industrial environments: a review. The Free Library. Retrieved March 22, 2011 from

http://www.thefreelibrary.com/Bioaerosols in industrial environments: a review.-a0216041834
Haqqi, T., X. Zhao, et al. (2002). "Sequencing in the presence of betaine: Improvement in sequencing of the localized repeat sequence regions." J Biomol Tech **13**(4): 265-271.
Hawkey, P. M. (2008). "The growing burden of antimicrobial resistance." J Antimicrob Chemother **62 Suppl 1**: i1-9.
Hirvonen, M. R., K. Huttunen, et al. (2005). "Bacterial strains from moldy buildings are highly potent inducers of inflammatory and cytotoxic effects." Indoor Air **15 Suppl 9**: 65-70.
Hope, A. P. and R. A. Simon (2007). "Excess dampness and mold growth in homes: an evidence-based review of the aeroirritant effect and its potential causes." Allergy Asthma Proc **28**(3): 262-270.
Inglesby, T. V., D. A. Henderson, et al. (1999). "Anthrax as a biological weapon: medical and public health management. Working Group on Civilian Biodefense." JAMA **281**(18): 1735-1745.
John Burke Sullivan and G. R. Krieger (2001). Clinical environmental health and toxic exposures. Philadelphia, Lippincott Williams & Wilkins.
Killingley, B., J. Greatorex, et al. (2010). "Virus shedding and environmental deposition of novel A (H1N1) pandemic influenza virus: interim findings." Health Technol Assess **14**(46): 237-354.
Krammer, F., S. Nakowitsch, et al. (2010). "Swine-origin pandemic H1N1 influenza virus-like particles produced in insect cells induce hemagglutination inhibiting antibodies in BALB/c mice." Biotechnol J **5**(1): 17-23.
Kreader, C. A. (1996). "Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein." Appl Environ Microbiol **62**(3): 1102-1106.
L.A. Fletcher, C.J. Noakes, et al. (2011). The importance of bioaerosols in hospital infections and the potential for control using germicidal ultraviolet irradiation, http://www.efm.leeds.ac.uk/CIVE/aerobiology/PDFs/uv-and-airborne-hospital-infection-fletcher.pdf.
Lane, D. J. (1991). "16S/23S rRNA sequencing." Nucleic Acid Techniques in Bacterial Systematics (ed Stackebrandt, E. and Goodfellow, M. Chichester, UK: John Wiley & Sons Ltd.): 115–175.
Maher, N., H. K. Dillon, et al. (2001). "Magnetic bead capture eliminates PCR inhibitors in samples collected from the airborne environment, permitting detection of Pneumocystis carinii DNA." Appl Environ Microbiol **67**(1): 449-452.
Michael T. Madigan and John M. Martinko (2006). Biology of Microorganisms. San Francisco, Pearson Benjamin Cummings.
Millie P. Schafer and P. A. Jensen (1998). Sampling and characterization of bioaerosols. New York.
Nasjonalt folkehelseinstitutt. (2011, 24.02.2011). "Influensaovervåking 2010-11, uke 7." Retrieved 25.02.2011, 10:27, from http://www.fhi.no/eway/default.aspx?pid=233&trg=MainLeft_5565&MainArea_5661=5565:0:15,3938:1:0:0:::0:0&MainLeft_5565=5544:88555::1:5569:9:::0:0.
National Research Council (2005). Sensor Systems for Biological Agent Attacks: Protecting Buildings and Military Bases. Washington, D.C., The National Academies Press.
Payment, P., E. Coffin, et al. (1994). "Blood agar to detect virulence factors in tap water heterotrophic bacteria." Appl Environ Microbiol **60**(4): 1179-1183.
Philip J. Wyatt (2009). "The Inverse Scattering Problem and Solutions for Addressing Bioterrorism." Journal of Homeland Security.

Rafal L. Gorny and Jacek Dutkiewicz (2002). "Bacterial and Fungal Aerosols in Indoor Environment in Central and Eastern European Countries." Ann Agric Environ Med **9**: 17-23.

Research International. (2011). "SASS 3100 Dry Air Sampler System." Retrieved 25.03.11, 14.15, 2011, from http://www.resrchintl.com/sass3100-air-sampler.html.

Rotz, L. D., A. S. Khan, et al. (2002). "Public health assessment of potential biological terrorism agents." Emerg Infect Dis **8**(2): 225-230.

Salaun, S., N. Kervarec, et al. (2010). "Whole-cell spectroscopy is a convenient tool to assist molecular identification of cultivatable marine bacteria and to investigate their adaptive metabolism." Talanta **80**(5): 1758-1770.

Seng, P., M. Drancourt, et al. (2009). "Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry." Clin Infect Dis **49**(4): 543-551.

Shiomori, T., H. Miyamoto, et al. (2001). "Significance of airborne transmission of methicillin-resistant Staphylococcus aureus in an otolaryngology-head and neck surgery unit." Arch Otolaryngol Head Neck Surg **127**(6): 644-648.

Silvy, M., G. Pic, et al. (2004). "Improvement of gene expression analysis by RQ-PCR technology: addition of BSA." Leukemia **18**(5): 1022-1025.

Song, Y., C. Liu, et al. (2003). "16S ribosomal DNA sequence-based analysis of clinically significant gram-positive anaerobic cocci." J Clin Microbiol **41**(4): 1363-1369.

Squires, Chang, et al. (2008). "BioHealthBase: informatics support in the elucidation of influenza virus host pathogen interactions and virulence." Nucleic Acids Research **vol. 36**(Database issue pp. D497).

Srikanth, Sudharsanam, et al. (2008). "Bio-aerosols in indoor environment: composition, health effects and analysis." Indian Journal of Medical Microbiology **26(4)**(4): 302-312.

Stellman, J. M. (1998). Encyclopaedia of occupational health and safety. Geneva, International Labour Office.

Stevens, D. L., A. L. Bisno, et al. (2005). "Practice guidelines for the diagnosis and management of skin and soft-tissue infections." Clin Infect Dis **41**(10): 1373-1406.

Sun, L., K. Teramoto, et al. (2006). "Characterization of ribosomal proteins as biomarkers for matrix-assisted laser desorption/ionization mass spectral identification of Lactobacillus plantarum." Rapid Commun Mass Spectrom **20**(24): 3789-3798.

Thomas, R. J., D. Webber, et al. (2008). "Characterization and deposition of respirable large- and small-particle bioaerosols." Appl Environ Microbiol **74**(20): 6437-6443.

van Elden, L. J. R., M. Nijhuis, et al. (2001). "Simultaneous Detection of Influenza Viruses A and B Using Real-Time Quantitative PCR." Journal of Clinical Microbiology **39**(1): 196-200.

Vellore, J., S. E. Moretz, et al. (2004). "A group II intron-type open reading frame from the thermophile Bacillus (Geobacillus) stearothermophilus encodes a heat-stable reverse transcriptase." Appl Environ Microbiol **70**(12): 7140-7147.

Verreault, D., S. Moineau, et al. (2008). "Methods for sampling of airborne viruses." Microbiol Mol Biol Rev **72**(3): 413-444.

Ward, C. L., M. H. Dempsey, et al. (2004). "Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement." J Clin Virol **29**(3): 179-188.

Weisburg, W. G., S. M. Barns, et al. (1991). "16S ribosomal DNA amplification for phylogenetic study." J Bacteriol **173**(2): 697-703.

Zipper, H., H. Brunner, et al. (2004). "Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications." Nucleic Acids Res **32**(12): e103.

Appendix list

Appendix A

Airborne particle monitoring

In order to investigate airborne particle size distribution profiles for samples collected indoor at day, indoor at night and outdoor, particle sizes and concentrations were monitored in parallel time intervals to sample collection in May – September 2011 (Table A.1).

Table A.1: Airborne particles monitored by Aerotrak 8220, expressed as numbers of aerosols detected during two hours sample collection

Sample name	Number of particles monitored per size interval (μm)					
	0.5-1.0	1.0-2.0	2.0-3.0	3.0-4.0	4.0-5.0	>5.0
180510D	91445915	13022425	2068093	802998	302477	313914
140610D	94423718	12920111	2059884	785216	293746	298059
280610D	70846300	9107893	1666468	662405	248388	258989
280610N	10201078	1498907	221267	101098	44295	58747
260710D	66412793	10278239	1750351	703699	273061	286669
260710N	6060589	739525	161850	74821	32474	39370
260710U	2842204	353375	111447	57307	30244	67769
160810D	116051253	18112949	2739787	1063510	397502	409296
160810N	2479204	180400	49005	27101	15387	31881
160810U	2279177	223025	76441	44935	25964	65822
300810D	97432099	8912054	2537407	1033805	400486	415790
300810N	10637705	1496836	450555	223226	102382	132374
300810U	2149048	167471	43700	26518	15737	36933
130910D	71659691	7834304	2627128	1098610	429928	432778
130910N	6234463	1081269	295429	117835	48503	53986
130910U	3465125	286624	104192	53571	28122	52911
270910D	40157745	5596006	1635227	712434	293294	332737
270910N	4399464	383719	193275	90372	40697	52497
270910U	1500513	117569	47316	26365	14376	33637

Appendix B:

Microbiological characterization

Colony morphologies were noted for each colony selected for classification (Table B.1).

Table B.1: Morphology observations for 402 isolates, noted after 48 hours incubation at 30 °C. _AS means aerobe endospore culturing and _ANS means anaerobe endospore culturing.

Sample name	Medium	Colour	Colony size	Colony shape	Surface
130910D-01	TSA	YELLOW	1mm	IRREGULAR	SMOOTH
130910D-02	TSA	YELLOW	5mm	IRREGULAR	RUFFLED
130910D-03	TSA	YELLOW	5mm	IRREGULAR	RUFFLES
130910D-04	TSA	BEIGE	5mm	ROUND	SMOOTH
130910D-05	TSA	YELLOW	1mm	ROUND	SMOOTH
130910D-06	TSA	BEIGE/CLEAR	1mm	ROUND	SMOOTH
130910D-07	TSA	ORANGE	5mm	IRREGULAR	RUFFLED
130910D-08	TSA	LIGHTYELLOW	5mm	IRREGULAR	RUFFLED
130910D-09	TSA	DARKORANGE	1mm	ROUND	SMOOTH
130910D-11	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
130910D-12	R2A	ORANGE	5mm	ROUND	TEXTURED
130910D-13	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
130910D-14	R2A	BEIGE/WHITE	5mm	IRREGULAR	TEXTURED
130910D-15	R2A	PINK	<1mm	IRREGULAR	SMOOTH
130910D-16	R2A	BEIGE/CLEAR	5mm	IRREGULAR	TEXTURED
130910D-17	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
130910D-18	R2A	BEIGE	1mm	IRREGULAR	SMOOTH
130910D-19	TSA_AS	BEIGE	5mm	IRREGULAR	TEXTURED
130910D-20	TSA_AS	BEIGE	5mm	IRREGULAR	TEXTURED
130910D-22	TSA_AS	BEIGE	1mm	ROUND	SMOOTH
130910D-25	R2A_AS	BEIGE	5mm	IRREGULAR	TEXTURED
130910D-27	R2A_AS	BEIGE	5mm	IRREGULAR	RUFFLED
130910D-28	R2A_AS	BEIGE/YELLOW	5mm	ROUND	SMOOTH
130910N-47	TSA	YELLOW	1mm	ROUND	SMOOTH
130910N-48	TSA	LIGHTYELLOW	1mm	ROUND	SMOOTH
130910N-49	TSA	BEIGE	1mm	ROUND	SMOOTH
130910N-50	R2A	PINK	1mm	ROUND	SMOOTH
130910N-51	R2A	BEIGE	1mm	ROUND	SMOOTH
130910N-52	R2A	BEIGE	1mm	ROUND	SMOOTH
130910U-29	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
130910U-30	TSA	YELLOW	1mm	IRREGULAR	RUFFLED
130910U-31	TSA	YELLOW	1mm	ROUND	SMOOTH
130910U-33	TSA	YELLOW	1mm	ROUND	SMOOTH
130910U-35	TSA	BEIGE	1mm	ROUND	SMOOTH
130910U-36	R2A	LIGHTPINK	1mm	ROUND	SMOOTH
130910U-37	TSA_AS	DARKBEIGE	5mm	IRREGULAR	TEXTURED
130910U-38	TSA_AS	BEIGE	1mm	IRREGULAR	TEXTURED
130910U-39	TSA_AS	BEIGE	5mm	IRREGULAR	TEXTURED
130910U-40	TSA_AS	YELLOW/CLEAR	1mm	IRREGULAR	SMOOTH
130910U-42	R2A_AS	BEIGE	5mm	IRREGULAR	TEXTURED

130910U-44	R2A_AS	BEIGE	1mm	ROUND	SMOOTH
130910U-45	R2A_AS	BEIGE	1mm	ROUND	SMOOTH
140610D-01	R2A_AS	BEIGE	1mm	IRREGULAR	RUFFLED
140610D-03	R2A_AS	BEIGE	5mm	ROUND	TEXTURED
140610D-04	R2A_AS	BEIGE	1mm	ROUND	SMOOTH
140610D05A	R2A_AS	WHITE/GREY	5mm	IRREGULAR	TEXTURED
140610D05B	R2A_AS	BEIGE	5mm	IRREGULAR	RUFFLED
140610D-06	R2A_AS	BEIGE/GREY	5mm	IRREGULAR	RUFFLED
140610D-07	R2A_AS	BEIGE	5mm	IRREGULAR/ROUND	TEXTURED
140610D-08	R2A_AS	BEIGE	5mm	IRREGULAR	TEXTURED
140610D-09	TSA_AS	BEIGE/PINK	5mm	IRREGULAR	RUFFLED
140610D-10	TSA_AS	ORANGE	5mm	IRREGULAR	TEXTURED
140610D-11	TSA_AS	BEIGE	5mm	ROUND	TEXTURED
140610D-12	TSA_AS	BEIGE	5mm	IRREGULAR	RUFFLED
140610D13A1	TSA	BEIGE	1mm	ROUND	SMOOTH
140610D13A2	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
140610D-13B	TSA	BEIGE	5mm	IRREGULAR	RUFFLED
140610D-14	TSA	BEIGE/ORANGE	5mm	IRREGULAR	RUFFLED
140610D-15	TSA	YELLOW	1mm	ROUND	SMOOTH
140610D-16	TSA	WHITE	1mm	ROUND	SMOOTH
140610D17A1	TSA	YELLOW	1mm	ROUND	SMOOTH
140610D17A2	TSA	BEIGE/WHITE	<1mm	ROUND	SMOOTH
140610D-17B	TSA	YELLOW	1mm	ROUND	SMOOTH
140610D-18	TSA	YELLOW	1mm	ROUND	SMOOTH
140610D-19	TSA	YELLOW	1mm	ROUND	SMOOTH
140610D-20	TSA	BEIGE/WHITE	1mm	IRREGULAR	TEXTURED
140610D-21	TSA	YELLOW	1mm	IRREGULAR/ROUND	RUFFLED
140610D-22	TSA	YELLOW	1mm	ROUND	SMOOTH
140610D-23	R2A	BEIGE	5mm	IRREGULAR	RUFFLED
140610D-24A	R2A	BEIGE	<1mm	ROUND	SMOOTH
140610D-24B	R2A	BEIGE	1mm	ROUND	SMOOTH
140610D-25A	R2A	YELLOW/GREY	1mm	ROUND	SMOOTH
140610D25B1	R2A	YELLOW	1mm	ROUND	SMOOTH
140610D25B2	R2A	YELLOW	5mm	IRREGULAR	RUFFLED
140610D-26	R2A	BEIGE	5mm	IRREGULAR/ROUND	SMOOTH
140610D-27	R2A	YELLOW/WHITE	1mm	ROUND	RUFFLED
140610D-28	R2A	BEIGE	5mm	ROUND	TEXTURED
140610D-29	R2A	BEIGE	5mm	ROUND	TEXTURED
140610D-30	R2A	BEIGE/GREY	1mm	ROUND	SMOOTH
140610D-31	R2A	YELLOW	1mm	ROUND	SMOOTH
140610D-32	R2A	YELLOW/WHITE	1mm	ROUND	RUFFLED
140610D-33A	R2A	ORANGE/GREY	1mm	ROUND	SMOOTH
140610D-33B	R2A	YELLOW	5mm	ROUND	SMOOTH
140610D-34	R2A	YELLOW	1mm	ROUND	SMOOTH
140610D-35	R2A	YELLOW/WHITE	1mm	IRREGULAR/ROUND	SMOOTH
160810D-37	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
160810D-38	TSA	YELLOW	1mm	IRREGULAR	RUFFLED
160810D-39	TSA	BEIGE	5mm	ROUND	SMOOTH
160810D-40	TSA	YELLOW	1mm	ROUND	SMOOTH
160810D-41	TSA	BEIGE/PINK	1mm	ROUND	SMOOTH
160810D-42	TSA	YELLOW	5mm	IRREGULAR	RUFFLED
160810D-43	TSA	YELLOW	1mm	ROUND	SMOOTH
160810D-44	TSA	YELLOW	1mm	ROUND	SMOOTH
160810D-45	TSA	BEIGE	1mm	IRREGULAR	SMOOTH
160810D-46	TSA	BEIGE	1mm	ROUND	SMOOTH

160810D-47	TSA	ORANGE	1mm	ROUND	SMOOTH
160810D-48	TSA	YELLOW	1mm	ROUND	SMOOTH
160810D-49	R2A	WHITE	5mm	IRREGULAR	TEXTURED
160810D-50	R2A	BEIGE/PINK	<1mm	ROUND	SMOOTH
160810D-51	R2A	YELLOW	1mm	ROUND	SMOOTH
160810D-52	R2A	WHITE	5mm	ROUND	SMOOTH
160810D-53	R2A	YELLOW	5mm	IRREGULAR	RUFFLED
160810D-54	R2A	YELLOW	5mm	ROUND	SMOOTH
160810D-55	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
160810D-56	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
160810D-57	R2A	YELLOW	1mm	ROUND	SMOOTH
160810D-58	R2A	WHITE	1mm	ROUND	SMOOTH
160810D-59	TSA_AS	BEIGE/YELLOW	5mm	IRREGULAR	TEXTURED
160810D-60	TSA_AS	BEIGE	5mm	IRREGULAR	TEXTURED
160810N-80	TSA	YELLOW	5mm	IRREGULAR	SMOOTH
160810N-81	TSA	BEIGE	1mm	ROUND	SMOOTH
160810N-82	TSA	YELLOW/CLEAR	5mm	IRREGULAR	TEXTURED
160810N-83	TSA	BEIGE	1mm	ROUND	SMOOTH
160810N-84	TSA	YELLOW/CLEAR	5mm	ROUND	SMOOTH
160810N-86	R2A	BEIGE	1mm	ROUND	SMOOTH
160810N-87	R2A	YELLOW/CLEAR	5mm	ROUND	SMOOTH
160810U-01	TSA	YELLOW/PINK	1mm	ROUND	SMOOTH
160810U-02	TSA	YELLOW	1mm	ROUND	SMOOTH
160810U-03	TSA	WHITE	1mm	ROUND	SMOOTH
160810U-04	TSA	BEIGE	1mm	ROUND	SMOOTH
160810U-05	TSA	BEIGE/PINK	1mm	ROUND	SMOOTH
160810U-06	TSA	YELLOW/CLEAR	1mm	ROUND	SMOOTH
160810U-08	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
160810U-09	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
160810U-10	R2A	WHITE/BEIGE	5mm	IRREGULAR	TEXTURED
160810U-11	R2A	BEIGE	5mm	ROUND	SMOOTH
160810U-13	R2A	BEIGE	5mm	IRREGULAR	SMOOTH
160810U-14	R2A	BEIGE	5mm	ROUND	TEXTURED
160810U-15	R2A	YELLOW	1mm	IRREGULAR	SMOOTH
160810U-16	R2A	YELLOW	<1mm	ROUND	SMOOTH
160810U-17	R2A	PINK	<1mm	ROUND	SMOOTH
160810U-18	R2A	YELLOW	1mm	ROUND	SMOOTH
160810U-19	R2A	YELLOW	1mm	ROUND	SMOOTH
160810U-20	R2A	BEIGE	1mm	ROUND	SMOOTH
160810U-21	R2A	BEIGE/DARK	5mm	IRREGULAR	RUFFLED
160810U-22	R2A	YELLOW	1mm	ROUND	SMOOTH
160810U-24	TSA_AS	BEIGE/YELLOW	1mm	ROUND	SMOOTH
160810U-25	TSA_AS	BEIGE/PINK	5mm	ROUND	SMOOTH
160810U-26	TSA_AS	WHITE/BEIGE	5mm	IRREGULAR	TEXTURED
160810U-27	TSA_AS	BEIGE	5mm	IRREGULAR	SMOOTH
160810U-28	R2A_AS	WHITE/BEIGE	5mm	IRREGULAR	TEXTURED
160810U-29	R2A_AS	BEIGE/DARK	5mm	IRREGULAR	RUFFLED
160810U-30	R2A_AS	BEIGE/DARK	1mm	IRREGULAR	TEXTURED
160810U-31	R2A_AS	WHITE/BEIGE	1mm	IRREGULAR	TEXTURED
160810U-33	TSA_ANS	BEIGE	5mm	IRREGULAR	TEXTURED
160810U-34	TSA_ANS	BEIGE	5mm	IRREGULAR	TEXTURED
160810U-35	TSA_ANS	BEIGE	5mm	IRREGULAR	TEXTURED
160810U-36	R2A_ANS	BEIGE	5mm	IRREGULAR	TEXTURED
180510-01	TSA_ANS	BEIGE	5mm	ROUND	SMOOTH
180510-02	R2A_AS	BEIGE/CLEAR	1mm	ROUND	SMOOTH

180510-03	R2A_AS	BEIGE	5mm	IRREGULAR	SMOOTH
180510-05	TSA	BEIGE	1mm	ROUND	SMOOTH
180510-06	TSA	ORANGE/CLEAR	<1mm	ROUND	SMOOTH
180510-07	TSA	WHITE	1mm	ROUND	SMOOTH
180510-08	TSA	BEIGE	1mm	ROUND	SMOOTH
180510-09	TSA	YELLOW	1mm	ROUND	SMOOTH
180510-10	TSA	WHITE	1mm	ROUND	SMOOTH
180510-11A	TSA	WHITE	1mm	ROUND	SMOOTH
180510-11B	TSA	YELLOW	1mm	IRREGULAR	RUFFLED
180510-12	TSA	YELLOW	1mm	ROUND	SMOOTH
180510-13	TSA	YELLOW	1mm	ROUND	SMOOTH
180510-14	TSA	WHITE/BEIGE	1mm	ROUND	RUFFLED
180510-15	TSA	BEIGE/CLEAR	1mm	ROUND	SMOOTH
180510-16	TSA	BEIGE	1mm	ROUND	SMOOTH
180510-17	TSA	YELLOW/BEIGE	1mm	ROUND	SMOOTH
180510-18	TSA	PINK	1mm	ROUND	SMOOTH
180510-19	TSA	ORANGE	1mm	ROUND	SMOOTH
180510-20	TSA	BEIGE	1mm	IRREGULAR	RUFFLED
180510-21	TSA	YELLOW/BEIGE	1mm	ROUND	SMOOTH
180510-22	TSA	WHITE/BEIGE	<1mm	ROUND	SMOOTH
180510-23	TSA	BEIGE	1mm	IRREGULAR	RUFFLED
180510-24	TSA	YELLOW	1mm	ROUND	SMOOTH
180510-25	TSA	YELLOW	1mm	ROUND	SMOOTH
180510-26	TSA	WHITE/BEIGE	1mm	ROUND	SMOOTH
180510-27	TSA	YELLOW	1mm	ROUND	TEXTURED
180510-28	R2A	WHITE/BEIGE	<1mm	ROUND	SMOOTH
180510-29	R2A	WHITE/BEIGE	5mm	IRREGULAR	TEXTURED
180510-30	R2A	ORANGE	<1mm	ROUND	SMOOTH
180510-31B	R2A	YELLOW	1mm	ROUND	SMOOTH
180510-33	R2A	PINK	1mm	ROUND	SMOOTH
180510-34	R2A	YELLOW	1mm	ROUND	SMOOTH
180510-35	R2A	YELLOW	1mm	ROUND	SMOOTH
180510-37A	R2A	ORANGE/YELLOW	<1mm	ROUND	SMOOTH
180510-37B	R2A	BEIGE	1mm	ROUND	SMOOTH
180510-38	R2A	YELLOW/BEIGE	1mm	ROUND	SMOOTH
180510-39	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
180510-40	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
180510-41	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
180510-43	R2A	YELLOW	1mm	IRREGULAR	RUFFLED
180510-44	R2A	YELLOW	1mm	ROUND	SMOOTH
180510-45	R2A	WHITE/BEIGE	1mm	ROUND	RUFFLED
180510-47	R2A	YELLOW	1mm	ROUND	SMOOTH
180510-48	R2A	WHITE/BEIGE	1mm	ROUND	SMOOTH
260710D-02	TSA	WHITE	1mm	ROUND	SMOOTH
260710D-04	TSA	YELLOW	1mm	ROUND	SMOOTH
260710D-05	TSA	YELLOW	1mm	ROUND	SMOOTH
260710D-06	TSA	LIGHTYELLOW	1mm	ROUND	SMOOTH
260710D-07	TSA	BEIGE	5mm	ROUND	TEXTURED
260710D-08	TSA	WHITE	1mm	ROUND	SMOOTH
260710D-10A	TSA	YELLOW	1mm	IRREGULAR	SMOOTH
260710D-10B	TSA	BEIGE/PINK	5mm	ROUND	SMOOTH
260710D-11	TSA	BEIGE	1mm	ROUND	SMOOTH
260710D-12	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
260710D-13	TSA	YELLOW	1mm	IRREGULAR	RUFFLED
260710D-14	TSA	YELLOW	1mm	IRREGULAR	RUFFLED

260710D-15	R2A	LIGHTYELLOW	5mm	IRREGULAR	SMOOTH
260710D-16	R2A	BEIGE/YELLOW	5mm	IRREGULAR	SMOOTH
260710D-17	R2A	YELLOW	5mm	ROUND	SMOOTH
260710D-18A	R2A	YELLOW	1mm	ROUND	SMOOTH
260710D-19	R2A	BEIGE/DARK	5mm	ROUND	SMOOTH
260710D-20	R2A	YELLOW	1mm	IRREGULAR	RUFFLED
260710D-21	R2A	BEIGE	1mm	ROUND	SMOOTH
260710D-22	R2A	BEIGE/WHITE	1mm	ROUND	SMOOTH
260710D-23	R2A	YELLOW	1mm	ROUND	SMOOTH
260710D-26	R2A	YELLOW	1mm	ROUND	SMOOTH
260710D-27	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
260710D-28	R2A	YELLOW	1mm	ROUND	TEXTURED
260710D-29	TSA_AS	YELLOW	1mm	ROUND	SMOOTH
260710D-30	R2A_AS	BEIGE	5mm	IRREGULAR	TEXTURED
260710N-36	TSA	BEIGE/WHITE	5mm	ROUND	SMOOTH
260710N-37	TSA	LIGHTYELLOW	1mm	ROUND	SMOOTH
260710N-38	TSA	BEIGE/WHITE	1mm	IRREGULAR	RUFFLED
260710N-39	TSA	BEIGE/WHITE	1mm	ROUND	SMOOTH
260710N-40	R2A	BEIGE/YELLOW	<1mm	IRREGULAR	SMOOTH
260710N-41	R2A	BEIGE	1mm	ROUND	SMOOTH
260710N-42	R2A	BEIGE/WHITE	1mm	IRREGULAR	RUFFLED
260710N-43	R2A	PINK	1mm	ROUND	SMOOTH
260710N-44	R2A	ORANGE	<1mm	ROUND	SMOOTH
260710N-45	R2A_AS	BEIGE/YELLOW	5mm	ROUND	SMOOTH
260710U-47	TSA	YELLOW	1mm	ROUND	SMOOTH
260710U-49	TSA	BEIGE	5mm	IRREGULAR	RUFFLED
260710U-50	TSA	WHITE	1mm	IRREGULAR	TEXTURED
260710U-51	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
260710U-52	R2A	BEIGE	1mm	IRREGULAR	RUFFLED
260710U-54	R2A	WHITE	1mm	ROUND	SMOOTH
260710U-55	R2A	YELLOW	1mm	IRREGULAR	RUFFLED
260710U-56	R2A	BEIGE	1mm	ROUND	SMOOTH
260710U-57	R2A	BEIGE	5mm	IRREGULAR	RUFFLED
260710U-59	TSA_AS	BEIGE	5mm	IRREGULAR	RUFFLED
260710U-60	TSA_AS	BEIGE	1mm	ROUND	SMOOTH
260710U-62	TSA_AS	YELLOW	1mm	ROUND	SMOOTH
260710U-63	TSA_AS	YELLOW	1mm	ROUND	SMOOTH
260710U-64	R2A_AS	BEIGE	5mm	IRREGULAR	RUFFLED
260710U-65	R2A_AS	BEIGE	5mm	IRREGULAR	TEXTURED
260710U-67	R2A_AS	BEIGE/BROWN	5mm	ROUND	TEXTURED
260710U-68	R2A_AS	BEIGE/PINK	1mm	ROUND	TEXTURED
260710U-69	R2A_AS	YELLOW/CLEAR	1mm	ROUND	TEXTURED
260710U-70	R2A_AS	WHITE	1mm	ROUND	SMOOTH
260710U-71	R2A_AS	BEIGE	5mm	IRREGULAR	RUFFLED
260710U-72	R2A_AS	BEIGE	5mm	IRREGULAR	RUFFLED
260710U-73	R2A_AS	BEIGE	5mm	IRREGULAR	RUFFLED
260710U-74	R2A_AS	BEIGE	1mm	IRREGULAR	RUFFLED
260710U-76	R2A_AS	BEIGE	5mm	IRREGULAR	RUFFLED
260710U-77	R2A_ANS	BEIGE/WHITE	1mm	ROUND	SMOOTH
260710U-78	R2A_ANS	BEIGE	5mm	IRREGULAR	TEXTURED
270910D-15	TSA	BEIGE	1mm	ROUND	SMOOTH
270910D-16	TSA	BEIGE	1mm	IRREGULAR	RUFFLED
270910D-17	TSA	YELLOW	1mm	ROUND	SMOOTH
270910D-18	TSA	ORANGE/CLEAR	1mm	ROUND	SMOOTH
270910D-20	TSA	YELLOW	1mm	IRREGULAR	RUFFLED

270910D-21	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
270910D-22	TSA	BEIGE	5mm	IRREGULAR	RUFFLED
270910D-23	TSA	YELLOW	5mm	IRREGULAR	RUFFLED
270910D-24	TSA	BEIGE	5mm	IRREGULAR	SMOOTH
270910D-25	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
270910D-26	R2A	WHITE	5mm	IRREGULAR	TEXTURED
270910D-27	R2A	BEIGE	5mm	IRREGULAR	SMOOTH
270910D-28	R2A	BEIGE	5mm	IRREGULAR	SMOOTH
270910D-29	R2A	BEIGE	<1mm	ROUND	SMOOTH
270910D-30	TSA_AS	BEIGE	5mm	ROUND	SMOOTH
270910D-31	TSA_AS	PINK	1mm	ROUND	SMOOTH
270910D-32	TSA_AS	BEIGE	1mm	ROUND	SMOOTH
270910D-33	TSA_AS	BEIGE	1mm	ROUND	SMOOTH
270910D-34	TSA_AS	BEIGE	5mm	IRREGULAR	TEXTURED
270910D-35	TSA_AS	BEIGE/WHITE	1mm	ROUND	RUFFLED
270910D-36	R2A_AS	BEIGE	5mm	IRREGULAR	TEXTURED
270910D-37	R2A_AS	ORANGE/CLEAR	1mm	IRREGULAR	SMOOTH
270910D-38	R2A_AS	BEIGE	<1mm	ROUND	SMOOTH
270910D-39	R2A_AS	BEIGE	1mm	ROUND	SMOOTH
270910D-41	TSA_ANS	BEIGE/WHITE	1mm	ROUND	SMOOTH
270910D-42	TSA_ANS	BEIGE/WHITE	1mm	ROUND	SMOOTH
270910D-43	TSA_ANS	BEIGE/WHITE	1mm	ROUND	SMOOTH
270910N-44	TSA	YELLOW	1mm	ROUND	SMOOTH
270910N-45	TSA	YELLOW/CLEAR	1mm	IRREGULAR	SMOOTH
270910U-01	TSA	ORANGE/CLEAR	5mm	IRREGULAR	SMOOTH
270910U-02	TSA	BEIGE	1mm	IRREGULAR	RUFFLED
270910U-03	TSA	YELLOW	1mm	ROUND	SMOOTH
270910U-04	TSA	BEIGE	1mm	ROUND	SMOOTH
270910U-05	TSA	PINK/BROWN	5mm	IRREGULAR	TEXTURED
270910U-06	TSA	ORANGE	1mm	ROUND	SMOOTH
270910U-07	TSA	BEIGE	1mm	IRREGULAR	SMOOTH
270910U-08	R2A	YELLOW	1mm	IRREGULAR	RUFFLED
270910U-09	TSA_AS	BEIGE	5mm	IRREGULAR	SMOOTH
270910U-10	TSA_AS	BEIGE	5mm	IRREGULAR	SMOOTH
270910U-11	TSA_AS	BEIGE	5mm	ROUND	SMOOTH
270910U-12	TSA_AS	BEIGE	1MM	ROUND	SMOOTH
270910U-13	R2A_AS	BEIGE	5mm	ROUND	SMOOTH
270910U-14	R2A_AS	BEIGE	5mm	IRREGULAR	TEXTURED
270910U-40	TSA_ANS	BEIGE/WHITE	1mm	ROUND	SMOOTH
280610D-07	TSA	BEIGE	1mm	IRREGULAR	RUFFLED
280610D-08	TSA	BEIGE	1mm	IRREGULAR	TEXTURED
280610D-09	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
280610D-10A	TSA	YELLOW/BEIGE	1mm	IRREGULAR	RUFFLED
280610D-10B	TSA	BEIGE/WHITE	1mm	IRREGULAR	SMOOTH
280610D-11	TSA	YELLOW	1mm	ROUND	SMOOTH
280610D-12	TSA	YELLOW	1mm	ROUND	TEXTURED
280610D-13	TSA	YELLOW	1mm	ROUND	SMOOTH
280610D-14	TSA	BEIGE	5mm	ROUND	SMOOTH
280610D-15	TSA	BEIGE/YELLOW	1mm	ROUND	SMOOTH
280610D-17	R2A	YELLOW	1mm	ROUND	SMOOTH
280610D-18	R2A	ORANGE	1mm	ROUND	RUFFLED
280610D-19	R2A	BEIGE/WHITE	1mm	ROUND	SMOOTH
280610D-20	R2A	YELLOW	1mm	ROUND	SMOOTH
280610D-21	R2A	BEIGE/PINK	5mm	ROUND	SMOOTH
280610D-22	R2A	BEIGE/YELLOW	<1mm	ROUND	SMOOTH

280610D-23	R2A	YELLOW	5mm	IRREGULAR	TEXTURED
280610D-24	R2A	BEIGE/WHITE	1mm	ROUND	SMOOTH
280610D-25	R2A	YELLOW	1mm	ROUND	SMOOTH
280610D-26	TSA_AS	BEIGE	5mm	IRREGULAR	RUFFLED
280610D-27A	TSA_AS	BEIGE/WHITE	1mm	ROUND	SMOOTH
280610D-27B	TSA_AS	BEIGE/TRANS	1mm	IRREGULAR	TEXTURED
280610D-28	TSA_AS	BEIGE	5mm	IRREGULAR	SMOOTH
280610D-29	TSA_AS	BEIGE/WHITE	5mm	IRREGULAR	RUFFLED
280610D-30	TSA_AS	BEIGE	5mm	IRREGULAR	TEXTURED
280610D-31	TSA_AS	BEIGE	5mm	IRREGULAR	TEXTURED
280610D-32	TSA_AS	BEIGE	5mm	IRREGULAR	RUFFLED
280610D-33	TSA_AS	BEIGE	5mm	ROUND	TEXTURED
280610D-34	R2A_AS	BEIGE	5mm	ROUND	TEXTURED
280610D-35	R2A_AS	BEIGE/YELLOW	5mm	IRREGULAR	TEXTURED
280610D-36	R2A_AS	WHITE/TRANS	5mm	IRREGULAR	TEXTURED
280610D-37	R2A_AS	BEIGE	5mm	ROUND	TEXTURED
280610N-2A	TSA	YELLOW/BEIGE	1mm	ROUND	SMOOTH
280610N-2B	TSA	BEIGE	<1mm	ROUND	SMOOTH
280610N-03	TSA	YELLOW	1mm	ROUND	SMOOTH
280610N-05	TSA	BEIGE	<1mm	ROUND	SMOOTH
280610N-06	R2A	WHITE	1mm	ROUND	SMOOTH
280610N-07	R2A	YELLOW	1mm	ROUND	SMOOTH
280610N-09	R2A	WHITE/BEIGE	1mm	ROUND	SMOOTH
280610N-10	R2A	YELLOW	<1mm	ROUND	SMOOTH
280610N-11	R2A	YELLOW	1mm	ROUND	SMOOTH
280610N-12	R2A	YELLOW	1mm	ROUND	SMOOTH
280610N-14	R2A	WHITE	1mm	ROUND	SMOOTH
280610N-15	R2A	ORANGE	<1mm	ROUND	SMOOTH
280610N-17	TSA	YELLOW	1mm	ROUND	SMOOTH
280610N-18	TSA	YELLOW	1mm	ROUND	SMOOTH
300810D-23	TSA	YELLOW	5mm	IRREGULAR	TEXTURED
300810D-24	TSA	YELLOW	1mm	ROUND	SMOOTH
300810D-25	TSA	BEIGE	1mm	ROUND	SMOOTH
300810D-27	TSA	YELLOW	1mm	ROUND	SMOOTH
300810D-28	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
300810D-29	TSA	BEIGE	1mm	ROUND	SMOOTH
300810D-30	TSA	YELLOW/CLEAR	5mm	IRREGULAR	SMOOTH
300810D-31	TSA	YELLOW/CLEAR	1mm	IRREGULAR	SMOOTH
300810D-32	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
300810D-33	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
300810D-34	TSA	BEIGE/CLEAR	1mm	ROUND	SMOOTH
300810D-35	R2A	YELLOW/BEIGE	5mm	ROUND	SMOOTH
300810D-36	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
300810D-37	R2A	YELLOW	1mm	ROUND	SMOOTH
300810D-38	R2A	BEIGE	5mm	ROUND	SMOOTH
300810D-39	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
300810D-40	R2A	ORANGE	1mm	ROUND	SMOOTH
300810D-41	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
300810D-42	R2A	YELLOW/CLEAR	<1mm	ROUND	SMOOTH
300810D-43	R2A	BEIGE	1mm	ROUND	SMOOTH
300810D-44	R2A	YELLOW/CLEAR	<1mm	ROUND	SMOOTH
300810D-45	TSA_AS	BEIGE	5mm	IRREGULAR	TEXTURED
300810D-46	TSA_AS	BEIGE	5mm	IRREGULAR	TEXTURED
300810D-47	TSA_AS	BEIGE	5mm	IRREGULAR	TEXTURED
300810D-48	TSA_AS	BEIGE	5mm	IRREGULAR	TEXTURED

300810D-49	TSA_AS	BEIGE	5mm	ROUND	SMOOTH
300810D-50	TSA_AS	BEIGE	5mm	ROUND	TEXTURED
300810D-51	R2A_AS	BEIGE	5mm	IRREGULAR	RUFFLED
300810D-52	R2A_AS	BEIGE	5mm	IRREGULAR	TEXTURED
300810D-53	R2A_AS	BEIGE	5mm	IRREGULAR	TEXTURED
300810N-61	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
300810N-64	TSA	YELLOW/CLEAR	1mm	ROUND	SMOOTH
300810N-65	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
300810N-67	R2A	YELLOW/WHITE	1mm	ROUND	SMOOTH
300810N-68	R2A	BEIGE	1mm	IRREGULAR	SMOOTH
300810N-69	R2A	BEIGE	1mm	ROUND	SMOOTH
300810N-71	R2A_AS	BEIGE	1mm	ROUND	SMOOTH
300810U-01	TSA	BEIGE	5mm	IRREGULAR	TEXTURED
300810U-02	TSA	WHITE/PINK	1mm	ROUND	SMOOTH
300810U-03	TSA	BEIGE	5mm	IRREGULAR	SMOOTH
300810U-04	TSA	YELLOW/CLEAR	1mm	IRREGULAR	SMOOTH
300810U-05	TSA	BEIGE/WHITE	1mm	ROUND	SMOOTH
300810U-06	TSA	BEIGE	5mm	ROUND	SMOOTH
300810U-07	TSA	BEIGE/WHITE	5mm	ROUND	SMOOTH
300810U-08	TSA	BEIGE	1mm	ROUND	SMOOTH
300810U-09	R2A	YELLOW	1mm	ROUND	SMOOTH
300810U-10	R2A	BEIGE	1mm	ROUND	SMOOTH
300810U-12	R2A	YELLOW/CLEAR	1mm	ROUND	SMOOTH
300810U-13	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
300810U-15	R2A	BEIGE	5mm	IRREGULAR	TEXTURED
300810U-16	R2A	WHITE/CLEAR	<1mm	IRREGULAR	SMOOTH
300810U-17	TSA_AS	BEIGE	1mm	ROUND	RUFFLED
300810U-18	TSA_AS	YELLOW/CLEAR	5mm	ROUND	SMOOTH
300810U-19	TSA_AS	BEIGE	1mm	ROUND	SMOOTH
300810U-20	R2A_AS	BEIGE	5mm	IRREGULAR	SMOOTH
300810U-21	R2A_AS	BEIGE	5mm	ROUND	SMOOTH
300810U-22	R2A_AS	BEIGE	5mm	IRREGULAR	TEXTURED

Appendix C

MALDI-TOF MS compared to RDP_SeqMatch

402 bacterial isolates were given a best match species identity by RDP_SeqMatch database search. In order to investigate the identification reliability of MALDI-TOF MS fingerprinting compared to RDP_SeqMatch, 84 bacterial isolates (OTUs) were analysed by MALDI-technology, and the results compared to the RDP_SeqMatch results (Table C.1).

Table C.1: Identification by MALDI-TOF MS compared to RDP_SeqMatch, where green colour represents corresponding species identifications, white colour corresponding genus identification and red colour no corresponding identification

OTU	RDP_SeqMatch identification	MALDI-spectra identification	MALDI Score*
1	*Paracoccus yeei*	no peaks found	0,000
2	*Sphingomonas sanguinis*	*Sphingomonas paucimobilis*	2,412
3	*Brevundimonas vesicularis*	*Brevundimonas vesicularis*	1,790
4	*Roseomonas mucosa*	*Roseomonas mucosa*	2,359
5	*Comamonas koreensis*	*Alcaligenes faecalis*	1,373
6	*Kocuria rosea*	*Kocuria rosea*	2,510
7	*Kocuria palustris*	*Kocuria palustris*	1,946
8	*Micrococcus luteus*	*Micrococcus luteus*	2,137
9	*Rothia nasimurium*	*Rothia nasimurium*	1,481
10	*Rothia amarae*	*Rothia amarae*	1,960
11	*Kocuria kristinae*	*Kocuria kristinae*	2,199
12	*Cellulosimicrobium funkei*	*Cellulosimicrobium cellulans*	2,156
13	*Arthrobacter koreensis*	*Arthrobacter gandavensis*	2,545
14	*Microbacterium esteraromaticum*	*Curtobacterium albidum*	1,372
15	*Curtobacterium pusillum*	*Curtobacterium flaccumfaciens*	1,443
16	*Plantibacter flavus*	*Kytococcus sedentarius*	1,399
17	*Dermacoccus nishinomiyaensis*	*Dermacoccus nishinomiyaensis*	1,938
18	*Streptomyces luridiscabiei*	*Streptomyces badius*	2,083
19	*Microbacterium oxydans*	*Microbacterium saperdae*	1,346
20	*Microbacterium oleivorans*	*Lactobacillus kimchii*	1,399
21	*Microbacterium phyllosphaerae*	*Microbacterium paludicola*	2,396
22	*Janibacter limosus*	*Staphylococcus saprophyticus*	2,064
23	*Microbacterium lacus*	*Arthrobacter castelli*	1,838
24	*Microbacterium hatanonis*	*Pseudomonas pictorum*	1,452
25	*Gordonia alkanivorans*	*Gordonia alkanivorans*	2,191
26	*Rhodococcus pyridinivorans*	*Rhodococcus rhodochrous*	2,366
27	*Rhodococcus qingshengii*	*Rhodococcus erythropolis*	2,319
28	*Dietzia cinnamea*	*Sphingobium cloacae*	1,329
29	*Corynebacterium callunae*	*Corynebacterium callunae*	2,300

30	*Arthrobacter humicola*	*Arthrobacter polychromogene*	1,832
31	*Arthrobacter tumbae*	*Arthrobacter ilicis*	1,689
32	*Pseudomonas asplenii*	*Lactobacillus vitulinus*	1,239
33	*Pseudomonas stutzeri*	*Pseudomonas stutzeriL*	2,228
34	*Pseudomonas psychrotolerans*	*Pseudomonas oryzihabitans*	1,866
35	*Pseudomonas poae*	*Pseudomonas poae*	2,142
36	*Pseudomonas asplenii*	*Pseudomonas brenneri*	1,822
37	*Pseudomonas fulva*	*Pseudomonas fuscovaginae*	1,823
38	*Enhydrobacter aerosaccus*	*Moraxella osloensis*	1,846
39	*Pectobacterium cypripedii*	*Escherichia coli*	1,661
40	*Erwinia tasmaniensis*	*Burkholderia sacchari*	1,474
41	*Serratia grimesii*	*Serratia liquefaciens*	1,835
42	*Bacillus simplex*	*Bacillus simplex*	1,901
43	*Bacillus asahii*	*Bacillus asahii*	2,193
44	*Bacillus flexus*	*Bacillus flexus*	1,927
45	*Bacillus megaterium*	*Bacillus megaterium*	1,933
46	*Bacillus luciferensis*	*Rhizobium rhizogenes*	1,427
47	*Bacillus litoralis*	*Aeromonas schubertii*	1,483
48	*Bacillus thuringiensis*	*Bacillus thuringiensis*	2,060
49	*Bacillus subtilis*	*Bacillus subtilis*	2,247
50	*Bacillus licheniformis*	*Bacillus endophyticus*	1,447
51	*Bacillus altitudinis*	*Bacillus pseudofirmus*	1,938
52	*Bacillus drentensis*	*Bacillus novalis*	1,756
53	*Bacillus bataviensis*	*Burkholderia xenovorans*	1,466
54	*Brevibacillus choshinensis*	*Brevibacillus choshinensis*	1,736
55	*Bacillus psychrodurans*	*Bacillus psychrodurans*	1,997
56	*Paenisporosarcina quisquiliarum*	*Bacillus bataviensis*	1,388
57	*Viridibacillus arvi*	*Viridibacillus neidei*	1,961
58	*Planococcus rifietoensis*	*Achromobacter xylosoxidans*	1,349
59	*Bacillus massiliensis*	*Lysinibacillus sphaericus*	1,438
60	*Lysinibacillus sphaericus*	*Lysinibacillus sphaericus*	1,809
61	*Sporosarcina ureae*	*Lactobacillus parabuchneri*	1,418
62	*Exiguobacterium indicum*	*Clostridium novyi*	1,377
63	*Weissella confusa*	*Bacillus asahii*	1,378
64	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	2,055
65	*Staphylococcus aureus*	*Staphylococcus aureus*	2,428
66	*Staphylococcus warneri*	*Staphylococcus warneri*	1,924
67	*Staphylococcus lentus*	*Staphylococcus lentus*	2,005
68	*Staphylococcus equorum*	*Staphylococcus equorum*	2,002
69	*Staphylococcus kloosii*	*Staphylococcus equorum*	1,718
70	*Staphylococcus cohnii*	*Staphylococcus cohnii*	1,815
71	*Staphylococcus saprophyticus*	*Staphylococcus saprophyticus*	2,149
72	*Staphylococcus haemolyticus*	*Staphylococcus haemolyticus*	2,000
73	*Staphylococcus succinus*	*Staphylococcus succinus*	1,849
74	*Tumebacillus permanentifrigoris*	*Staphylococcus auricularis*	1,383

75	*Paenibacillus glucanolyticus*	*Paenibacillus glucanolyticus*	2,392
76	*Paenibacillus konsidensis*	*Paenibacillus glucanolyticus*	1,695
77	*Paenibacillus favisporus*	*Paenibacillus rhizosphaerae*	2,055
78	*Paenibacillus odorifer*	*Paenibacillus odorifer*	1,829
79	*Paenibacillus peoriae*	*Paenibacillus polymyxa*	1,972
80	*Paenibacillus woosongensis*	*Pandoraea pnomenusa*	1,414
81	*Paenibacillus turicensis*	*Lactobacillus curvatus*	1,459
82	*Paenibacillus kobensis*	*Paenibacillus agaridevorans*	1,536
83	*Paenibacillus pabuli*	*Paenibacillus amylolyticus*	1,758
84	*Paenibacillus hodogayensis*	*Staphylococcus cohnii*	1,597

*The MALDI score values were given by the MALDI identification software program

50 % of the isolates identified with non-corresponding species names were found to be lacking in the MALDI-database (Table C.2).

Table C.2: Lack of identification spectra for 25 of the OTUs identified with non-corresponding species by MALDI-TOF MS compared to RDP_SeqMatch

OTU	MALDI compared to RDP	Identification spectra lacking in MALDI-database	
		genus	Species
5	incorrect		*Comamonas koreensis*
12	correct genus		*Cellulosimicrobium funkei*
14	incorrect		*Microbacterium esteraromaticum*
15	correct genus		*Curtobacterium pusillum*
16	incorrect	*Plantibacter*	*Plantibacter flavus*
18	correct genus		*Streptomyces luridiscabiei*
22	incorrect	*Janibacter*	*Janibacter limosus*
23	incorrect		*Microbacterium lacus*
24	incorrect		*Microbacterium hatanonis*
27	correct genus		*Rhodococcus qingshengii*
28	incorrect		*Dietzia cinnamea*
30	correct genus		*Arthrobacter humicola*
34	correct genus		*Pseudomonas psychrotolerans*
38	incorrect	*Enhydrobacter*	*Enhydrobacter aerosaccus*
51	correct genus		*Bacillus altitudinis*
56	incorrect	*Paenisporosarcina*	*Paenisporosarcina quisquiliarum*
58	incorrect	*Planococcus*	*Planococcus rifietoensis*
59	incorrect		*Bacillus massiliensis*
61	incorrect		*Sporosarcina ureae*
62	incorrect	*Exiguobacterium*	*Exiguobacterium indicum*
74	incorrect	*Tumebacillus*	*Tumebacillus permanentifrigoris*
76	correct genus		*Paenibacillus konsidensis*
80	incorrect		*Paenibacillus woosongensis*
81	incorrect		*Paenibacillus turicensis*
84	incorrect		*Paenibacillus hodogayensis*

Appendix D

Bead mill homogenization

A method for extraction of microbial DNA was optimized for samples containing both endospores and vegetative bacteria cells. Bead mill homogenization was chosen as the method for cell lysis prior to DNA isolation, and different amounts of beads and duration of bead beating were tested separately on samples containing endospores *(B. atrophaeus)* and vegetative bacteria cells *(E. coli)*.

The optimal amount of beads and duration of bead beating was found to vary for *B. atrophaeus* and *E. coli*. 1.0 gram beads and bead beating for maximum one minute gave the best results for *E. coli*, whereas 2.0 gram beads and bead beating for minimum three minutes gave the best results for *B. atrophaeus*, as investigated by PCR (data not shown). A compromise that seemed to disfavor *E. coli* and *B. atrophaeus* equally was chosen; 1.5 gram beads and bead beating for two minutes. The effect of these conditions on equally initial numbers of *B. atrophaeus* and *E. coli* were investigated, and compared to non-bead beaten samples (Table D.1).

Table D.1: Comparison of DNA level in bead beaten and non-bead beaten samples, containing *B. atrophaeus* and *E. coli.* Low crossing point (Cp) means high initial concentration of DNA

Sample	Cp	Parallel (Cp)	Average	St.dev.
Bead beaten *B. atrophaeus* ($1.0*10^6$ spores/µl)	16.76	17.18	16.97	0.297
B. atrophaeus ($1.0*10^6$ spores/µl)	25.70	25.49	25.60	0.148
negative control (water)	30.00	30.00	30.00	0.000
Bead beaten *E. coli* ($1.0*10^6$ cells/µl)	13.51	13.46	13.49	0.035
E. coli ($1.0*10^6$ cells/µl)	13.44	14.43	13.94	0.700
negative control (water)	30.00	30.00	30.00	0.000

For *B. atrophaeus*, the amount of DNA in the bead beaten samples was higher than in the non-bead beaten samples, verified by lower Cp (Table D.1). More DNA was released in the bead beaten *E. coli* samples than in the bead beaten *B. atrophaeus* samples, as expected due to weaker cell walls for vegetative cells. For *E. coli*, equal amount of DNA was measured in the non-bead beaten and the bead beaten samples, implying that the initial heat step in PCR was sufficient for lysis of these cells. The results implied that no loss of DNA due to fragmentation was observed in the *E. coli* samples after two minutes bead beating.