Marius Dybwad

Characterization of Airborne Bacteria at a Subway Station: Implications for Testing and Evaluation of Biological Detection, Identification, and Monitoring Systems

Thesis for the degree of Philosophiae Doctor

Trondheim, March 2014

Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
Department of Biotechnology

NTNU – Trondheim
Norwegian University of
Science and Technology

NTNU
Norwegian University of Science and Technology

Thesis for the degree of Philosophiae Doctor

Faculty of Natural Sciences and Technology
Department of Biotechnology

© Marius Dybwad

ISBN 978-82-326-0022-9 (printed ver.)
ISBN 978-82-326-0023-6 (electronic ver.)
ISSN 1503-8181

Doctoral theses at NTNU, 2014:48

Printed by NTNU-trykk

Acknowledgements

This PhD study was financed by and performed at the Norwegian Defence Research Establishment (FFI) in collaboration with the Department of Biotechnology at the Norwegian University of Science and Technology (NTNU). The study was supervised by Dr. Janet Martha Blatny (FFI) and Prof. Svein Valla (NTNU), and co-supervised by Prof. Per Einar Granum at the Norwegian School of Veterinary Sciences.

This study involved laboratory work at FFI and field work at the Nationaltheatret subway station in Oslo, as well as nearly six months of laboratory work at NTNU in collaboration with Ass. Prof. Per Bruheim at the Department of Biotechnology. The study also consisted of an eight-month stay at the Netherlands Organization for Applied Scientific Research (TNO) in Rijswijk, The Netherlands, involving biosafety level-3 laboratory work and scientific collaboration with Dr. Armand Paauw at TNO.

I would like to thank my supervisors, co-authors and everyone else who have contributed to the success of this study. My dear friends and colleagues at FFI deserve a special thank, and especially those in the microbiology group spearheaded by my inspiring, demanding and knowledgeable supervisor Janet. I have also had the privilege of supervising several MSc students: Anja Valen, Maria Hedenstad, and Hanne Grydeland, all of whom have contributed greatly to inspire, challenge and help me throughout the study. Last but not least I must thank those closest to me, my beloved parents for their unconditional support and for providing me with a solid foundation to start from, my beautiful girlfriend for being there through a rollercoaster ride of ups and downs, and several others that have taken part in shaping me into the man and scientist I am today.

I started at FFI in April 2009, completely inexperienced in the field of aerobiology and bioaerosol research, but extremely motivated to dive deep and follow through. Now, almost five years later I hand in my thesis. These past years have opened my eyes to previously unappreciated aspects of both my scientific and personal life, and hopefully awarded me with the tools needed to succeed in both.

This is hopefully just the beginning. Many pages are still left blank. The sky's the limit.

Marius Dybwad, Kjeller, December 2013

"The difficult is what takes a little time. The impossible is what takes a little longer."

~ Fridtjof Nansen

Abstract

Biological detection, identification, and monitoring (BioDIM) systems that are able to provide rapid and reliable early-warning in the event of a bioterrorism attack may contribute to reduce the impact of such incidents. Currently, few if any available BioDIM systems have been able to meet all the users' requirements with respect to reliable, sensitive, and selective detect-to-warn capabilities in different operational environments. BioDIM efforts at most real life sites must be accomplished against a naturally occurring biological aerosol (bioaerosol) background. The bioaerosol background may be both complex and variable, and could challenge the operational performance of BioDIM systems, potentially resulting in the triggering of false alarms, or even worse, the failure to respond to a real incident. One way to improve the operational performance of BioDIM systems is to increase our understanding of relevant bioaerosol backgrounds. Subway stations are enclosed and crowded public environments which may be regarded as potential bioterrorism targets, and therefore also as a relevant operating environment for BioDIM systems.

In order to improve our understanding of the bioaerosol background at subway stations, and especially how it may challenge the operational performance of BioDIM systems, the airborne bacterial background at the Nationaltheatret subway station in Oslo, Norway, was characterized in this study. Information about the concentration level, diversity, size distribution, and temporal variability of the airborne bacterial background was obtained. In addition various virulence- and survival-associated airborne bacterial characteristics such as hemolytic activity, antibiotic resistance, pigmentation, and spore fraction were investigated.

The obtained bioaerosol background characteristics were consolidated with similar and different types of existing characteristics from other subway stations, and used to define a set of realistic subway station background characteristics. Such background characteristics may be valuable when applied in a BioDIM context for several reasons. The information may be used to improve the operational performance of BioDIM systems (e.g. by optimizing alarm algorithms), but also to develop more-realistic methods for testing and evaluation (T&E) of BioDIM systems that take into account the real life background. The defined background characteristics may be used to guide the construction of realistic synthetic subway station bioaerosol backgrounds that can be recreated together with a biological threat agent aerosol challenge during simulated operational T&E of BioDIM systems in aerosol chambers. While

the airborne bacterial background information mainly was intended for use in a BioDIM context in this study, it may also be relevant when viewed in the context of public and occupational health as well as microbial ecology.

An important part of this study also involved testing and implementation of sampling and analysis methods for airborne bacteria. Based on the recognized need for air samplers with well-defined performance criteria, comparative T&E of air samplers was performed in an aerosol chamber to establish their physical and biological sampling efficiencies. The obtained results revealed significant differences between the samplers, which were used to assess their suitability for various bioaerosol sampling applications.

A matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) bacterial identification method (MALDI Biotyper) was evaluated and implemented as a rapid and cost-effective screening tool for airborne bacterial isolate collections. The identification results obtained with the MALDI Biotyper were shown to correspond well with 16S rRNA gene sequencing-based results. However, the MALDI Biotyper failed to obtain reliable identifications when the reference database did not contain library entries at the corresponding species or genus level, and it was suggested that the coverage of environmental airborne bacterial taxa in the reference database should be increased.

Another BioDIM-relevant topic addressed as part of this study was rapid identification methods for *Bacillus anthracis* spores in suspicious powders (e.g. letters). A MALDI-TOF MS-based identification method for *B. anthracis* spores in powders was developed and validated. The observed performance of the analysis method demonstrated its potential applicability as a rapid, specific, sensitive, robust, and cost-effective analysis tool for resolving incidents involving suspicious powders in less than 30 min.

The work presented in this thesis contributes to a deeper understanding of the bioaerosol background, especially at subway stations. It similarly highlights the potential importance of the bioaerosol background in a BioDIM context, and emphasizes the need for increased research efforts to close existing knowledge gaps. This thesis may also serve to highlight the research efforts that will be needed before real life bioaerosol background information may be fully exploited in a BioDIM context.

Abbreviations

ATOFMS	Aerosol time-of-flight mass spectrometry
BioDIM	Biological DIM
BSE	Biological sampling efficiency
BTA	Biological threat agent
CAB	Cultivable airborne bacteria
CBRN	Chemical, biological, radiological, and nuclear
DIM	Detection, identification, and monitoring
FCM	Flow cytometry
FFI	Norwegian Defence Research Establishment
FISH	Fluorescence *in situ* hybridization
FM	(Epi-)Fluorescence microscopy
MALDI	Matrix-assisted laser desorption ionization
NTNU	Norwegian University of Science and Technology
PhD	*Philosophiae Doctor*
PM10	Particulate matter <10 µm
PM2.5	Particulate matter <2.5 µm
PSE	Physical sampling efficiency
qPCR	Quantitative polymerase chain reaction
R&D	Research and development
RH	Relative humidity
ROC	Receiver operating characteristic
SASP	Small acid-soluble protein
T&E	Testing and evaluation
TAB	Total airborne bacteria
TNO	Netherlands Organization for Applied Scientific Research
TSP	Total suspended particulates
UV-LIF	Ultraviolet laser-induced fluorescence

List of publications

This thesis is based on the following peer-reviewed journal publications:

Paper I
Dybwad, M, PE Granum, P Bruheim, and JM Blatny. 2012. Characterization of Airborne Bacteria at an Underground Subway Station. Applied and Environmental Microbiology 78(6):1917-1929.

Paper II
Dybwad, M, G Skogan, and JM Blatny. 2014. Temporal Variability of the Bioaerosol Background at a Subway Station: Concentration Level, Size Distribution and Diversity of Airborne Bacteria. Applied and Environmental Microbiology 80(1):257-270.

Paper III
Dybwad, M, G Skogan, and JM Blatny. 2014. Comparative Testing and Evaluation of Nine Different Air Samplers: End-to-End Sampling Efficiencies as Specific Performance Measurements for Bioaerosol Applications. Aerosol Science and Technology 48(3):281–294.

Paper IV
Dybwad, M, AL van der Laaken, JM Blatny, and A Paauw. 2013. Rapid Identification of *Bacillus anthracis* Spores in Suspicious Powder Samples by Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS). Applied and Environmental Microbiology 79(17):5372-5383.

The following peer-reviewed journal publications were contributed to during the PhD study, but not included in the thesis proper:

Paper V

Stafsnes, MH, M Dybwad, A Brunsvik, and P Bruheim. 2013. Large scale MALDI-TOF MS based taxa identification to identify novel pigment producers in a marine bacterial culture collection. Antonie van Leeuwenhoek 103(3):603-615.

Table of contents

Acknowledgements .... **i**

Abstract .... **iii**

Abbreviations .... **v**

List of publications .... **vii**

Table of contents .... **ix**

1. Introduction **1**

1.1. Biological warfare and terrorism 1

1.2. Defense against biological threats 5

1.3. Biological detection, identification, and monitoring (BioDIM) 6

1.4. Autonomous early-warning BioDIM systems 9

1.5. Testing and Evaluation of BioDIM systems 14

1.6. Environmental backgrounds 23

1.6.1. Aerosols 23

1.6.2. Bioaerosols 24

1.6.3. Airborne bacteria 26

1.7. Characterization of airborne bacterial backgrounds 29

1.7.1. Sampling and analysis of airborne bacteria 29

1.7.2. Airborne bacteria in outdoor and indoor air 36

1.8. Subway transportation systems 38

1.8.1. Airborne bacteria in subway environments 40

1.8.2. The Nationaltheatret subway station, Oslo, Norway 41

1.9. MALDI-TOF MS-based identification of airborne bacteria 43

1.10 Background of the study 44

2. Objectives **47**

3. Summary of publications **49**

4. Discussion **53**

4.1. General discussion 53

4.1.1. BioDIM-relevant background characteristics 57

4.1.2. Proposed generic set of subway station background characteristics 60

4.1.3. General recommendations for the construction of synthetic backgrounds 63

4.2. Methodological considerations 68
4.2.1. Technical challenges associated with TAB quantification methods 69
4.2.2. MALDI-TOF MS as a cost-effective method for CAB identification 71
4.3. Future perspectives 72
5. Conclusions 73
References 79
Papers I-IV 99

1. Introduction

1.1. Biological warfare and terrorism

Biological warfare, also known as germ warfare, is by no means a modern invention and has at least in some form been waged since ancient times [1]. The history of biological warfare has recently been reviewed [2, 3]. During the 20th century, several state-sponsored biological warfare programs (e.g. in the US and the former Soviet Union) developed and stockpiled massive arsenals of biological weapons [4]. Most biological warfare programs were discontinued prior to the 1972 Biological Weapons Convention, also known as the Biological and Toxin Weapons Convention [4]. However, the Soviet Union violated the treaty by developing offensive capabilities and stockpiling biological weapons at least until its fall, and in 1992 the President of Russia, Boris Yeltsin, acknowledged that there had been a delay in his country's implementation of the Biological Weapons Convention [4-6]. The available information regarding the Soviet biological warfare program is still today far from complete, and uncertainties exist concerning the full fate of the program, including its employees, facilities, and stockpiles [4, 5, 7]. Other countries including Iraq also admittedly developed and stockpiled biological weapons at least into the 1990's [4, 8].

In the wake of the era with state-sponsored biological warfare programs soon followed a new reality: the birth of biological terrorism (bioterrorism). Bioterrorism is the deliberate use of pathogenic microorganisms (e.g. bacteria and viruses) or other harmful biological substances (e.g. bacterial toxins) to cause illness or death in people, animals, or plants [9].

Pathogenic microorganisms that may be used for bioterrorism or biological warfare do not by themselves constitute biological weapons, and are generally referred to as biological threat agents (BTAs) or alternatively biological warfare agents. A biological weapon is inherently a two-part system, consisting of a weapons-grade BTA (e.g. weaponized *Bacillus anthracis* spores) mated with a well-defined delivery system (e.g. artillery shells or vehicle-mounted sprayer systems).

The term biological warfare is typically used to describe state-sponsored deployments of biological weapons as part of a tactical or strategic military campaign, while the term bioterrorism more generally describes the intentional use of BTAs or biological weapons by

non-state individuals or terrorist groups. The Merriam-Webster dictionary defines "terror" as "something that causes very strong feelings of fear" and "violence that is committed by a person, group, or government in order to frighten people and achieve a political goal" [10]. In reference to the proposed requirement of a political goal in the "terror" definition, the intentional non-military use of BTAs or biological weapons in the absence of a political goal may be considered acts of biological crime rather than bioterrorism. However, the term bioterrorism is commonly used to describe any intentional non-military use of BTAs or biological weapons [11].

BTAs may be disseminated in a variety of different ways, including through air, water, food, and direct contact [9]. Most BTAs are naturally occurring disease-causing pathogens [9]. However, recent advances in biotechnology, genetics, and synthetic biology have dramatically increased our understanding of microorganisms and our ability to manipulate them, knowledge which may be used to alter the natural state of BTAs, including their virulence, medical countermeasure resilience, and dispersal and persistence characteristics [8, 12]. Some BTAs cause highly contagious diseases that can be transmitted from person to person (e.g. smallpox), while others do not (e.g. anthrax) [9]. The US Centers for Disease Control and Prevention has prioritized BTAs into three categories (A, B, and C) based on their perceived risk to public and national security [9]. A summary of the Bioterrorism Agents List is provided in Table 1, while examples of BTAs from Category A are shown in Figure 1.

As the 20th century drew to a close, most biological defense professionals, both military and civilian, were in agreement that the likelihood of a bioterrorism event occurring in the US was not a matter of *if*, but *when* [13]. In 1984 the Rajneeshee cult in Oregon had contaminated restaurant salad bars with *Salmonella typhimurium*, sickening more than 700 people with food poisoning, some of whom were seriously affected although no fatalities were reported [14]. In 1993 the Japanese Aum Shinrikyo cult had aerosolized *B. anthracis* spores from a rooftop in Tokyo, but no cases of anthrax were reported and largely attributed to the use of an attenuated vaccine strain [15].

The 21th century had barely begun when the "not *if*, but *when*" prediction was proven right. After terrorist attacks on the World Trade Center and the Pentagon in the fall of 2001, envelopes containing *B. anthracis* spores were mailed to several news media and government officials, leading to the first known bioterrorism-related cases of anthrax in the US [16, 17]. The bioterrorism event, commonly termed the "Amerithrax" incident, demonstrated *B.*

anthracis as a bioterrorism agent, killed five people, and sickened an additional seventeen [16, 17]. Although the number of casualties was relatively limited, the societal consequences were massive, including public fear and panic, disruption of the mail service, distrust in the US government's ability to protect its citizens, and economic disbursements of more than one billion US dollars [13, 16, 17]. This serves to demonstrate that although bioterrorism by definition is intended to cause illness or death, the societal consequences of a bioterrorism incident may be far from restricted or proportional to its direct health implications. Acts of bioterrorism are prone to trigger fear and panic in humans because disease outbreaks and the causative BTAs are inherently associated with images of pain, suffering, and death. The human society has throughout history experienced several devastating natural outbreaks with diseases such as plague [18], smallpox [19] and influenza [20]. Microorganisms will generally be invisible to the naked eye due to their small size even when present in life-threatening amounts, and the fact that BTAs may unnoticeably be present in the air we breathe, the water we drink, and the food we eat constitute another potentially important psychological aspect of bioterrorism [21]. Furthermore, BTAs may also be extremely difficult to detect and do not cause clinical symptoms in those exposed until several hours, days, or even weeks after exposure [22].

The future of bioterrorism in the 21th century is extremely difficult to predict. Uncertainties exist even with respect to state-sponsored biological warfare programs since monitoring of clandestine offensive efforts is complicated by the inherent dual-use potential of the know-how, equipment, and facilities involved [7, 8]. Compared to state actors, terrorists and terrorist organizations may be harder to deter and are not bound by the Biological Weapons Convention, and thus have fewer constraints on the use of BTAs although their technical capabilities are presumably more limited [8]. Biological weapons have been described as the "poor man's atom bomb" because the comparative costs associated with producing civilian casualties have been estimated at 2000 USD per square kilometer with conventional weapons, 800 USD with nuclear weapons and 1 USD with biological weapons [23, 24]. A warning of what the future might bring may be read from recent reports suggesting that terrorist organizations such as al-Qaeda have requested for trained microbiologists and chemists to answer the call to arms [12]. An important lesson from human history is the observation that whatever technology is available will eventually be applied in warfare as one side seeks to gain an advantage over the other [25], which may be an especially relevant principle during asymmetric conflicts.

Table 1. The Bioterrorism Agents List [9].

Disease/Agent	Criteria
Category A agents	
	Highest priority because they may:
Anthrax (*B. anthracis*)	- pose the highest risk to the public and national security
Botulism (botulinum toxin of *Clostridium botulinum*)	- be easily spread or transmitted from person to person
Plague (*Yersinia pestis*)	- result in high death rates
Smallpox (variola major virus)	- have potential for major public health impact
Tularemia (*Francisella tularensis*)	- cause public panic and social disruption
Viral hemorrhagic fevers (e.g. Ebola virus)	- require special actions for public health preparedness
Category B agents	
	Second highest priority because they may:
Brucellosis (*Brucella* spp.)	- be moderately easy to spread
Glanders (*Burkholderia mallei*)	- result in moderate illness rates and low death rates
Melioidosis (*Burkholderia pseudomallei*)	- require specific enhancements of CDC's laboratory capacity
Psittacosis (*Chlamydia psittaci*)	- require enhanced disease monitoring
Q fever (*Coxiella burnetii*)	
Typhus fever (*Rickettsia prowazekii*)	
Viral encephalitis (e.g. Venezuelan equine encephalitis)	
Food safety threats (e.g. *Escherichia coli* O157:H7)	
Water safety threats (e.g. *Vibrio cholerae*)	
Epsilon toxin of *Clostridium perfringens*	
Staphylococcal enterotoxin B of *Staphylococcus aureus*	
Category C agents	
	Third highest priority because they may:
Nipha virus	- be considered emerging pathogens
Hantaviruses	- be engineered for mass spread in the future
	- be easily available, produced, and spread
	- have potential for high morbidity and mortality rates
	- have potential for major public health impact

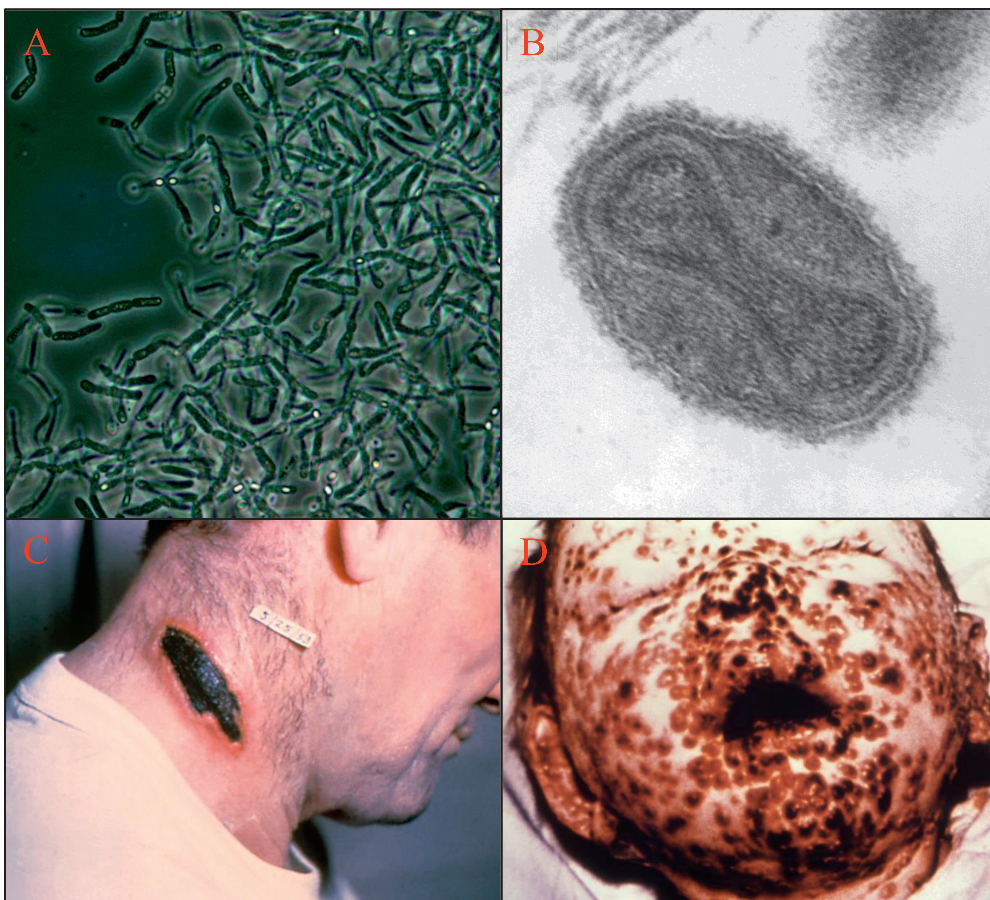

Figure 1. Examples of BTAs in Category A. Phase-contrast microscopy image (A) showing rod-shaped vegetative cells, some with phase-bright endospores, of the Gram-positive bacterium *B. anthracis,* the causative agent of anthrax (C, cutaneous anthrax). Transmission electron microscopy image (B, ~370 000x magnification) showing a virion of the variola virus, a dsDNA enveloped virus in the *Orthopoxvirus* genus, the causative agent of smallpox (D). Image credit: Centers for Disease Control and Prevention. The images are in the public domain.

1.2. Defense against biological threats

Defense against biological threats (biodefense) is both a military and civilian objective, aiming at protecting troops and military operations, and public health and civilian operations, respectively. Biodefense is an integral part of the broader defense against chemical, biological, radiological, and nuclear threats (CBRN). Recent statements made by several civilian and military organizations, including the North Atlantic Treaty Organization, the

European Union, the United Nations, and the US government, serve to highlight the well-recognized importance of having CBRN defense capabilities [26-31]. Although the scenarios, concepts, and operational requirements may differ between various military and civilian organizations, the fundamental objective of biodefense is the same, namely avoiding or reducing the impact of biological threat incidents.

Biodefense strategies are generally based on various combinations of core principles or capabilities, sometimes referred to as pillars, and which may include but are not limited to principles such as: prevention, preparedness, protection, response, and recovery [26, 27, 29, 30, 32]. Biodefense efforts typically consist of a variety of different activity types, for example: intelligence work, stockpiling of medical countermeasures, response scheme planning, and training of involved stakeholders.

The term biodefense is generally used in association with incidents involving an intentional release of BTAs. However, since accidents and natural disease outbreaks involving BTAs may have similar manifestations as intentional releases, most biodefense efforts, except maybe those that are exclusively directed towards prevention, may provide beneficial synergies independently of whether the biological threat originates from an intentional act, accident, or natural disease outbreak.

Aerial dispersion of BTAs is generally recognized as the most efficient way to target a high number of people and potentially cause mass casualties, and bioterrorism and biological warfare incidents involving aerosolized BTAs may therefore be proposed to represent the largest health and security threat [2, 33, 34].

1.3. Biological detection, identification, and monitoring (BioDIM)

Accurate, timely, and reliable detection, identification, and monitoring (DIM) capabilities are important assets in any civilian and military CBRN defense strategy [26, 27, 29, 30, 32, 35]. However, the currently existing early-warning DIM capabilities for biological threats (BioDIM) have been shown to lag behind their C, R, and N counterparts [23, 36]. CBRN DIM concepts have been formulated by various organizations, including the North Atlantic Treaty Organization, the European Union, the European Defence Agency, and the US Department of Defense [32, 35, 37, 38]. The individual concepts are generally not identical, and which

probably reflect different needs of the various organizations due to their structure and mission. However, the overall CBRN DIM concept appears to be largely consistent, and when formulated in a generic BioDIM context the following DIM definition seems appropriate:

The primary objective of ***detection*** is to discover the presence of a biological threat as early as possible, preferably before it has negative effects, and provide timely information (alarming/warning) which permit an appropriate level of individual and collective protection to be adopted [32, 35]. Detection may be categorized depending on where the detection event takes place relative to the site of protection: i) point detection at the site of protection by a detector at the same site, ii) stand-off detection at a distance from the site of protection by a detector at the site of protection, and iii) remote detection at a distance from the site of protection by a detector at the distant site [32, 35].

The purpose of ***identification*** is to characterize and determine the nature of the biological threat in quality and quantity, in order to provide information on the type and nature of the hazard, optimize physical protection levels, and select appropriate hazard management and medical countermeasures [32, 35]. Identification may be categorized according to the level of confidence achieved: i) provisional identification when based on one recognized immunological (i.e. antibody-based), nucleic acid (e.g. PCR-based), or *in vitro* culture/multi-metabolic identification method, ii) confirmed identification when based on two of the three method classes, and iii) unambiguous identification when based on all three method classes in combination with an accepted *in vivo* model [39].

A ***monitoring*** capability refers to the process of continuously or periodically determining whether or not a biological threat is present [32, 35].

Several reviews of BioDIM equipment, including commercial off-the-shelf, emerging, and developmental products, have recently been published [36, 40-42]. It is however important to recognize that the collective term BioDIM inherently covers a broad range of different capability and equipment classes, and which may be associated with very different operational requirements and intended uses. As an example the various BioDIM equipment classes may be categorized based on:

- Intended user (e.g. first responders, warfighters, civilian or military CBRN specialists, and clinical diagnosticians).

- Location of use (e.g. on-site/field use, mobile response laboratories, reach-back CBRN specialist laboratories, and clinical diagnostics laboratories).
- Mode of operation (e.g. manual, semi-automated, autonomous, handheld, mobile, and stationary).
- Sample type (e.g. air, soil, water, powder, surface, and clinical samples).

The benefit of BioDIM capabilities will inherently be closely linked to the responsive actions they trigger and the timeliness of their implementation. Responsive actions are commonly categorized as either low or high regret actions based on their disruptive effects [43]. In a military context, low regret actions may include donning of personal protective equipment, initiation of additional BioDIM efforts, and halting of non-critical operations, while high regret actions may include administering medical countermeasures that could compromise warfighter combat readiness and mission-critical operations. In a civilian context, low regret actions may include ventilation system adjustments and initiation of additional BioDIM efforts, while high regret actions may involve facility shutdown, evacuation, and prophylactic treatment with medical countermeasures.

A response time-centric approach may be used to categorize BioDIM assets into at least two different time-dependent capability classes: i) detect-to-warn, sometimes referred to as detect-to-protect, and ii) detect-to-treat [43, 44].

A detect-to-warn capability must respond in sufficient time to allow implementation of responsive actions that prevent or reduce exposure of a significant portion of the at-risk population (e.g. through evacuation or avoidance) [43]. The timeframe of a detect-to-warn capability will depend on the type and location of the incident, and may range from hours in the context of remote detection and outdoor incidents, to minutes or even seconds in the context of point detection and indoor incidents [43].

A detect-to-treat capability must respond in sufficient time to allow implementation of responsive actions that result in avoidance or reduction of the health implications that may be associated with BTA exposures (e.g. early medical treatment that results in improved prognosis) [43]. The timeframe of a detect-to-treat capability will typically range from several hours to several days or even weeks, and depend on the type of BTA involved, the level of exposure, and the time-dependent efficacy and availability of medical countermeasures [43].

After the detect-to-treat timeframe has passed, the benefits associated with BioDIM capabilities will generally be limited to forensic and reclamation activities (e.g. source identification and decontamination) [43].

1.4. Autonomous early-warning BioDIM systems

Traditional clinical diagnostics, including disease surveillance and outbreak recognition systems, will play a central role in any biodefense strategy. However, a biodefense strategy based solely on clinical recognition of symptomatic individuals, several hours, days, or even weeks after an incident, may result in: i) exposed individuals that have travelled worldwide before the incident is unraveled, ii) maximized number of exposed individuals and exposure levels, iii) minimized applicability of prophylactic treatment, and iv) potentially reduced disease prognosis due to late initiation of medical countermeasures. Based on this it becomes apparent that a detection timeframe based solely on clinical recognition may complicate post-incident response efforts, and potentially result in the unnecessary loss of human lives and increased societal and economic burdens [33, 45]. Adding to the challenge is the fact that most BTAs result in diseases that initially present with unspecific symptoms, including flu-like symptoms such as fever, body aches, cough, and tiredness, and which may further delay clinical recognition and subsequent response efforts [33].

Autonomous early-warning BioDIM systems* for BTA aerosols are therefore likely to play an important role in any biodefense strategy because they are probably the only enablers of responsive actions (e.g. evacuation and prophylactic medical treatment) within the exposure (i.e. detect-to-warn) or disease incubation (i.e. detect-to-treat) timeframe of a biological threat incident [33, 45, 46].

****Note: The Author recognizes that different types of BioDIM systems exist and that they are in no way restricted to autonomous, early-warning, or aerosol-dedicated systems. However for the purpose of this report, the term BioDIM will from here on be used consistently in the context of, and should be taken to suggest, autonomous early-warning BioDIM systems for BTA aerosols.***

Some inherent and to some extent also unique challenges associated with BioDIM are:

- The presence of even extremely low quantities of most BTAs in the air may result in significant health hazards due to their infectious nature (i.e. the multiplier effect).
- The inherent complexity and diversity of microorganisms combined with their ubiquity in nature, and which may include environmental nonpathogenic near-neighbors of BTAs, may contribute to an omnipresent and potentially highly variable natural background.
- The response time will be a critical factor since the time delay between exposure and health effects (i.e. the incubation period) that are associated with most BTAs may result in incidents that remain undiscovered until symptomatic individuals seek medical attention several hours, days, or even weeks later.
- The list of potential BTAs is long, and even when only the highest priority BTAs are considered, the compositional and structural diversity of the BTAs that may be encountered is vast (e.g. bacterial spores, Gram-negative vegetative bacteria, DNA and RNA viruses, and protein toxins).
- The range of potential target locations and attack scenarios is almost endless.

In combination the various challenges have made it difficult to meet all the operational requirements that will be associated with BioDIM, and especially detect-to-warn capabilities. The individual requirements (e.g. sensitivity, selectivity, and response time) have however been shown to be within the reach of various already existing BioDIM technologies and products, but that being said, successful integration of the combined requirements into a single system has proven much more challenging and typically resulted in significant performance trade-offs [36, 43]. An ideal BioDIM asset should [34]:

- Respond to all or at least all high-priority BTAs in real or near-real time.
- Have sufficient sensitivity to react to the lowest life-threatening hazard levels.
- Have sufficient selectivity to avoid false alarms independently of the operating environment.
- Be capable of autonomous operation for extended periods of time with minimal maintenance.
- Have acquisition and operating costs that are harmonized to the benefit of the system.

An urgent need for BioDIM systems capable of operating in complex environmental backgrounds has been expressed by both military and civilian authorities, exemplified by the Joint Biological Point Detection System program of the US Department of Defense and the BioWatch Generation 3 program of the US Department of Homeland Security.

While BioDIM systems for point detection are available, few if any systems have currently been fielded for stand-off detection [43]. The system that may be considered closest to fielding is probably the Joint Biological Stand-off Detection System program of the US Department of Defense [43]. However, based on several limitations that are inherent to stand-off detection, in combination with the wide variety of environmental conditions that may be encountered and the low BTA aerosol concentrations needed to present a hazard, it has been suggested that it may not be possible to develop a stand-off system with acceptable performance in terms of reliably detecting small but still potentially hazardous biological threat incidents [46].

Several multi-step architectures have been proposed for BioDIM systems [43, 46, 47]. Most concepts typically involve the use of one or more front-end units that serve to initiate low regret actions and trigger one or more back-end units, which in turn confirm the front-end response and initiate high regret actions. The front-end typically consists of one or several real or near-real time detectors/triggers (usually spectroscopy-based), while the back-end consists of a combination of air collectors and identifiers (usually immunoassay- or PCR-based). Although the back-end identifiers achieve a higher selectivity than the front-end detectors/triggers, and which typically is at the level of provisional (immunological- or nucleic acid-based) or even confirmed (immunological- and nucleic acid-based) identifications, the increased selectivity generally results in longer response times and high-cost systems.

Currently, few if any available BioDIM systems have been able to meet all the users' requirements with respect to reliable, sensitive, and selective detect-to-warn capabilities in different operational environments [36, 43]. However, ongoing research and development (R&D) efforts by the private industry and governmental research organizations are continuously working to close the capability gaps by implementing new technologies and refining existing ones. Examples of current and emerging BioDIM systems are shown in Figure 2.

Figure 2. Examples of current and emerging BioDIM systems. Panel A: C-FLAPS from Dycor Technologies. Panel B: BioHawk from Research International. Panel C: Resource Effective Bio-Identification System from Battelle. Panel D: Joint Biological Point Detection System program of the US Department of Defense. Image credit: Dycor Technologies (A), Research International (B), Battelle (C), and the US Joint Program Executive Office for Chemical and Biological Defense (D). Printed with permission.

BioDIM systems operating in real or near-real time were initially based on light scattering techniques (i.e. particle counting and sizing), but have gradually evolved to incorporate more advanced spectroscopy-based techniques (e.g. ultraviolet laser-induced fluorescence [UV-LIF] and laser- or spark-induced breakdown spectroscopy) in an effort to address inherent selectivity and sensitivity limitations [46-49]. Various attempts have been made to further improve the performance of spectroscopy-based systems by combining several orthogonal techniques in a single detector (e.g. the Rapid Agent Aerosol Detector based on multi-band elastic scattering and UV-LIF in combination with laser-induced breakdown spectroscopy), as well as attempts to reduce the instrument costs (e.g. the Bioaerosol Agent Sensor and Trigger which utilizes low-cost UV-LEDs instead of UV-lasers) [46, 47].

In addition to spectroscopy, various aerosol time-of-flight mass spectrometry (ATOFMS) techniques have been investigated for use as real or near-real time BioDIM systems (e.g. the Single-Particle Aerosol Mass Spectrometry system, formerly known as the Bio-Aerosol Mass Spectrometry system) [50-52].

In recently years, several identification technologies (e.g. nucleic acid- and antibody-based techniques) previously restricted to laboratory-based systems have been automated, miniaturized, and ruggedized for field use. These R&D efforts have resulted in BioDIM systems that are based on identification technologies, such as the Autonomous Pathogen Detection System utilizing continuous air sampling followed by PCR- and immunoassay-based identifications [34], and the Joint Biological Point Detection System utilizing spectroscopy-based detection followed by triggered air sampling and immunoassay-based identifications. When these systems have been successfully validated for autonomous field applications they may with their current response times allow for on-site BTA identifications within the early detect-to-treat timeframe, but typically not within the detect-to-warn timeframe. However, future progress in the fields of microfluidics, nanotechnology, and molecular biology may someday result in BioDIM systems that are capable of obtaining BTA identifications within the detect-to-warn timeframe. Such systems may then at least in principle merge all DIM tasks into a single process, and reduce the need for a separate and less discriminatory (e.g. spectroscopy-based) detection step. With this in mind it is however important to remember that even if the technological basis for a detect-to-warn BioDIM system based on one or several identification technologies becomes available, several additional factors must be carefully considered when assessing the cost-benefit of any BioDIM capability (e.g. availability of responsive actions, cost, and ease-of-use). Furthermore, it is similarly important to remember that since the fundamental objective of a detection capability by definition is to rapidly warn about the presence of a biological hazard, the inherent capability need stops at the ability to confidently determine whether there is a biological hazard present or not. In contrast to detection, an identification capability must inherently involve a higher level of characterisation with respect to discerning the exact nature of the biological hazard. It is therefore essential for the understanding of the BioDIM concept that detection, identification, and monitoring remain closely inter-related but separate processes by which an incident involving a biological hazard is discovered [32, 35, 39], although they may at least in principle be performed as a single process.

1.5. Testing and Evaluation of BioDIM systems

A lot of effort is being put into R&D of BioDIM systems. Prior to qualification and commissioning these systems must fulfill stringent requirements before they can be relied upon in the context of a biodefense strategy, including decision-making in terms of high and low regret responsive actions. Testing and evaluation (T&E) of BioDIM systems will therefore be a critical task to ensure that the operational performance (e.g. sensitivity and false alarm rate) is harmonized to the user's operational needs.

The potential benefits of BioDIM systems will inherently be linked to their operational performance and credibility, and erroneous responses, including responding when no biological threat is present (false positives) or failing to respond when a biological threat is present (false negatives), may severely damage a system's credibility and potentially result in terrible consequences. While false positives generally will be restricted to economic and psychological implications, false negatives may on the other hand potentially result in the unnecessary loss of human lives.

T&E of BioDIM systems may be performed in a variety of ways and for different purposes, but the overall approach may broadly be categorized as either developmental testing or operational testing [53]. Operational testing is performed in the real environments where the system will be used (e.g. on the battlefield) during execution of realistic missions (e.g. warfighters in combat-like situations), while developmental testing is testing performed in the absence of realistic operational conditions (e.g. in aerosol test chambers). In addition to the large economic costs and logistical challenges that may be associated with operational testing, one of the major hurdles in achieving realistic operational testing is the fact that BTAs cannot be used due to concern for public safety, laws, and treaties [53]. T&E involving BTAs is therefore inherently restricted to containment laboratory facilities, and as a consequence developmental testing is currently the only available option for live agent testing.

T&E of BioDIM systems that consist of an integrated architecture of detectors, air collectors, and identifiers may be performed as either component testing (i.e. the individual components are tested separately) or whole system testing (i.e. the integrated system is tested as a whole).

The T&E scheme used for BioDIM systems generally involves subjecting the system to a well-characterized aerosol challenge and observing its response. The challenge typically

consists of an artificially generated aerosol containing live or inactivated BTAs or simulants, but may also consist of potentially interfering substances including dirt particulates (e.g. Arizona road dust), smoke, kaolin, fungal spores, pollen, and others [47]. Inactivation of BTAs and simulants is typically achieved using ionizing radiation, heat, or chemicals. Common examples of simulants used for T&E of BioDIM systems include [43, 54, 55]:

- *Bacillus atrophaeus* spores (syn. *Bacillus globigii*, BG, and *Bacillus subtilis* var *niger*) as surrogate for bacterial spores (e.g. *B. anthracis* spores).
- *Pantoea agglomerans* (syn. *Erwinia herbicola*) as surrogate for Gram-negative vegetative bacteria (e.g. *Yersinia pestis* and *Francisella tularensis*).
- Bacteriophage MS2 (syn. Male-specific coliphage 2 and MS2) as surrogate for viruses (e.g. variola virus and filoviruses).
- Ovalbumin (syn. egg white protein) as surrogate for protein toxins (e.g. botulinum toxin).

It has been proposed that the currently used simulants may have several shortcomings and fail to sufficiently represent the whole range of potential BTAs in terms of physical, physiological, chemical, and antigenic properties [55]. The appropriateness and utility of certain organisms as simulants is an ongoing topic of debate [54]. In an attempt to circumvent the potentially limited relevance of simulants, as well as the inherent use-restrictions associated with BTAs, an additional class of biological test materials termed agent-like organisms has been developed. Agent-like organisms may be used in their live or inactivated form, and typically consist of organisms that are closely related to BTAs (e.g. attenuated vaccine strains) but that have reduced or nonexistent associated health risks [53, 54, 56].

Several different types of test facilities (Figure 3) may be used for T&E of BioDIM systems:

- Laboratory testing (e.g. aerosol test chambers and wind tunnels).
- Field testing (e.g. open-air test ranges and ambient breeze tunnels).
- Operational testing (e.g. real life operational environments).

During laboratory testing both the challenge (e.g. BTA aerosol) and the environment (e.g. meteorological conditions) may be tightly controlled and predicted, and thus offer the possibility for reproducible and cost-effective T&E. Field testing will typically be more expensive, logistically demanding, and time-consuming than laboratory testing, but allows T&E to be performed under real ambient environmental conditions. Due to the inherent lack

of control with respect to environmental conditions, both the challenge and the environmental background presented to the BioDIM system is generally less predictable and reproducible during field testing than laboratory testing. Field testing involving the use of ambient breeze tunnels instead of open-air releases may allow improved control of the challenge while retaining realistic environmental conditions. However, because of the use-restrictions and potential hazards that are associated with releasing microorganisms into the environment, field testing facilities are generally located far away from urban or otherwise densely populated areas, and which may result in environmental backgrounds that are operationally representative only for desolate locations with a limited or nonexistent presence of anthropogenic influences. Operational testing is therefore generally restricted to testing without artificially generated challenges (i.e. limited to evaluation of false positive rates) as long as the operationally realistic environments for the BioDIM system differ from those that are available at existing field testing facilities.

Figure 3. Examples of aerosol test facilities that may be used for T&E of BioDIM systems. Panel A-B: Open-air test ranges. Panel C-D: Ambient breeze tunnels. Panel E: Containment aerosol test chambers. Image credit: Dugway Proving Ground. Printed with permission.

Examples of commonly used equipment for aerosol challenge generation and characterization during T&E of BioDIM systems (Figure 4) include [54]:

Challenge generation (aerosolization)

- Wet dissemination (e.g. nebulizers and ultrasonic atomizers)
- Dry dissemination (e.g. fluidized bed aerosol generators)

Challenge characterization (reference measurements)

- Particle counting and sizing (e.g. optical particle counters and aerodynamic particle sizers)

- Air sampling (e.g. slit-to-agar samplers, liquid impingers, and filter samplers)
- Sample analysis (e.g. cultivation, qPCR, flow cytometry, and electrochemiluminescence)

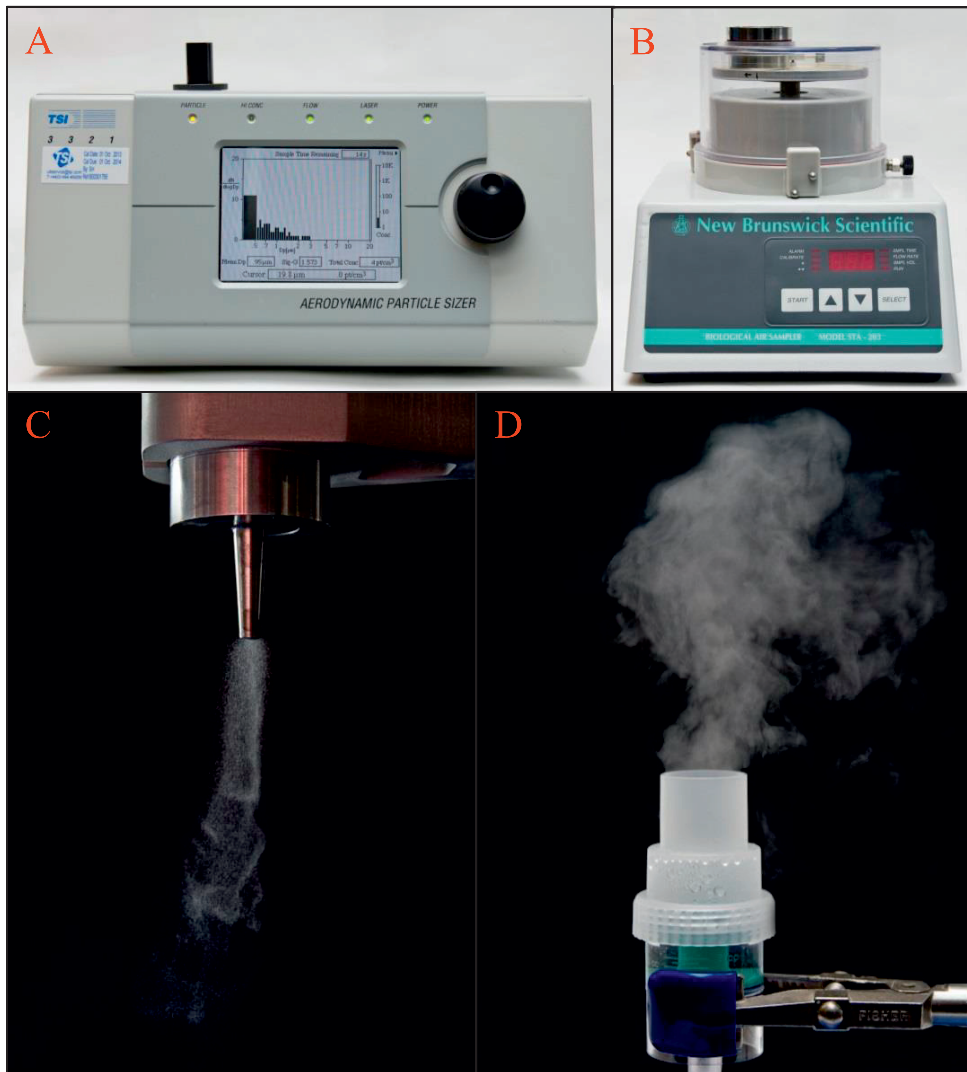

Figure 4. Examples of aerosol challenge generation and characterization equipment that are commonly used during T&E of BioDIM systems. Panel A: Aerodynamic particle sizer APS3321 (TSI, Shoreview, MN). Panel B: Slit-to-agar sampler STA-203 (New Brunswick, Edison, NJ). Panel C: Ultrasonic atomizer nozzle (Sonotek, Milton, NY). Panel D: Hudson-type MicroMist nebulizer (Hudson RCI, Temecula, CA). Image credit: Norwegian Defence Research Establishment.

At least three different reference standards have been proposed for use in T&E of BioDIM systems [54, 57]:

- ACPLA (Agent containing particles per liter of air).
- $BAULA_{Dae}$ (Biologically active units per liter of air) with particle size information.
- TALAp (Total agents per liter of air) with particle size information.

Despite several decades of R&D and T&E of BioDIM systems we may still remain vulnerable to biological threat incidents that are not uncovered until those exposed and infected are either sick, dying, or dead [47]. An unfortunate but nevertheless common trend shows that BioDIM systems performing well under laboratory conditions may fail when faced with real-world challenges [56, 58]. The importance of rigorous T&E, and especially the importance of performing T&E under realistic conditions, is highlighted by capability gaps that are commonly associated with current BioDIM systems. During operation in realistic environmental backgrounds existing detect-to-warn systems are typically prone to having high false positive rates, or alternatively low detection probabilities if the sensitivity is leveraged to reduce the false positive rate [58]. The limited selectivity of existing detect-to-warn systems, which typically is at or below the level of discriminating biological aerosols (bioaerosols) from non-biological aerosols, may result in an inherent vulnerability to natural fluctuations in the environmental background [58, 59]. Even static background conditions may present a challenge to BioDIM systems with limited selectivity (e.g. bioaerosol-selective systems). If the concentration level of BTA aerosols that represents a health hazard is much lower than the natural background level of bioaerosols, obvious sensitivity challenges are likely to be associated with bioaerosol-selective BioDIM systems (exemplified in Figure 5).

The measured response of a BioDIM system may be decomposed into three components [60]:

- Noise = measured response due to intrinsic factors not dependent on the external environment.
- Clutter = measured response due to all external environmental factors (interferents) other than the intended BTA aerosol target.
- Signal = measured response due to the intended BTA aerosol target.

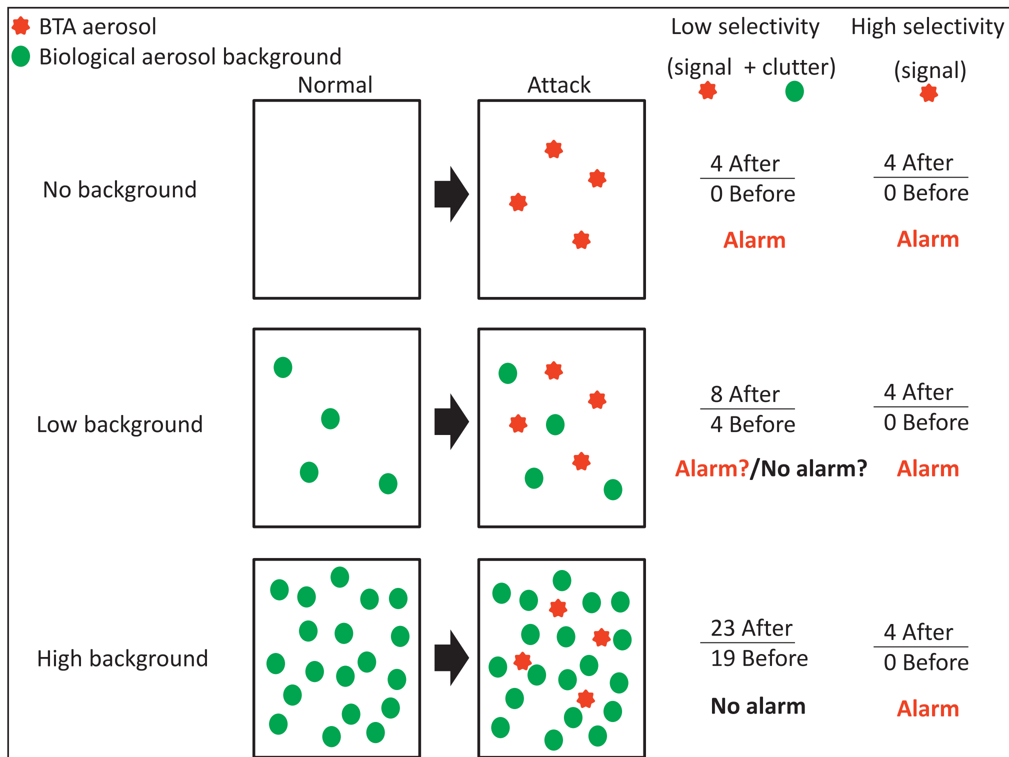

Figure 5. Hypothetical response of a BioDIM system with low (e.g. bioaerosol-selective) and high (e.g. BTA aerosol-selective) selectivity when challenged to a BTA aerosol in different static bioaerosol backgrounds. While the response (i.e. difference between attack and normal conditions) of a BTA aerosol-selective BioDIM system will be largely independent of the bioaerosol background (i.e. measures signal only), the response of a bioaerosol-selective system will be closely linked to the concentration level of the bioaerosol background (i.e. measures both signal and clutter). The non-biological aerosol background was not included in the figure, but could have been incorporated without affecting the predicted outcome.

It has been proposed that the performance of a BioDIM system is most properly characterized by four inter-related parameters: sensitivity (i.e. lower detection limit), probability of detection, false positive rate, and response time [60]. These performance attributes may be assessed by developing Receiver Operating Characteristic (ROC) curves [55]. It has been suggested that ROC curves should be constructed at several stages during R&D and T&E to assess the readiness of a system before moving to the next development or testing stage [60]. The ROC formalism stems from signal detection theories refined in the 1970's by the Radio Detection and Ranging community for the development of low signal-to-noise detection systems [55]. A BioDIM system's ROC curves will be able to quantitatively capture and

visualize performance trade-offs between sensitivity, detection probability, false positive rate, and response time [60], as exemplified in Figure 6.

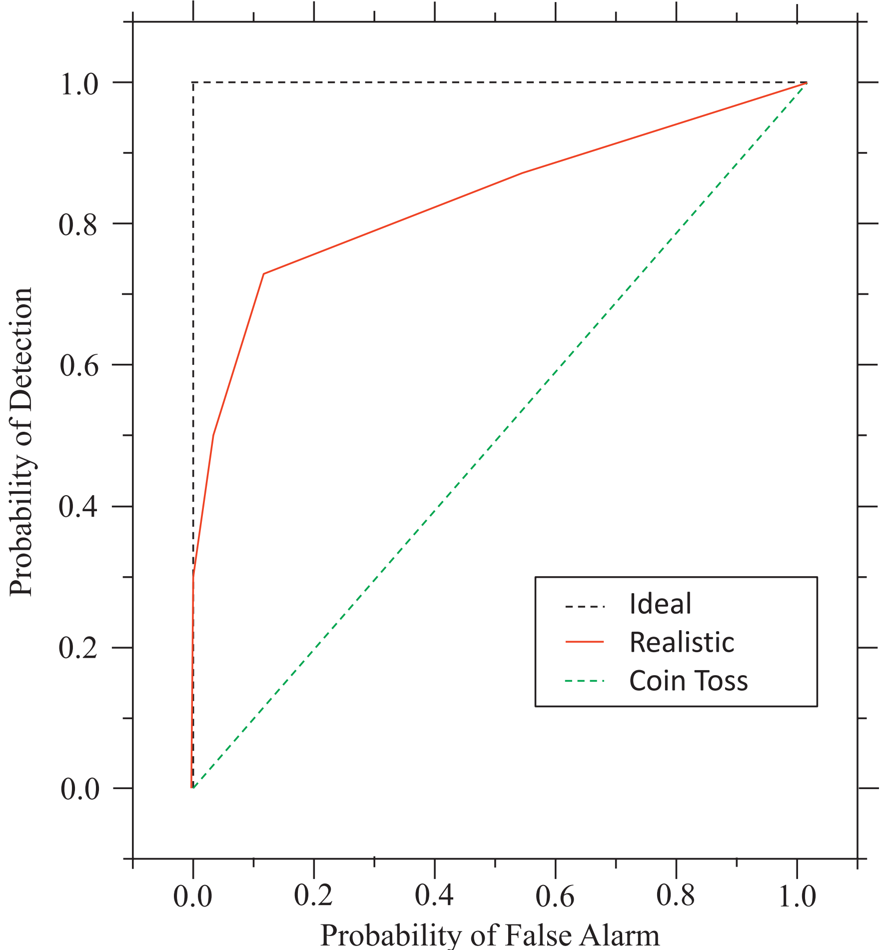

Figure 6. Example of a Receiver Operating Characteristic (ROC) curve visualizing performance trade-offs between the false alarm probability and the detection probability for an ideal BioDIM system (black dashed line), a hypothetic "realistic" BioDIM system (red solid line), and a completely random response (green dashed line). The figure was adapted from [55] and printed with permission.

The performance attributes of BioDIM systems commonly depend on their operating environment, and ROC curves are therefore generally operationally meaningful only when based on realistic environmental conditions [60]. However, as already pointed out, several limitations associated with operational testing may render such an approach impractical,

especially when a representative challenge may not be used in a sufficiently representative operational environment. To circumvent these challenges, an alternative approach proposed for ROC curve construction involves directly merging challenge data from laboratory testing (i.e. signal + noise) with background data from operational testing (i.e. clutter + noise) [60]. However, not all BioDIM technologies will be compatible with raw data fusion and conducting operational testing on a large number of BioDIM systems to record background data for extended periods of time prior to technology pre-screening and down-selection may not be feasible in light of the involved economic costs.

By considering the fact that laboratory testing (e.g. aerosol chamber-based) inherently will be likely to outperform field testing in several ways (e.g. cost effectiveness, reproducibility, and potential for live agent testing), except for the absence of realistic operational conditions, a possibility could be to mimic realistic environmental conditions during laboratory testing [59]. Most aerosol chambers already have controllable meteorological conditions (e.g. temperature and humidity), and the suggested approach would therefore only require the added technical capability of artificially generating realistic bioaerosol and non-biological aerosol backgrounds (e.g. concentration level, composition, particle size distribution, and temporal variability). An example of such a technical capability, the DyCAG (Dynamic Concentration Aerosol Generator), has recently been demonstrated [59]. However, in addition to the technical capability, it will also be necessary to obtain detailed information about the environmental backgrounds that are to be mimicked [59, 60]. This information may be used to define and standardize a set of representative synthetic operational backgrounds that can be recreated together with a challenge (e.g. BTA aerosol) during aerosol chamber-based T&E [59, 60]. Such an approach may then at least in principle possess all the properties that will be needed to enable cost-effective, reproducible, and standardized simulated operational T&E of BioDIM systems with BTA aerosols in background-enabled aerosol chambers.

Although the bioaerosol and non-biological aerosol backgrounds encountered at various operational locations may be both complex and variable, the different constituents that make up these backgrounds are probably not all equally important or relevant for the operation of BioDIM systems, and even simplified synthetic backgrounds could therefore potentially allow for improved screening and down-selection of BioDIM technologies and systems.

The current standards and methods used for T&E of BioDIM systems are not adequate, and this inadequacy hampers the R&D of new systems as well as the proper T&E of existing ones

[60]. The recognized need for increased standardization and harmonization of the operational requirements, reference standards, and T&E concepts and methods that are employed within the BioDIM community has been highlighted by several recent efforts that have attempted to address such topics [28, 43, 54, 56, 60].

1.6. Environmental backgrounds

1.6.1. Aerosols

The scientific term *aerosol* was coined in 1920 to describe liquid or solid particles suspended in a gaseous medium, typically air, as an analog to the term *hydrosol* describing solid particles suspended in a liquid [61, 62]. Aerosols are ubiquitous in both natural and man-made environments (Figure 7), originate from a myriad of different sources including sea spray, re-suspension of soil and dust, vehicle exhaust, and volcanic eruptions, and span a broad particle size range from a few nm to more than 100 µm [61, 63].

During the last few decades the interest in aerosol research has increased within several scientific disciplines including public and occupational health, atmospheric science, microbial ecology, and biodefense [61]. The recent interest could probably be ascribed to an increased awareness of the various effects aerosols may have on our health and surrounding environment, as well as the increasing availability of new and improved analytical tools. Nevertheless, the history of aerosol science and much of the fundamental knowledge stretches back to early pioneers in the field such as Victor K. LaMer, David Sinclair, Nicolai A. Fuchs, and Charles Norman Davies [64].

Important academic literature for practitioners in the field of aerosol science include pivotal works by Fuchs [65], Hidy [66], and Hinds [62], as well as several edited books [61, 63]. Peer-review journals dedicated to the field of aerosol science include Aerosol Science and Technology in association with the American Association for Aerosol Research and the Journal of Aerosol Science in association with the European Aerosol Assembly.

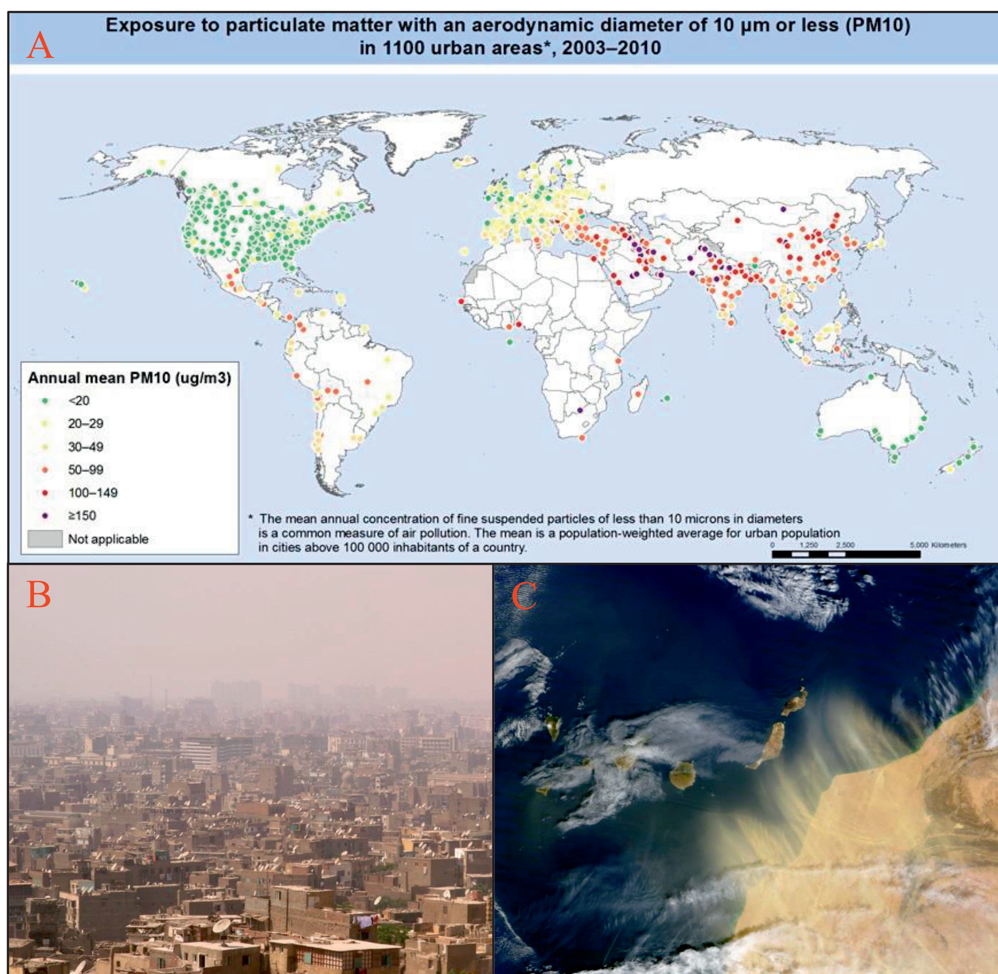

Figure 7. Panel A: Mean annual mass concentration level of aerosols with an aerodynamic diameter of 10 µm or less at 1100 urban locations. Image credit: World Health Organization. Printed with permission. Panel B: Air pollution (smog) in the city of Cairo, Egypt. Image credit: Wikimedia/Sturm58. Panel C: A dust storm from the Sahara desert travelling towards the Canary Islands. Image credit: National Aeronautics and Space Administration. The image is in the public domain.

1.6.2. Bioaerosols

Bioaerosols are aerosols of a biological origin which may include bacteria, viruses, fungi, protozoa, plant and animal debris, as well as their fragments, toxins, and metabolic products [67-69]. The particle sizes of bioaerosols span most of the aerosol size range, with the restrictions in terms of upper and lower particle size generally defined by the circumstances of

bioaerosol generation [69]. Among all aerosols larger than 0.2 μm in outdoor ambient air, 5-50% appears to be of a biological origin [70].

Common sources of bioaerosols include soil, water, vegetation, and living organisms, and the generation mechanisms include wind action, anthropogenic activities, active release processes, and several others [62, 63, 68, 69, 71]. Various industrial processes have been shown to generate bioaerosols, for example wastewater treatment [72-74], composting [75], and land application of biosolids [76]. The composition of bioaerosols may vary depending on their source and generation mechanism, and range in complexity from a single biological substance or microorganism, to aggregates and mixtures of such, and even more complex composite particles containing the aforementioned constituents in combination with other biological and non-biological materials including mucus residues, skin flakes, and soil and dust particulates [62, 63, 68, 69, 71].

Bioaerosol studies have been performed in several different indoor and outdoor environments during the last two centuries, often addressing public and occupational health-related questions (e.g. indoor air quality control and health hazard assessments), as well as microbial ecology, atmospheric science, and biodefense topics [43, 77-81]. The study of bioaerosols is inherently a multidisciplinary field that brings together and require expertise from a variety of scientific disciplines including aerosol physics, microbiology, molecular biology, atmospheric science, and occupational and public health [71]. All aerosols are subject to the same physical laws, and the aerodynamic behavior of bioaerosols is therefore primarily governed by physical principles and generic aerosol properties (e.g. particle size, shape, and density) [69].

Exposure to bioaerosols may result in a range of adverse health effects, including infectious disease, acute toxic effects, allergy, cancer, and other health impairments, and has received increasing attention in recent years [82]. Some of the earliest recorded effects of aerosols and bioaerosols can be found as far back as in ancient Greek literature [69]. Bioaerosols that can penetrate into the lower respiratory tract and deposit in the alveolar region are generally considered to be of primary concern [67, 69, 83, 84].

Recent pandemic and epidemic disease outbreaks caused by influenza viruses (e.g. swine flu and bird flu) and coronaviruses (e.g. severe acute respiratory syndrome), as well as the emerging bioterrorism threat demonstrated by the 2001 “Amerithrax” incident, have contributed to highlight the importance of bioaerosol research [71].

The increasing attention paid to bioaerosol research has resulted in a rapid increase in peer-reviewed publications and conference presentation related to bioaerosols [71]. The Journal of Aerosol Science has allotted three special issues to the measurement and characterization of bioaerosols, edited by Ho and Griffiths in 1994 [85], Lacey in 1997 [86], and Grinshpun and Clark in 2005 [87], while the interdisciplinary journal CLEAN – Soil, Air, Water had a special issue on bioaerosols edited by Grinshpun and Agranovski in 2008 [88]. In addition to peer-reviewed journals and the highlighted academic aerosol literature, important works dedicated to bioaerosols include books edited by Cox and Wathes [69], Burge [89], Macher [90], and Dimmick and Akers [91], as well as several book chapters [68, 71, 84, 92-94]. Since bioaerosol research typically contains appreciable elements of both aerosol science and microbiology, the resulting scientific publications are generally not restricted to aerosol-dedicated journals, and are frequently also published in microbiology-dedicated journals (e.g. Applied and Environmental Microbiology by the American Society of Microbiology).

1.6.3. Airborne bacteria

Bacteria are unicellular prokaryotic microorganisms ranging in physical size from about 0.5 to 30 μm, and may be found in almost any natural or man-made environment [68]. Due to the ubiquitous presence of bacteria in the environment (e.g. soil, water, plants, and animals), airborne bacteria are also encountered almost everywhere [69]. Some examples of airborne bacteria are shown in Figure 8. The most important source of airborne bacteria in outdoor air has been suggested to be the surfaces of living and dead plants [68]. Industrial processes involving mechanical handling of plant materials, soil, sewage, and wastewater may all be strong sources of airborne bacteria, while the most important source in non-industrial indoor environments is usually the presence of human occupants [68].

The majority of airborne bacteria are generally not harmful to healthy humans, and predominantly consist of environmental, saprophytic, and commensal bacteria [95]. However, bioaerosols that contain pathogenic airborne bacteria may represent a serious health threat and have been shown to cause infectious disease in humans, exemplified by *Mycobacterium tuberculosis* (tuberculosis), *Legionella pneumophila* (Legionnaires' disease) and *B. anthracis* (anthrax) [17, 96]. Several other bacterial pathogens, including species in the *Escherichia*, *Salmonella*, *Legionella*, *Neisseria*, *Bacillus*, *Francisella*, *Burkholderia*, *Clostridium*, *Brucella*,

and *Yersinia* genera, may also cause important health and ecological issues upon aerial dispersal [97]. The predicted increase in the number of immunosuppressed individuals in the global population due to HIV infections, chemotherapy, and genetic disorders [98], may contribute to an increased prevalence of nosocomial and community-acquired infections by opportunistic pathogens (e.g. *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*) [99], possibly through aerial dispersal of bacteria. Adding to the challenge is the observed increase in bacterial antibiotic resistance (e.g. methicillin-resistant *Staphylococcus aureus* [100], vancomycin-resistant enterococci [101], and multidrug-resistant tuberculosis [102]), which may have important implications with respect to health risks associated with exposure to pathogenic and opportunistic airborne bacteria. In addition to infectious diseases, airborne bacteria have also been associated with several other negative health effects, including occupational lung diseases such as non-allergic asthma and organic dust toxic syndrome [82]. Endotoxins consist of lipopolysaccharides and are non-allergenic cell wall components of Gram-negative bacteria with strong pro-inflammatory properties [82]. Peptidoglycans are on the other hand more abundant in Gram-positive bacteria and have been proposed to cause similar health effects as those associated with endotoxins [82].

The atmosphere is generally considered to be a hostile environment for microorganisms, and the survival of airborne bacteria have been shown to depend on a range of different atmospheric conditions, including solar radiation, relative humidity (RH), temperature, oxygen concentration, and the open air factor [69, 84]. The mechanisms contributing to the biological decay of airborne bacteria are highly complex, and different classes, species, or even strains of bacteria may have to be considered separately with respect to their survival characteristics in air [69, 84, 103]. Some generalizations have however been proposed [69, 84, 103, 104]:

- Bacterial spores are more resistant to atmospheric conditions than vegetative cells.
- Some, but not all, Gram-negative bacteria are inactivated by oxygen after desiccation.
- RH appears to be a critical parameter with respect to the aerosol stability of bacteria.
- Complicated RH-dependent inactivation profiles are commonly observed.
- The various inactivation mechanisms appear to be highly interdependent.
- Several factors related to intrinsic bacterial properties, environmental conditions prior to aerial dispersion (e.g. growth phase and suspending medium), and the

aerosolization process (e.g. generation mechanism, particle size, and particle composition) have been shown to strongly influence the aerosol stability of bacteria.

The biological state of airborne bacteria is commonly categorized as being viable or nonviable (i.e. dead) [68]. The definition of bacterial viability is not explicit [105], although viable bacteria are generally defined as those having a retained potential for reproduction and/or metabolic activity. Viable airborne bacteria are commonly further categorized as being cultivable or non-cultivable (i.e. viable-but-not-cultivable), depending on their ability to reproduce *in vitro* [68]. All cultivable airborne bacteria are therefore viable, while the opposite is not necessarily true. The biological state of airborne pathogenic bacteria may directly affect their potential health impact since exposure to nonviable bacteria will not result in infectious disease, although nonviable bacteria and bacterial fragments may still induce inflammatory and toxic effects [103, 106]. Viability is a prerequisite for bacterial infectivity, and all infective airborne bacteria are therefore inherently viable, while the converse is not necessarily true [107]. Obtaining information about the biological state of airborne pathogenic bacteria is therefore essential to accurately assess their potential health implications. Airborne pathogenic bacteria with a retained potential to cause infection in susceptible hosts (i.e. 'the infective fraction') may be considered the most relevant in terms of health hazard, but our ability to efficiently and selectively quantify this fraction is currently also the most limited. Viability is frequently used as a convenient proxy for infectivity, while cultivability is similarly used as a proxy for viability. It is however challenging to assess the relevance and accuracy of such proxy measurements, especially for the purpose of estimating health hazards, and efforts to develop and validate methods that are able to obtain relevant, reliable, and accurate exposure estimates for airborne bacteria are urgently needed [82, 90, 103, 108].

Taken together, it is apparent that the study of airborne bacteria is a challenging and complex multidisciplinary science. Nevertheless, the increasing interest in the field highlights the importance and societal relevance of extending our knowledge about airborne bacteria, and how they impact on our health and surrounding environment.

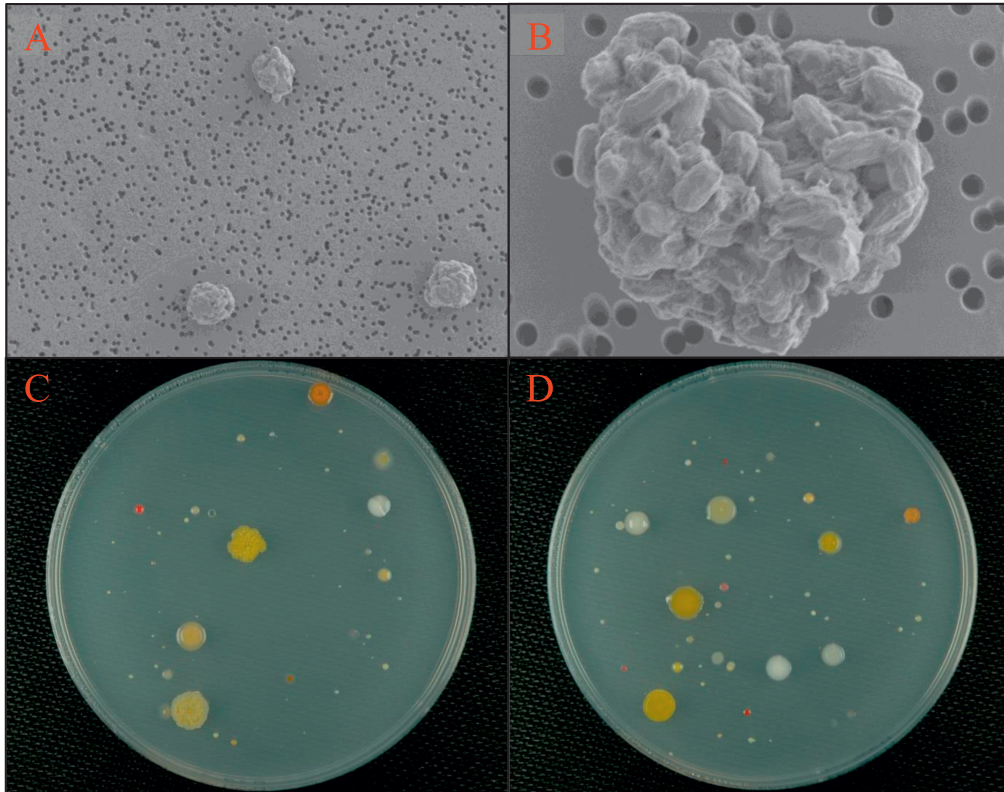

Figure 8. Examples of airborne bacteria. Panels A-B: Scanning electron microscopy images of artificially generated bioaerosols (~3-4 µm droplet nuclei) containing clusters of *B. atrophaeus* spores. The bioaerosols were sampled from an aerosol test chamber onto polycarbonate filters (0.4 µm pore size), coated with 2 nm Pt/Pd, and imaged with a Hitachi SU6600 field-emission scanning electron microscope. Panels C-D: R2A agar plates with bacterial colonies cultured from air samples collected at a Norwegian subway station showing variable colony morphologies and pigmentations. Image credit: Norwegian Defence Research Establishment.

1.7. Characterization of airborne bacterial backgrounds

1.7.1. Sampling and analysis of airborne bacteria

Accurate and reliable quantification and identification of airborne bacteria depends on several factors, including the use of air sampling equipment that efficiently capture representative samples and appropriate downstream analysis methods [108]. Sampling of airborne bacteria is usually achieved through common aerosol collection principles such as impaction,

impingement, cyclonic separation, filtration, and thermal or electrostatic precipitation [68, 71, 93].

With respect to sampling of airborne bacteria, the performance characteristics of air sampling equipment may be described in at least two fundamentally different ways: physical sampling efficiency and biological sampling efficiency. The physical sampling efficiency describes the air sampler's ability to physically collect and retain aerosols, and is therefore mainly dependent on generic aerosol characteristics largely independent of the type of aerosol being sampled (e.g. inert aerosols or stress-sensitive airborne bacteria). On the other hand, the biological sampling efficiency additionally takes into account the air sampler's ability to preserve the biological state of the sampled material and will therefore also depend on the aerosol's sensitivity to sampling-associated stress (e.g. shear forces and desiccation).

Some air samplers used to collect airborne bacteria are the same as those used for non-microbial aerosols, while others have been specifically developed or adapted for sampling of airborne microorganisms. Sieve and slit impactors (e.g. the Andersen sampler and slit-to-agar samplers, respectively) may be used to deposit aerosols directly onto the surface of agar plates and are examples of samplers that have been specifically developed or adapted for sampling of airborne microorganisms [109, 110]. Some other well-known examples include various liquid impingers such as the all-glass-impingers (e.g. AGI-30) and the BioSampler [111, 112]. Since impingers employ a liquid collection media the downstream analysis will not be restricted to direct plate cultivation, allowing them to be used for other types of aerosols and analysis methods as well.

A myriad of different air samplers has been developed and offer a wide range of instrument properties in terms of sampling efficiency, particle collection size range, airflow rate, collection medium type and volume, physical instrument properties (e.g. size, weight, ruggedness, power requirements, and automation), and inflicted sampling-associated microbial stress (e.g. shear forces and desiccation) [113-122]. Some examples of air samplers that are commonly used to collect airborne bacteria are shown in Figure 9.

Figure 9. Examples of air samplers that may be used to collect airborne bacteria. Panel A: Andersen-type six-stage cascade agar impactor (Tisch Environmental, Cleves, OH). Panel B: MAS-100 agar impactor (Merck, Billerica, MA). Panel C: BioSampler swirling liquid impinger (SKC, Eighty Four, PA). Panel D: AGI-30 all-glass-impinger (Ace Glass, Vineland, NJ). Panel E: SASS 3100 electret filter sampler (Research International, Monroe, WA). Panel F: SASS 2300 wetted-wall cyclone (Research International). Panel G: OMNI-3000 wetted-wall cyclone/contactor (InnovaPrep, Drexel, MO). Image credit: Norwegian Defence Research Establishment.

The process of selecting an appropriate air sampler and sampling scheme for use in airborne bacterial investigations is a nontrivial task that must be well-harmonized not only to the scope of the study (e.g. targeting of all bacteria or specific subset, quantitative or qualitative

assessment, and biological state-dependent or -independent assessment), but also the meteorological conditions (e.g. temperature, RH, and wind speed), the analysis methods (e.g. cultivation, microscopy, and qPCR), and various characteristics of the targeted airborne bacterium-containing particle population (e.g. concentration level, size distribution, and stress sensitivity). Thus, no single air sampler is likely to be optimal, or even suitable, for all airborne bacterial sampling purposes [123, 124]. Additional factors that may influence the choice of air sampler and sampling scheme include the need for personal sampling (i.e. on-body sampler) or static area sampling (i.e. stationary sampler), as well as requirements related to the use of size-selective sampling conventions. Two main types of size-selective sampling conventions are commonly used in aerosol studies:

- The US Environmental Protection Agency (www.epa.gov) conventions
    - TSP (total suspended particulates with aerodynamic diameter ≤100 μm)
    - PM10 (particulate matter with aerodynamic diameter ≤10 μm)
    - PM2.5 (particulate matter with aerodynamic diameter ≤2.5 μm)
- The American Conference of Governmental Industrial Hygienists (www.acgih.org) conventions
    - Inhalable fraction (aerosols with aerodynamic diameter ≤100 μm)
    - Thoracic fraction (aerosols with aerodynamic diameter ≤10 μm)
    - Respirable fraction (aerosols with aerodynamic diameter ≤4 μm)

Air samples will typically be subjected to quantitative and/or qualitative analyses based on microscopy, microbiological, biochemical, immunological, or molecular techniques [68, 71, 93]. Some selected examples of analysis methods that may be used to quantify and/or identify airborne bacteria are shown in Figure 10.

Figure 10. Examples of analysis methods that may be used for quantification and/or identification of airborne bacteria. Panel A: Plate cultivation may be used to quantify airborne bacteria and to obtain bacterial isolate collections (R2A agar plate showing bacterial colonies cultured from a subway station air sample). Panel B: Identification of airborne bacteria may be done using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of bacterial isolates (Microflex LT instrument from Bruker Daltonics, Bremen, Germany). Panel C: Identification of airborne bacteria may be done using Sanger sequencing of 16S rRNA genes from bacterial isolates or clone libraries (ABI 310 Genetic analyzer, Applied Biosystems, Foster City, CA). Panel D: Quantification (qPCR) and identification (e.g. PCR-based identification assays) of airborne bacteria may be done using real-time PCR (LightCycler 480 real-time PCR instrument, Roche Diagnostics, Indianapolis, IN). Image credit: Norwegian Defence Research Establishment.

Cultivation has traditionally been the predominant analysis method for airborne bacteria [125, 126], and continue to be of importance and in frequent use [43, 127]. Most alternative methods cannot readily obtain information about the biological state of airborne bacteria, and

cultivation-based methods are commonly preferred when the focus of the study is on viable microorganisms only [128]. The ability of airborne bacteria to reproduce *in vitro* after sampling is commonly used to infer the presence of viable and thus potentially infectious airborne bacteria. The choice of culture conditions (e.g. temperature, humidity, and duration) depends on scope of the study, and a range of different growth media formulations (e.g. trypticase soy agar and Reasoner's 2a agar) with and without additional supplements (e.g. antibiotics and antifungals) have been used to study airborne bacteria [92]. A common feature of plate count methods is their reliance on *in vitro* bacterial reproduction resulting in colonies that may be enumerated [92]. The use of cultivation-based methods allow bacterial isolates to be recovered and subjected to further characterization (e.g. pathogenic traits, antibiotic resistance, and genotyping), and is still the "gold standard" for many applications, including forensic and epidemiological investigations [97].

However, a major drawback with cultivation-based methods is the observation that bacterial cells may be damaged by the aerosolization process, exposure to atmospheric conditions, or even by sampling, and which could change their biological state [107, 115, 129-131]. It has also been suggested that less than 1% of bacteria in any environment are readily cultured with standard laboratory methods [132]. The concentration level of cultivable airborne bacteria (CAB) has been showed to correspond to 0.02-75% of total airborne bacteria (TAB = CAB + viable-but-not-cultivable airborne bacteria + nonviable airborne bacteria) [133, 134]. Although the TAB diversity typically is more diverse than the CAB diversity, it has at least in some environments been shown to largely consist of cultivable bacterial taxa [135, 136].

Cultivation-independent analyses based on microscopy, and especially epifluorescence microscopy (FM) in combination with various staining techniques (e.g. 4′,6-diamidino-2-phenylindole, SYBR green, and acridine orange), have traditionally been the predominant methods used to quantify TAB [137, 138]. However, microscopy-based methods are typically labor intensive and associated with sensitivity and selectivity limitations [92]. Recently, the implementation of flow cytometry (FCM) as an alternative to FM has contributed to reduce the workload by automating the measurement process, while the use of fluorescence *in situ* hybridization (FISH) involving labeled nucleic acid probes instead of generic staining techniques has been shown to increase the selectivity of both FM and FCM analyses [139].

The need for rapid and reliable quantification and identification of airborne bacterial pathogens, especially within the public health and biodefense community, has led to an

increased interest in cultivation-independent molecular analysis methods (e.g. PCR-based techniques) [140]. Quantitative real-time PCR (qPCR) has recently been demonstrated for quantification of TAB [141]. PCR-based methods have previously been used to detect specific airborne bacteria [142], as well as in TAB diversity studies where PCR may serve as the starting point for various other molecular techniques including Sanger sequencing of 16S rRNA gene clone libraries and community fingerprinting methods such as denaturing gradient gel electrophoresis [143, 144].

The recent implementation of high-throughput molecular techniques to study TAB diversity (e.g. next-generation sequencing [145-150] and PhyloChip 16S rRNA gene-based microarrays [151, 152]) has expanded our knowledge about airborne bacteria in various environments. These high-throughput methods typically enable a deeper probing of the TAB diversity compared to traditional methods, which allows even the previously largely unrecognized "rare" bacterial diversity to be elucidated.

Despite the increasing use of cultivation-independent methods to study airborne bacteria, several challenges remain to be solved with these methods since they: i) generally cannot discriminate between viable and nonviable bacteria, ii) may be sensitive to environmental interferents (e.g. PCR inhibitors and autofluorescent particulates), and iii) may have variable sensitivity and selectivity compared to cultivation-based methods [43, 141, 142, 153-157]. Since information about the biological state of airborne bacteria is frequently required, cultivation-based methods will probably continue to be of importance, at least until validated cultivation-independent methods that are able to reliably discriminate between viable and nonviable airborne bacteria become readily available [140]. However, the increasing implementation of techniques such as differential staining (e.g. live/dead staining) in combination with FM and FCM, as well as ethidium monoazide in combinations with qPCR, have shown promising results that may allow improved cultivation-independent discrimination between viable and nonviable airborne bacteria [158, 159].

The inherent challenges that are associated with sampling and analysis of airborne bacteria, in combination with study-specific sampling and analysis requirements, have made community-wide standardization and harmonization difficult, and consequently led to the use of several different air samplers and analysis methods in previous studies. Inter-study data comparisons may therefore be difficult to perform since the various sampling and analysis methods have been shown to provide different results even when subjected to the same bioaerosol challenge

[113, 119, 122, 160-163]. Investigations of airborne bacteria are therefore hampered by the lack of standardized and harmonized methods that are able to provide accurate, reliable, and comparable results, especially when the study objective is to obtain exposure estimates for use in health hazard assessments [90].

1.7.2. Airborne bacteria in outdoor and indoor air

The current state of knowledge regarding airborne bacteria in indoor and outdoor environments has recently been extensively reviewed [77-79, 81, 128, 133, 134, 164-172]. Various airborne bacterial characteristics such as the concentration level, diversity, and spatiotemporal variability of CAB and TAB have been investigated in a myriad of different environments, including urban [145, 149, 151, 173], rural [137, 138, 174], coastal [126, 136, 175], marine [176-178], polar [179-181], and alpine [146] outdoor locations, in the troposphere above the atmospheric boundary layer [182, 183], the stratosphere [184], and mesosphere [185], as well as at several indoor and industrial locations including hospitals [186-188], educational facilities [150, 189, 190], libraries [191], shopping centers [192], museums [193, 194], churches [195], office buildings [196, 197], residential homes [198-200], subway stations [201-213], aircraft cabins [214, 215], space stations [216, 217], clean rooms [218, 219], animal farms [155, 220, 221], composting facilities [75, 222, 223], wastewater and sewage treatment plants [73, 74, 224, 225], and during land application of biosolids [76, 226]. A variety of different topics related to airborne bacteria have been addressed in these studies, including exposure and health hazard assessments [72, 227-230], microbial ecology and background characterizations [125, 126, 133, 138], and efforts to elucidate the potential impact of airborne bacteria on atmospheric processes and climate (e.g. by serving as fog, cloud, and ice nucleators) [81, 231-233].

The CAB level in typical outdoor and indoor environments has been shown to range from not detectable to ~10^4 cfu m^{-3} [81, 128, 134], although higher levels have been observed in the presence of strong local sources, especially at industrial locations (e.g. animal barns, compositing facilities, and sewage and wastewater treatment plants) [77, 78]. The TAB level has been shown to range from ~10^1 to ~10^7 cells m^{-3}, although the presence of strong local sources may, as for CAB, also result in higher TAB levels [77, 78]. The TAB levels have been shown to be approximately two orders of magnitude higher than the CAB levels [134],

although the ratio will probably vary depending on the type of predominant sources, the distance from these sources, and the atmospheric conditions.

The CAB diversity is generally dominated by Gram-positive bacteria, particularly the phyla *Firmicutes* (e.g. *Bacillus* and *Staphylococcus* spp.) and *Actinobacteria* (e.g. *Micrococcus* spp.) [81, 128, 134]. The TAB diversity, however, typically shows a higher relative abundance of Gram-negative bacteria, mainly belonging to *Alpha*- (e.g. *Sphingomonas* spp.), *Beta*-, and *Gammaproteobacteria* (e.g. *Pseudomonas* spp.) as well as *Bacteroidetes,* although Gram-positive bacteria such as *Firmicutes* and *Actinobacteria* are commonly also among the most abundantly observed TAB phyla [81, 128].

Substantial temporal and spatial variability are commonly observed with respect to the concentration level and diversity of both CAB and TAB, and thus make it challenging to present a single unified background estimate [135]. Although some exceptions have been reported, several generalized trends concerning the spatiotemporal variability of airborne bacteria have been proposed [81, 128, 134, 135]:

- The seasonal variation in outdoor ambient air shows summertime maximum and wintertime minimum levels.
- The diurnal variation in outdoor ambient air shows early morning and afternoon maximum levels and nighttime minimum levels.
- Increased airborne bacterial levels are observed at urban compared to rural locations, and similarly at inland sites compared to coastal sites.
- Cultivation-based results suggest that Gram-negative bacteria dominate in marine air, while Gram-positive bacteria dominate in continental air.
- The composition of airborne bacterial communities commonly show substantial temporal (e.g. diurnal, day-to-day, and seasonal) and spatial (e.g. land-type and land-use) variability.
- At least in continental and temperate climate regions, plant-associated bacteria often dominate during the warm seasons, while soil-inhabiting bacteria commonly dominate during the cold seasons.
- Various meteorological events including dust storms and rain showers may dramatically change both the concentration level and composition of airborne bacteria.

1.8. Subway transportation systems

Subway transportation systems, commonly referred to as subways or metros, are found in at least 133 cities worldwide and transport about 200 million passengers daily (www.uitp.org). Subways are rapid underground transit systems that allow a large number of people to be transported at a high frequency over relatively short distances, and may therefore be considered as critical infrastructure for public transportation [234]. Subway stations are typically confined and crowded underground public environments (Figure 11). The architectural layout of subway stations may vary between countries, cities, and even within the same subway network. In addition to the station design, several other building-related properties may differ between stations, including: i) depth of the station below street level, ii) natural (passive) or fan-driven mechanical (active) station ventilation, and iii) presence or absence of platform screen doors which serve to physically separate the passenger concourses from the tunnel network.

Subway stations operating without fan-driven mechanical ventilation (e.g. heating, ventilation, and air condition systems) generally rely on air flows generated by the movement of trains in the tunnel network (i.e. the "piston effect") [235], although convection-driven air flows may also occur when a temperature differential exists between the underground tunnel network and the outdoor atmosphere. The term "piston effect" refers to the air cushion created in front and the suction created behind a train traveling through a narrow tunnel [235]. The cross-section of subway trains typically covers the majority of the tunnel cross-section, resulting in air cushions that push air forward and out of the subway system in front of trains, and negative pressure zones that draw ambient air into the subway system behind trains. Air may escape or enter subway systems through tunnel and station entrances, as well as through active or passive ventilation ports.

Although no deliberate dispersion of a BTA aerosol has so far been reported in a subway environment, several terrorist attacks with explosives (e.g. in Spain, the UK, and Russia) or chemical threat agents (e.g. in Japan) have demonstrated subway stations as terrorism targets [234]. The dispersion of sarin, a chemical threat agent, by the Japanese religious cult Aum Shinrikyo in the Tokyo subway in 1995 resulted in 11 casualties and over 5,000 injuries [236], and served as a powerful reminder of the massive consequences that may be associated with CBRN incidents in subway environments.

Figure 11. Panel A: The Moscow subway is one of the busiest in the world and known for its deep and beautifully decorated stations. Photo credit: Wikimedia/Christophe Meneboeuf. Panel B: A station in the Taipei subway with passenger screen doors. Photo credit: Wikimedia/Howard61313.

1.8.1. Airborne bacteria in subway environments

Airborne bacteria have been investigated at subway stations in several countries worldwide, including: the US [202, 209], Japan [213], South Korea [204, 206, 207], China [237], Russia [238], Egypt [201], the UK [203], Hungary [211, 212], and Iran [208]. These studies have reported CAB levels ranging from not detectable to 10^4 cfu m^{-3}. The CAB diversity has been shown to predominantly consist of the bacterial genera *Micrococcus*, *Staphylococcus,* and *Bacillus*, while several other genera such as *Pseudomonas*, *Corynebacterium*, *Paracoccus*, *Kocuria*, *Aerococcus*, *Moraxella,* and *Enterococcus* have also been observed, although generally less consistently and abundantly.

Only two of the previous works [202, 209] have addressed TAB levels at subway stations. A study performed in the New York City subway [209] reported TAB levels ranging from 10^3 to 10^4 cells m^{-3}, while a study performed in the Washington DC subway [202] investigated but did not establish TAB levels due to issues related to an erratic air sampler. Previous TAB diversity studies in subway environments are similarly restricted to these two studies [202, 209]. Compared to the CAB diversity, a higher relative abundance of Gram-negative bacteria from the phyla *Proteobacteria* and *Bacteriodetes* was observed, while Gram-positive bacteria from the phyla *Firmicutes* and *Actinobacteria* were still among the most abundant taxa.

Previous investigations of airborne bacteria at subway stations have employed a variety of different air sampling methods, including passive sedimentation techniques, agar impactors, liquid impingers, wetted-wall cyclones, and filtration samplers. The sampling and analysis schemes have also differed with respect to the number of collected samples, collected particle size range, duration of sampling, and culture conditions. In combination these differences have made it difficult to predict the accuracy and relevance of inter-study data comparisons and result generalizations.

In addition to airborne bacteria, several other types of bioaerosols and non-biological aerosols may be encountered at subway stations. Airborne fungi have been investigated at subway stations in several countries around the world, including: Iran [239], India [240], South Korea [206, 241], Japan [242], Russia [238], Italy [243], Egypt [201], China [237], Brazil [244], and the UK [203]. These studies have reported airborne cultivable fungal levels ranging from not detectable to 10^3 cfu m^{-3}. The fungal diversity has been shown to mainly consist of the genera *Penicillium*, *Cladosporium*, and *Aspergillus*, although *Alternaria*, *Chrysosporium*, *Fusarium*,

several non-sporulating fungi (sterile mycelium), and yeasts are also commonly observed. The airborne fungal levels observed at subway stations have generally been shown to be lower than the airborne bacterial levels [203, 206, 237, 238].

Total aerosol concentration levels have similarly been investigated at subway stations in several countries, including: Finland [245], Sweden [246-248], the Czech Republic [249], Taiwan [250-253], Japan [254], South Korea [255-259], the US [202, 260, 261], Mexico [262, 263], Argentina [264], Turkey [265], France [266], Hungary [267], China [268], Egypt [201], Italy [269], the UK [270-272], and Brazil [244]. Measurements of total aerosols are commonly presented as either number (e.g. particles m^{-3}) or mass (e.g. µg m^{-3}) concentrations [273]. The previous studies at subway stations have reported mass concentrations from: 3-480 (PM2.5), 6-1500 (PM10), and 208-1610 (TSP) µg m^{-3}, while the number concentrations have been shown to range from 10^9 to 10^{10} and 10^6 to 10^8 particles m^{-3} for particles <1 and >1 µm, respectively. The elemental composition of subway station aerosols commonly shows a strong enrichment of Fe-containing particles, which has been attributed to sources associated with train operations (e.g. wear and tear of rails, wheels, and brakes) [273].

Several factors have been proposed to influence the bioaerosol and non-biological aerosol background encountered at subway stations, including:

- Depth of station below street level [203, 204, 238]
- Type of ventilation system [201, 238]
- Number of passengers [201, 208, 238, 239]
- Amount of settled dust [240, 241]
- Presence or absence of platform screen doors [204, 255, 257]
- The outdoor bioaerosol and non-biological aerosol background [209, 250]

1.8.2. The Nationaltheatret subway station, Oslo, Norway

Oslo is the capital city of Norway and has a population of approximately 630,000 inhabitants. The subway transportation system in Oslo has six bidirectional lines transporting more than 80 million passengers annually (www.ruter.no). The Nationaltheatret subway station (Figure 12) is one of the busiest subway stations in Oslo, and when it opened in 1928 it was the first underground subway station in Scandinavia. The subway station is co-located with an

underground railway station which is situated deeper than the subway station. The subway station consists of a single double-tracked tunnel hall which houses both the eastbound and westbound passenger concourses. All six subway lines in Oslo pass through the station, and the average departure frequency is approximately one per minute during its operating hours. The station does not rely on active mechanical ventilation or passenger screen door systems. The station is non-operative and closed from the last evening train (~01:15) until the first morning train (~05:10). During the non-operative hours, diesel-powered trains and subway personnel regularly perform maintenance activities at the station and in the tunnel network.

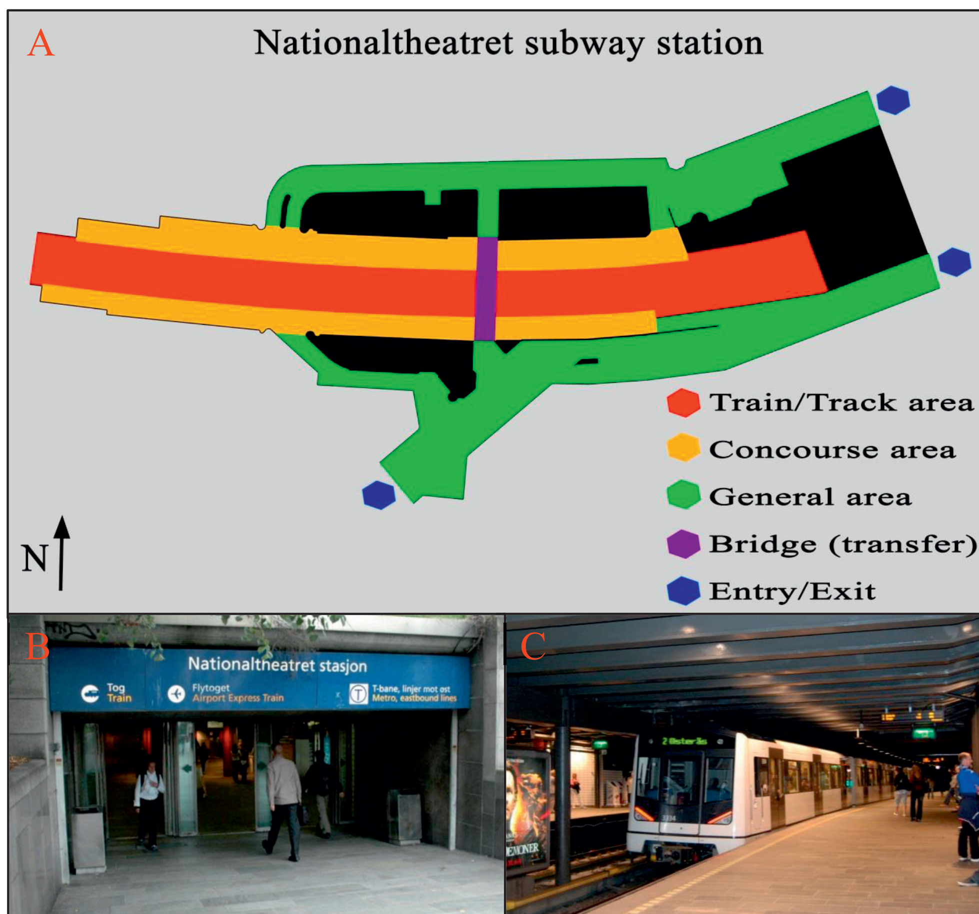

Figure 12. Panel A: Layout of the Nationaltheatret subway station. The two entrances on the right side connect to an outdoor square at street level, while the third entrance connects to the main hall of an underground railway station. Panel B: Entrance to subway lines from the outdoor square. Photo credit: Wikimedia/Ekko. Panel C: Westbound subway concourse. Photo credit: Wikimedia/Maxxii.

1.9. MALDI-TOF MS-based identification of airborne bacteria

The use of mass spectrometry (MS) in combination with whole-cell analysis or whole-cell extract analysis for rapid bacterial identification was proposed and demonstrated more than 30 years ago [274]. The introduction of soft ionization techniques such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization which allow MS analysis of intact organic macromolecules (e.g. proteins) has led to an increased interest in MS-based microorganism identification methods, especially within infectious disease diagnostics and biodefense [275-278].

Commercial MALDI-time-of-flight (TOF) MS analysis platforms for rapid microbial identifications have recently been introduced, including the MALDI Biotyper from Bruker Daltonics and SARAMIS from AnagnosTec, and are rapidly becoming commonplace in hospital diagnostic laboratories [279-283]. These systems have been shown to be capable of obtaining bacterial identification results both faster and more cost-effective than traditional diagnostic testing which typically involves Gram staining, culture and growth characteristics, and biochemical patterns [282].

MALDI-TOF MS has recently been implicated as a rapid and cost-effective de-replication tool during screening of environmental bacterial culture collections [284], and may potentially be used as an alternative to 16S rRNA gene sequencing-based methods. Various MS techniques, including MALDI-TOF MS, have also been used to investigate small acid-soluble proteins (SASPs) as candidate biomarkers for rapid identification of *Bacillus* sp. spores including *B. anthracis* [285-290]. As previously mentioned, several ATOFMS techniques, including single-particle MALDI-ATOFMS [52], have recently been investigated for use as real or near-real time BioDIM systems.

Taken together it may therefore be suggested that several different MALDI-TOF MS-based methods represent novel and potentially valuable analysis tools that could allow for rapid and cost-effective qualification (e.g. detection, identification, classification, and de-replication) of airborne bacteria. However, limited information is currently available concerning the use of MALDI-TOF MS methods for airborne bacterial applications, and the potential benefits and limitations that will be associated with these methods are therefore largely unknown.

1.10 Background of the study

Subway stations are crowded and enclosed underground public environments that may be considered as potential bioterrorism targets, and therefore also as a relevant operational environment for BioDIM systems. Limited information is available about the bioaerosol backgrounds that can challenge BioDIM systems at operational sites. The characterization of bioaerosol backgrounds in subway environments assists in obtaining information that can be used by the biodefense community to define and standardize a set of representative subway station bioaerosol backgrounds. Realistic bioaerosol background conditions may then be reproduced together with a BTA aerosol challenge during aerosol chamber-based T&E, and potentially allow for improved performance measurements that more-accurately predict the operational performance of BioDIM systems at subway stations to be established. Bioaerosol background characterization studies may also obtain public and occupational health- and microbial ecology-relevant information.

The bioaerosol background at subway stations is a complex assemblage of different microorganisms and biological substances that are likely to include, but not limited to, airborne bacteria and fungi. Most types of bioaerosols may at least in principle influence the operational performance of BioDIM systems. However, several factors will likely affect the interfering potential of different bioaerosols, including their concentration level, particle composition, and particle size distribution, as well as the selectivity level of the BioDIM system.

Most BTAs are highly pathogenic bacteria or viruses, and which often share a high degree of structural and chemical similarity with several non-BTA bacterial or viral microorganisms. It may therefore be proposed that bioaerosols which contain bacterial or viral microorganisms constitute the fraction of the bioaerosol background that is most likely to impact the operational performance of BioDIM systems. However, the majority of previous studies that have characterized the bioaerosol background at subway stations have exclusively addressed airborne bacteria or fungi. This is probably due to the known public and occupational health implications that may be associated with exposure to airborne bacteria and fungi, and also the fact that these organisms are relatively easily studied using culture methods compared to viruses which require a suitable host for reproduction. While bacteria and fungi typically are

ubiquitously observed in air, viruses, and at least human viruses, are generally not detected in air in the absence of strong local sources (e.g. actively shedding infected hosts).

Taken together it may therefore be suggested that airborne bacteria, based on their ubiquitous presence in air and shared structural and chemical similarity with several BTAs, could be considered as the fraction of the bioaerosol background with the highest potential for interfering with the operational performance of BioDIM systems.

In conclusion this inspired us to investigate the airborne bacterial environment at the Nationaltheatret subway station in Oslo, Norway, with particular emphasis placed on obtaining relevant information for the operation and T&E of BioDIM systems. Emphasis was also placed on acquiring information that may be interesting when viewed in the context of public and occupational health as well as microbial ecology.

Based on the bioaerosol community's call for improved air sampling and analysis methods, as well as increased standardization and harmonization needs, we were also inspired to address issues related to the selection and T&E of air sampling methods. In a similar fashion, this also led us to evaluate and implement MALDI-TOF MS as a novel classification/identification method for use in airborne bacterial background characterization studies.

The study also involved an eight-month scientific exchange to TNO in the Netherlands. The aim of the collaborative research was to investigate MALDI-TOF MS as a rapid and cost-effective identification method for *Bacillus anthracis* spores in powders (e.g. letters).

2. Objectives

The main objective of this PhD study was to perform an in-depth characterization of the cultivable airborne bacterial background at the Nationaltheatret subway station in Oslo, Norway, with special emphasis on obtaining information that may allow realistic subway station conditions to be reproduced during aerosol chamber-based T&E of BioDIM systems (**Paper I+II**).

To this end, the current literature status concerning airborne bacteria in subway environments was reviewed (section 1.8.1) to identify existing knowledge gaps, resulting in the formulation of specific study objectives aimed at obtaining information from the subway station about:

- Concentration level of airborne bacteria (**Paper I+II**)
- Diversity of airborne bacteria (**Paper I+II**)
- Size distribution of airborne bacterium-containing particles (**Paper II**)
- Concentration level and size distribution of total aerosols (**Paper I+II**)
- Meteorological conditions such as temperature and RH (**Paper I+II**)
- Temporal variability of the airborne bacterial environment (**Paper II**)
- Relationship between airborne bacteria and other background characteristics (**Paper I+II**)
- Survival- and virulence-associated characteristics of airborne bacteria (**Paper I**)

Furthermore, in an effort to respond to the bioaerosol community's call for improved air sampling and analysis methods, and especially the call for increased standardization and harmonization, emphasis was also placed on evaluating and establishing in-house sampling and analysis schemes for use in airborne bacterial background characterization studies, resulting in the formulation of three specific sub-objectives:

- Perform aerosol chamber-based comparative T&E of air samplers (**Paper III**)
- Implement and evaluate MALDI-TOF MS as a rapid and cost-effective identification method for use in airborne bacterial background studies (**Paper I+II**)
- Develop and validate a MALDI-TOF MS-based identification assay that is capable of identifying *B. anthracis* spores in powder samples (**Paper IV**)

3. Summary of publications

Paper I

Characterization of Airborne Bacteria at an Underground Subway Station

Dybwad, M, PE Granum, P Bruheim, and JM Blatny

Applied and Environmental Microbiology, 2012;78(6):1917-1929

The airborne bacterial environment at the Nationaltheatret subway station in Oslo, Norway, was characterized during the spring, summer, and fall seasons of 2010 (May to September). Emphasis was placed on obtaining information about the airborne bacterial concentration level and diversity at the station, as well as virulence- and survival-associated properties of the airborne bacteria, including hemolytic activity, antibiotic resistance, pigmentation, and spore fraction. The use of a high volume electret filter air sampler (SASS 3100) and a MALDI-TOF MS-based bacterial isolate identification method (MALDI Biotyper) was demonstrated for the first time in an airborne bacterial background study. Air samples were collected with two- to three-week intervals throughout the study period, and each sampling event included collection of a two-hour daytime and nighttime sample at the subway station as well as a two-hour daytime sample at an outdoor reference location. The daytime level of airborne bacteria at the station (~400 cfu m^{-3}) was shown to be higher than the nighttime (~20 cfu m^{-3}) and outdoor levels (~110 cfu m^{-3}). The airborne bacterial spore fraction was shown to be higher in outdoor air (~16%) than at the station (~3%). The majority of airborne bacteria belonged to the genera *Bacillus*, *Micrococcus*, and *Staphylococcus*, while a total of 37 different bacterial genera were observed. The concentration level and diversity of airborne bacteria, as well as the total aerosol concentration level and size distribution, was shown to be stable within each environment (i.e. daytime station, nighttime station, and daytime outdoor location) throughout the study. About 22% of the airborne bacterial isolates displayed hemolytic activity on sheep blood agar (mainly beta-hemolysis), and were predominantly distributed between the bacterial genera *Bacillus* (87%) and *Staphylococcus* (10%). About 27% of the isolates showed resistance against at least two of the five antibiotics tested (ampicillin, streptomycin, nalidixic acid, tetracycline, and chloramphenicol), while 10% of the isolates did not show antibiotic resistance. About 48% of the isolates showed visual colony pigmentation with the predominant pigmentation being various shades of yellow, although orange, pink, and red pigmentations were also commonly observed. The airborne bacterial

diversity was studied using both the MALDI-TOF MS-based MALDI Biotyper and partial 16S rRNA gene sequencing. The identification results were shown to correspond well between the two methods, although the MALDI-TOF MS method failed to obtain confident classifications when the reference database (Bruker Taxonomy) did not contain representative species- or genus-level library entries that corresponded to the analyzed bacterial isolate.

Paper II

Temporal Variability of the Bioaerosol Background at a Subway Station: Concentration Level, Size Distribution and Diversity of Airborne Bacteria

Dybwad, M, G Skogan, and JM Blatny

Applied and Environmental Microbiology, 2014;80(1):257-270

The airborne bacterial environment at the Nationaltheatret subway station in Oslo, Norway, was characterized during a 72-hour continuous sampling campaign in February 2011. Emphasis was placed on obtaining information about the temporal variability of the concentration level, size distribution, and diversity of airborne bacteria at the station. Three air samplers were used to obtain different characteristics of the airborne bacterial environment: i) the SASS 3100 high volume electret filter sampler which allowed the results to be directly compared with those obtained in **Paper I**, ii) the Andersen six-stage cascade agar impactor which obtained particle size-resolved airborne bacterial results, and iii) the MAS-100 high volume agar impactor which obtained airborne bacterial results with a higher temporal resolution (10-min sampling frequency) than the other samplers. The concentration level and diversity of airborne bacteria at the station was shown to be conserved both between days and seasons, while the intraday variability was found to substantial, although generally associated with a highly consistent diurnal pattern. The airborne bacterial levels ranged from not detectable to 10^3 cfu m^{-3}, and typically showed increased levels during the daytime compared to the nighttime, as well as during rush hours compared to non-rush hours. The airborne bacterial levels showed rapid temporal variation on some occasions (up to 270-fold), which were both consistent and inconsistent with the typical diurnal profile. The majority of the airborne bacteria belonged to the genera *Micrococcus*, *Rhodococcus*, *Arthrobacter*, *Moraxella*, *Staphylococcus*, *Bacillus*, *Pseudomonas*, *Dermacoccus*, and *Kocuria*, and a higher relative abundance of Gram-negative bacteria was observed with the Andersen agar impactor (~17%) compared to the SASS 3100 electret filter sampler (~4%). Airborne bacterium-

containing particles were distributed between different sizes for particles of >1.1 µm, although ~50% were between 1.1-3.3 µm. Anthropogenic activities (mainly passengers) were demonstrated as a major source of airborne bacteria at the station, and shown to predominantly contribute to bacterium-containing particles between 1.1-3.3 µm.

Paper III

Comparative Testing and Evaluation of Nine Different Air Samplers: End-to-End Sampling Efficiencies as Specific Performance Measurements for Bioaerosol Applications

Dybwad, M, G Skogan, and JM Blatny

Aerosol Science and Technology, 2014;48(3):281–294

Based on more than 100 aerosol experiments, a common aerosol chamber-based T&E scheme capable of obtaining specific performance measurements for bioaerosol sampling applications was demonstrated and used to evaluate and compare the performance of nine different air samplers, including impactors, impingers, cyclones, electrostatic precipitators, and filter samplers. The air samplers' end-to-end cultivation-based biological sampling efficiencies (BSEs) and qPCR-/microscopy-based physical sampling efficiencies (PSEs) relative to a reference sampler (BioSampler) were determined for Gram-negative and Gram-positive vegetative bacteria, bacterial spores, viruses, and non-biological polystyrene latex spheres. Significant differences were revealed among the samplers and shown to depend on the stress-sensitivity and particle size of the sampled bioaerosol. Air samplers employing dry collection methods were found to have reduced BSEs for stress-sensitive bioaerosols compared to samplers that were based on wet collection methods. Air samplers that were not filter-based showed reduced PSEs for 1 µm compared to 4 µm bioaerosols. The results also revealed that while several of the evaluated samplers were prone to underestimate bioaerosol concentration levels relative to the BioSampler due to having lower end-to-end sampling efficiencies, they generally obtain more-concentrated samples due to having higher end-to-end concentration factors. Based on the consolidated T&E results several generalized features regarding the evaluated air samplers' suitability for various bioaerosol sampling applications were proposed.

Paper IV

Rapid Identification of *Bacillus anthracis* Spores in Suspicious Powder Samples by Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS)

Dybwad, M, AL van der Laaken, JM Blatny, and A Paauw

Applied and Environmental Microbiology, 2013;79(17):5372-5383

A rapid and reliable MALDI-TOF MS-based analysis method for identification of *B. anthracis* spores in powders was developed and validated. A reference library containing 22 different *Bacillus* sp. strains or hoax materials was constructed and coupled with a novel classification algorithm and standardized processing protocol for various powder samples. The recorded mass spectra revealed that the predominant biomarkers of *Bacillus* sp. spores could be related to small acid-soluble proteins (SASPs), including α-, β-, and γ-SASPs. The developed method's limit of *B. anthracis* detection was determined to be 2.5 x 10^6 spores, equivalent to a 55-µg sample size of the crudest *B. anthracis*-containing powder discovered during the 2001 "Amerithrax" incident. The end-to-end analysis method was able to successfully discriminate among samples containing *B. anthracis* spores, closely related *Bacillus* sp. spores, and commonly encountered hoax materials. No false positive or negative classifications of *B. anthracis* spores were observed, even when the method was challenged to a wide range of other bacterial agents. The robustness of the method was demonstrated by analyzing samples: i) at an external facility using a different MALDI-TOF MS instrument, ii) using an untrained operator, and iii) using mixtures of *Bacillus* sp. spores and various hoax materials. Taken together, the observed performance of the analysis method demonstrated its potential applicability as a rapid, selective, sensitive, robust, and cost-effective laboratory-based analysis tool for resolving incidents involving powders suspected of containing *B. anthracis* spores in less than 30 min.

4. Discussion

4.1. General discussion

BioDIM activities at most real life sites must be accomplished against a naturally occurring bioaerosol background and a more-detailed understanding of this background may be valuable when applied in a BioDIM context for several reasons, including:

- The instrument settings and software algorithms of existing BioDIM systems may be optimized and adjusted to reduce the impact of the operating environment.
- Future BioDIM systems may be specifically designed to minimize the impact of the operating environment, and could even be tailored to achieve optimized performance in specific environmental backgrounds.
- May contribute to establish BioDIM-relevant background generalizations that could assist in predicting T&E requirements for BioDIM systems and with the development of more-accurate modeling and simulation tools.
- May be used to guide the construction of realistic synthetic bioaerosol backgrounds that can be introduced together with a BTA aerosol challenge during simulated operational T&E of BioDIM systems in background-enabled aerosol chambers.

In order to improve our understanding of the bioaerosol background at subway stations, and especially how it may challenge the performance of BioDIM systems, the main part of this PhD study was designed to obtain information about the airborne bacterial background at the Nationaltheatret subway station in Oslo, Norway (**Paper I+II**). No studies of airborne bacteria or other types of bioaerosols have to the author's knowledge previously been performed in a Norwegian subway environment.

While information about the airborne bacterial background may in itself be interesting and have more than one uses, the final goal of this PhD study was to apply the acquired knowledge in a BioDIM context. The conceptual idea was to combine the results from the Norwegian station with similar and different kinds of existing results from other stations, and use the consolidated information to define a set of realistic subway station bioaerosol background characteristics. The defined background characteristics could then serve as a

framework to guide the construction of synthetic subway station backgrounds that may be introduced together with a BTA aerosol challenge during aerosol chamber-based simulated operational T&E of BioDIM systems.

It should be pointed out that a substantial part of this PhD study also involved testing and implementation of sampling and analysis methods for airborne bacteria (**Paper I+II+III**) in order to develop a subway station-compatible airborne bacterial background characterization scheme. Several of the methods that were employed, including the SASS 3100 electret filter air sampler and the MALDI-TOF MS-based MALDI Biotyper microbial identification system, had never before been used in a subway environment or for the purpose of characterizing airborne bacterial backgrounds. To ensure the success of this PhD study it was also necessary to put a great deal of effort into establishing the in-house expertise and know-how needed to design and perform airborne bacterial background characterization studies in a subway environment.

Substantial efforts were also put into the development and validation of the MALDI-TOF MS-based identification assay for *B. anthracis* and other *Bacillus* sp. spores in powders (**Paper IV**). Several topics addressed in **Paper IV** may be linked to those that were addressed in the other publications of this PhD study, including:

- MALDI-TOF MS was used as a bacterial identification method in most publications (**Paper I+II+IV**, and also in **Paper V** which was not included in the thesis proper).
- As demonstrated by the 2001 "Amerithrax" incident, *B. anthracis* spores in powders (e.g. letters) may become aerosolized and present a significant aerosol threat (e.g. may cause inhalation anthrax).
- *B. anthracis*-containing powders are considered as a relevant bioterrorism threat and therefore also as an important BioDIM task.
- At the Nationaltheatret subway station, airborne bacteria belonging to the *Bacillus* genus were abundantly observed, including *Bacillus* sp. spores (**Paper I+II**).
- Although the MALDI-TOF MS-based identification method for *Bacillus* sp. spores was specifically developed to analyze powder samples in this study (**Paper IV**), it may still be adaptable to other sample types (e.g. air samples).

The background characterization studies performed at the Nationaltheatret subway station as part of this PhD study (**Paper I+II**) generated several interesting results that warrant discussion. However, these results have already been extensively discussed and compared to existing literature in the respective publications. The following discussion will therefore be centered on the potential applications and implications of the acquired knowledge in a BioDIM context, and more precisely in the context of simulated operational T&E of BioDIM systems in background-enabled aerosol chambers.

While it may in itself be interesting to understand the airborne bacterial background of Norwegian subway stations from a national perspective, this knowledge may also be relevant from an international perspective. Efforts to compare the background at different stations (e.g. in different cities and geographic regions) will be needed to elucidate similarities and differences on a global scale, and may assist in the formulation of generalizations that can be used to predict the background of uncharacterized stations and also to develop generic modeling and simulation tools.

Knowledge about how conserved the background will be at different stations may similarly assist in predicting T&E requirements for BioDIM systems, both in terms of the need for real life operational T&E at subway stations and the feasibility of achieving sufficiently realistic simulated operational T&E in background-enabled aerosol chambers. The ideal situation would be that the environmental background, and especially the properties that may have an impact on the performance of BioDIM systems, turns out to be conserved between stations. It could then be proposed that operational testing at a single subway station, or simulated operational testing in a single synthetic background, might be sufficient to predict the operational performance of BioDIM systems in most or all subway environments. On the other hand, if the environmental background turns out to differ substantially between stations, it may be necessary to suggest that the operational performance of BioDIM systems can only be accurately assessed through operational testing at each station, or alternatively through simulated operational testing in synthetic backgrounds that are tailored to match each individual station. The economic costs and practical challenges that will be associated with these “best case” and “worst case” scenarios are obviously very different, and efforts to elucidate the actual real world situation may therefore be considered as an important task. It should however be noted that even though a single “generic” background turns out not to be sufficiently representative to accurately predict the performance of BioDIM systems at most or all subway stations during late-stage T&E (e.g. for commissioning purposes), it might still

be sufficiently representative to predict the operational performance of BioDIM systems during R&D and initial T&E (e.g. for technology screening and down-selection purposes).

To the Author's knowledge, this PhD study is also the first to address the airborne bacterial background of subway stations specifically in a BioDIM context. That being said, it should be emphasized that even though the previous characterization studies have not focused on BioDIM applications, the obtained results may still be useful in a BioDIM context in the same way as the results from the current work may be applied in a public and occupational health as well as microbial ecology context. Even when all environments are considered, only a limited number of studies have investigated bioaerosol backgrounds or reviewed existing bioaerosol background information specifically in a BioDIM context [43, 58, 291, 292]. A common theme in these reports appears to be a call for increased efforts to close existing knowledge gaps, in combination with a call for the BioDIM community to better recognize the potential importance of the bioaerosol background and to strengthen the integration of this knowledge into all phases of R&D and T&E of BioDIM systems.

An encouraging observation in light of this has been what appears to be a growing emphasis on the potential importance of the bioaerosol background in recent years. This may be exemplified by the development of aerosol generation systems which could allow for realistic bioaerosol background conditions to be recreated during aerosol chamber-based T&E of BioDIM systems [59], in combination with increased efforts to characterize real life bioaerosol backgrounds [43, 58, 291, 292].

Recent efforts to perform T&E of BioDIM systems at real life operational locations may also suggest that there is a growing recognition of the potential importance of the bioaerosol background within the BioDIM community. In 2013, at least two different T&E campaigns were performed at subway stations, further strengthening the view that such sites may be considered relevant for the deployment and operation of BioDIM systems. One campaign was performed in the Boston subway by the US Department of Homeland Security [293], while the other campaign was performed in the Prague subway as part of the TWOBIAS project (Two Stage Rapid Biological Surveillance and Alarm System for Airborne Threats) which was funded through the 7th Framework Program of the European Union (www.twobias.com). The fact that subway stations were chosen as the real life test sites in both campaigns could be taken to suggest that subway environments may present challenges to BioDIM systems that

will not be sufficiently addressed through T&E at existing test facilities (e.g. open-air ranges and ambient breeze tunnels).

4.1.1. BioDIM-relevant background characteristics

It is a challenging task to predict which constituents of the bioaerosol background, and similarly which characteristics of the individual constituents and the background as a whole, that will be relevant in a BioDIM context. A major contributing factor that makes such predictions challenging is the fact that various BioDIM technologies may respond very differently to the same bioaerosol background, in combination with the fact that the backgrounds themselves may be both complex and variable. In addition to the bioaerosol background, various other environmental parameters, including the total aerosol background and the meteorological conditions, may also have an impact on the operational performance of BioDIM systems, both independently and in concert with the bioaerosol background.

The selectivity level of BioDIM systems (e.g. bioaerosol- or BTA aerosol-selective systems) may obviously have a strong impact on the interfering (i.e. clutter) potential of the bioaerosol background (as exemplified in Figure 5 in section 1.5). While a bioaerosol-selective system inherently will "see" most or all of the bioaerosol background, a BTA aerosol-selective system will on the other hand be blinded to most or all of the bioaerosol background, at least as long as BTA aerosols are not a natural part of the background. In light of this, it should however be noted that although the selectivity level of a BTA aerosol-selective system dictates that it will be blinded to the background, both bioaerosols and non-biological aerosols may contribute to matrix effects that can negatively impact the operational performance of even highly sensitive and selective BioDIM systems. In the context of PCR-based BioDIM technologies, such matrix effects may be exemplified by the inhibition of essential enzymes (e.g. DNA polymerases) due to the presence of PCR inhibitors (e.g. humic-like substances), and by the loss of sensitivity and selectivity that may occur in the presence of too high amounts of various non-target materials (e.g. non-target nucleic acids).

In addition to the selectivity level, another potentially important difference between BioDIM systems that may impact on how they are affected by the bioaerosol background is their fundamental operating principles (e.g. single-particle analysis or bulk sample analysis). Most

detect-to-warn technologies are currently based on single-particle analyses (e.g. on-line particle counting and sizing combined with UV-LIF), while most detect-to-threat technologies are based on bulk sample analyses (e.g. air sampling followed by PCR- or immunoassay-based identification assays).

The use of size-selective air sampling techniques to specifically capture aerosols within the relevant particle size range may also have an impact on how BioDIM systems are challenged by the bioaerosol background. BTA aerosols between 1 and 10 µm are generally considered to be of primary concern since they may penetrate deep into and deposit in the human respiratory tract [43]. However, the exact upper and lower limits of this size range are still subject to debate, and should in any case not be interpreted as absolute limits [43]. BioDIM systems may utilize specialized air sampling techniques, including virtual impactors and size-selective inlets, to specifically capture aerosols in the relevant size range and to exclude aerosols below or above this range. All bioaerosol background constituents that are successfully excluded from entering the BioDIM system may at least in principle be disregarded, even if they are known to have a strong interfering potential and are present in the air in high concentrations. This is an important concept to highlight since it shows that it is only the fraction of the bioaerosol background that reaches the analytical part of the BioDIM system that may have an impact on its performance. A good example to use in this context could be pollen. Pollen grains have been shown to be present in outdoor air in very high concentrations under certain conditions [43], but due to their relatively large unit size, which generally is >10 um [43, 294], pollen may be excluded from the analytical part of BioDIM systems with the use of appropriate size-selective air sampling techniques. It should however be noted that when broken up into fragments, pollen and other large-sized biological substances may still contribute to bioaerosols that are within the relevant particle size range.

This PhD study exclusively investigated the airborne bacterial fraction of the bioaerosol background (see section 1.10 for the rationale behind this decision). In light of the large number of factors that may influence on how different BioDIM systems "see" and get affected by the bioaerosol background, it was decided that the best course of action would be to define a generic set of bioaerosol background characteristics without pre-selecting or tailoring the choice of characteristics to fit with or favor any particular BioDIM technology or system. The rationale behind this decision was that as long as the defined set of generic background characteristics represented the real life background in sufficient detail, it should allow for simulated operational T&E of BioDIM systems independently of their technological

basis and operating principle. However, the technological basis and operating principle for a selection of commonly used BioDIM systems were still reviewed to ensure that the defined set of characteristics covered most of the real life characteristics which may be expected to be particularly important in a BioDIM context. The characteristics that were proposed to have the highest potential for interfering with the operational performance of BioDIM systems based on this non-exhaustive and non-systematic review included, but were not limited to, the concentration level, diversity, size distribution, and temporal variability of the relevant bioaerosol background. What actually constitutes the relevant bioaerosol background will obviously depend on the BioDIM system, and it may therefore be preferable to divide the bioaerosol background into separate constituent classes based on their relative abundance and predicted relevance. The various background characteristics may then be established separately for each constituent class (e.g. bacteria, fungi, and pollen), and then later be applied in a BioDIM context both individually and combined.

In addition to the bioaerosol background characteristics, the fact that several BioDIM systems (e.g. UV-LIF-based systems) frequently utilize the bioaerosol (i.e. aerosols that upon UV-irradiation display certain fluorescent properties) to total aerosol ratio, rather than the absolute bioaerosol concentration alone, could suggest that total aerosol characteristics may also have an impact on the operational performance of BioDIM systems. Total aerosol background characteristics could thus be proposed to be BioDIM-relevant background characteristics that should be established.

In addition to bioaerosols and aerosols, various meteorological conditions may similarly be suggested to be relevant background characteristics that could have an impact on the operational performance of BioDIM systems. Temperature, humidity, and wind speed conditions outside the intended operating range can directly impact the technical operation of BioDIM systems, but may also indirectly affect the operational performance of BioDIM systems by influencing the aerodynamic and biological properties of BTA aerosols and background aerosols. Humidity- and temperature-dependent changes in desiccation and condensation processes may for example alter the size distribution of aerosols and thus impact their aerodynamic behavior, but may also change the inactivation rates of aerosolized BTAs which could alter their associated health hazard.

4.1.2. Proposed generic set of subway station background characteristics

A generic set of realistic subway station background characteristics was proposed (Table 2) based on the results from the Norwegian subway station (**Paper I+II**) in combination with results from other stations (section 1.8.1). It should however be noted that several of the proposed characteristics only tentatively represent a "typical" subway station at this point, since they had to be based on information that is currently available only from a single subway station. In addition to provide useful information on its own, the proposed set of background characteristics may also serve to highlight areas where additional data will be needed to close still existing knowledge gaps. The proposed background characteristics were limited to a core set of generic characteristics (e.g. concentration level, diversity, size distribution, and temporal variability) for the purpose of this discussion. However, several additional background characteristics, especially airborne bacterial characteristics (e.g. antibiotic resistance, pigmentation, and hemolytic activity), may be taken directly from the publications (**Paper I+II**).

Since information about the characteristics of pollen and viral aerosols in subway environments is currently lacking, these bioaerosol constituent classes could not be included in the proposed set of background characteristics, and will only be briefly discussed. Pollen grains have been proposed to not represent a major challenge to BioDIM systems in indoor environments since their sources are generally limited to the outdoor environment [43]. However, in naturally ventilated indoor locations (e.g. the Nationaltheatret subway station) where the outdoor-to-indoor transport of aerosols can be substantial, pollen may still be introduced into the indoor air and constitute a relevant component of the bioaerosol background. Nevertheless, for pollen aerosols it may be sufficient to know their characteristics in outdoor air in combination with a detailed understanding of outdoor-to-indoor transport mechanisms [43]. Information about the background characteristics of viral aerosols is currently nonexistent or scarce from any environment [43]. However, the presence of various specific viral agents in air (e.g. influenza viruses) is increasingly being studied and reported, especially in hospital environments [295]. Taken together, it may therefore be proposed that efforts to obtain information about pollen and viral aerosols at subway stations could be an interesting research topic.

Detailed information was obtained from the Norwegian subway station about the meteorological "background" conditions (**Paper I+II**), and most reports from other stations have usually contained meteorological information (e.g. temperature and RH). It is however difficult to establish a set of "typical" subway station meteorological conditions since a substantial variability is seen both within (e.g. seasonal and diurnal) and between stations (e.g. different climate regions and presence/absence of heating, ventilation, and air conditioning systems). The temperature at the Norwegian subway station ranged from ~22°C in the summer (**Paper I**) to ~6°C in the winter (**Paper II**). The relative humidity at the Norwegian subway station ranged from about 40-80%RH without a clear seasonal pattern (**Paper I+II**), and on at least one occasion the diurnal variation spanned almost the entire range (**Paper II**). The wind speed at the Norwegian subway station ranged from about 0 to 2 m s^{-1} while trains were operating and from about 0 to 0.5 m s^{-1} when trains were not operating (**Paper II**).

Table 2. Proposed generic set of realistic subways station background characteristics.

Airborne bacterial characteristics	
Concentration level	Cultivable bacteria: 0-10^4 cfu m^{-3} (**Paper I+II** and section 1.8.1). Total bacteria: 10^3-10^4 cells m^{-3} (section 1.8.1). Bacterial spore level corresponding to less than 2-3.4% of the bacterial level (**Paper I+II**).
Diversity	Mainly *Micrococcus*, *Staphylococcus*, *Bacillus*, *Pseudomonas*, *Rhodococcus*, *Arthrobacter*, *Moraxella*, *Kocuria*, and *Dermacoccus* spp. (**Paper I+II** and section 1.8.1).
Size distribution	Airborne bacterium-containing particles distributed between different sizes for particles of >1.1 μm with about 50% between 1.1 and 3.3 μm (**Paper II**).
Temporal variability	Limited seasonal and day-to-day variability, and substantial diurnal variability (**Paper II**). Consistent diurnal profile with increased bacterial levels during daytime compared to nighttime and during rush hours compared to non-rush hours (**Paper II**). Rapid temporal variability in the bacterial level (up to 270-fold within a 10-min period), both consistent and inconsistent with the typical diurnal profile (**Paper II**).
Airborne fungal characteristics	
Concentration level	Cultivable fungi: 0-10^3 cfu m^{-3} (section 1.8.1). Generally lower airborne fungal levels than airborne bacterial levels (section 1.8.1).
Diversity	Mainly *Penicillium*, *Cladosporium*, and *Aspergillus* spp. (section 1.8.1.).
Temporal variability	The diurnal concentration level profile for fungal aerosols may resemble the diurnal airborne bacterial concentration level profile established in **Paper II** [241].
Total aerosol characteristics	
Concentration level	Mass concentrations: 3-480 (PM2.5), 6-1500 (PM10), and 208-1610 (TSP) μg m^{-3} (section 1.8.1). Number concentrations: 10^9-10^{10} (<1 μm) and 10^6-10^8 (>1 μm) particles m^{-3} (**Paper I+II** and section 1.8.1).
Diversity	Strongly enriched in iron-containing particles (section 1.8.1). Sodium chloride-containing particles may also be present at high concentrations [202].
Temporal variability	The diurnal concentration level profile for total aerosols may resemble the diurnal airborne bacterial concentration level profile established in **Paper II** [267].

4.1.3. General recommendations for the construction of synthetic backgrounds

Simulated operational T&E in background-enabled aerosol chambers could be an efficient way to predict the operational performance of BioDIM systems without the need for expensive and logistically demanding on-site testing. However, the process of defining and recreating realistic synthetic environmental backgrounds is far from straightforward, largely due to the complexity and variability of the real life backgrounds. The fact that the relevance of different background constituents and their characteristics will depend on the technological basis and operating principles of the BioDIM systems may further complicate the process of defining which constituents and characteristics that should be included in a generic synthetic background to make it sufficiently realistic. Similarly, the exact level of operational realism that will be required during simulated operational T&E of BioDIM systems is currently largely unknown, and will likely directly depend on the specific T&E objective (e.g. technology down-selection, qualification, or commissioning).

The level of realism needed to accurately predict the operational performance of BioDIM systems during late-stage T&E may require the use of synthetic backgrounds that are both complex and dynamic. Depending on the actual level of operational realism needed, the economic costs and technological challenges associated with defining and recreating such detailed synthetic backgrounds may at some point become so large as to favor real life operational T&E instead of simulated operational T&E. However, during R&D and early-stage T&E of BioDIM systems, the inherent benefits associated with aerosol chamber-based T&E (e.g. low cost and reproducibility) in combination with a presumably reduced need for complete operational realism, may allow for cost-effective simulated operational T&E of BioDIM systems that offers a substantial benefit compared to real life operational T&E.

It may be reasonable to suggest that even simplified synthetic backgrounds could allow for a selection of the most important BioDIM-relevant background challenges to be simulated in an aerosol chamber. Even though it is unlikely that all possible challenges can be recreated in sufficient detail with a simplified background, it may still be enough to discern whether the performance of a candidate BioDIM system is sufficient to warrant further R&D and T&E efforts. As an example it could be proposed that if a BioDIM system fails to qualify during T&E in a simplified and static synthetic background (i.e. without temporal variation), it may already at that point be excluded from further T&E, even though the real life background is

complex and dynamic. A T&E scheme that is able to screen and down-select candidate BioDIM systems based on their empirically predicted operational performance already during R&D and early-stage T&E could offer a substantial economic benefit and allow for an efficient allocation of resources and funding.

The generic set of realistic subway station background characteristics that was established in this PhD study may assist in the construction of synthetic subway station backgrounds, and while the construction process itself was outside the scope of the study, some general recommendations and synthetic background examples were still formulated based on the obtained results.

The process of artificially generating a high number of different aerosols and bioaerosols to recreate realistic environmental background characteristics (e.g. concentration level and particle size distribution) in an aerosol chamber may obviously be a challenging task. However, the technical challenges that will be associated with aerosol chamber-based simulated operational T&E of BioDIM systems were considered to be outside the scope of this PhD study and will therefore not be further addressed.

While an idealized synthetic background should mirror all aspects of the real life background in complete detail, the construction of synthetic backgrounds that approach this level of detail is probably not achievable, at least not in the foreseeable future. Furthermore, synthetic backgrounds with this level of detail may not even be warranted due to the economic costs that would be involved and the uncertainties that exist regarding the level of operational realism that will be required during simulated operational T&E of BioDIM systems.

It may therefore be reasonable to suggest that initial efforts to construct a synthetic subway station background could benefit from a bottom-up approach, starting with the construction of a simplified background involving only a limited number of background constituents and characteristics as a proof-of-concept. Additional background constituents and characteristics may then be added to the "core" background at a later time point if the level of operational realism associated with this background turns out to be insufficient.

Based on the generic set of subway station background characteristics that was established in this PhD study (Table 2), the following step-by-step process may be suggested as a reasonable approach in terms of initial efforts to construct a synthetic subway station "core" background:

1. **Recreate the total aerosol background**
    - The concentration level, diversity, and size distribution of total aerosols should reflect the background characteristics that were defined in Table 2.
    - A certified reference material such as urban dust (CRM-1649b) from the US National Institute of Standards and Technology or British Rail Dust (BR/1008/1989) from the UK-based company Particle Technologies may be proposed as candidate test materials.
    - Iron-containing particles should be included to mimic the observed enrichment of this element in subway aerosols if a non-subway-based test material is used (e.g. urban dust). No certified reference materials that could serve as an appropriate subway station iron-only simulant was identified. A candidate test material for subway station iron may possibly be constructed by magnetic extraction of iron-containing particles from a subway station air sample.
2. **Recreate the airborne bacterial background**
    - The concentration level, diversity, and size distribution of airborne bacteria should reflect the background characteristics that were defined in Table 2.
    - A mixture of cultured bacterial cells may be suggested as a candidate test material. A selection of conventional bacterial simulants or bacterial isolates derived from a subway station (e.g. *Bacillus*, *Micrococcus*, *Staphylococcus*, and *Pseudomonas* spp.) may be used to mimic an authentic airborne bacterial diversity (Table 2).
    - *Bacillus* sp. spores should be included to recreate a realistic spore fraction (Table 2). A conventional simulant (e.g. *B. atrophaeus*) or *Bacillus* sp. isolates derived from a subway station may be used to produce the spores.
3. **Recreate the airborne fungal background**
    - The concentration level and diversity of airborne fungi should reflect the background characteristics that were defined in Table 2. Since no information about the size distribution of airborne fungi in subway air was identified, it may be reasonable to target a particle size distribution that reflects the aerodynamic unit size of fungal spores.
    - A mixture of fungal spores may be suggested as a candidate test material. Fungal spores produced from a selection of model organisms or fungal isolates obtained at a subway station (e.g. *Penicillium*, *Cladosporium*, and *Aspergillus* spp.) may be used to recreate a realistic airborne fungal diversity (Table 2).

The proposed three-step approach will allow for the selected background constituent classes (i.e. total aerosols, airborne bacteria, and airborne fungi) to be simulated independently of each other. Each constituent class may also be further subdivided as needed to allow independent simulation of different sub-constituents (e.g. vegetative bacteria and bacterial spores, and different bacterial types such as Gram-positive and Gram-negative bacteria). Such an approach will be needed when the aerosol characteristics (e.g. concentration level, size distribution, and particle composition) associated with different sub-constituents within a constituent class differ substantially from each other. While the proposed "core" background is currently static, the ability to independently manipulate the aerosol characteristics of different background constituent classes and sub-constituents will also be required to incorporate their dynamic characteristics (i.e. temporal variability).

An alternative approach to the proposed three-step process could be to use a bulk air sample from a subway station as a stand-alone test material. While this approach will allow for each constituent of the subway station background to be present in realistic amounts in the synthetic background, it will unfortunately also result in an irreversible averaging/blending of the real life background, which means that critical information about the real life particle size distribution and particle composition of the individual constituent classes and sub-constituents will be lost. A bulk sample approach will similarly not allow for any of the individual constituents or their characteristics to be varied independently of each other.

It should however be noted that although the bulk sample approach probably is far from optimal, and may not even be suitable for T&E of BioDIM systems that are based on single-particle analyses, it could still be suitable for T&E of BioDIM systems that are based on bulk sample analyses as long as the test material is obtained using an air sampling technique with similar size-selective properties as the BioDIM system. However, since the bulk air sample represents the sampled material as it would be presented to the analytical part of the BioDIM system after air sampling, there is basically no need to recreate an aerosol and the test material may instead be presented directly to BioDIM system as a liquid sample.

The subway station background will obviously not be a static environment in real life, and this PhD study has documented that at least the total aerosol and airborne bacterial levels will be associated with rapid temporal variability on some occasions (**Paper II**). It may therefore be suggested that a synthetic subway station background must at some point adopt dynamic characteristics if it is to truly reflect the real life background. The "core" background may

however still be useful as a static baseline, on top of which dynamic characteristics of the various constituent classes and sub-constituents may be added.

Efforts directed towards the initial construction of a synthetic subway station background could probably benefit from a stepwise development process, which may be proposed to consist of the following phases:

- Phase 1 Static "core" background
    - In accordance with the proposed three-step construction approach.
- Phase 2 Static complex background
    - Add additional constituent classes (e.g. pollen and viruses) to the background.
    - Split existing constituent classes into sub-constituents to allow for a more-accurate reproduction of their individual characteristics.
- Phase 3 Dynamic (complex) background
    - Start to adjust the individual characteristics (e.g. concentration level) of one or several constituent classes or sub-constituents as a function of time to recreate the temporal dynamics of the real life background.
    - The dynamic background characteristics may be added on top of the static "core" background, or the individual constituents classes and sub-constituents that make up the "core" background may themselves be varied.

The proposed phases should only be considered as tentative suggestions, and it may for example be warranted to introduce dynamic background characteristics earlier in the construction process (e.g. the order of phase 2 and phase 3 could be reversed).

4.2. Methodological considerations

Detailed information about the airborne bacterial background at the Nationaltheatret subway station was obtained in this PhD study. However, the study should be considered as one piece of a bigger puzzle since several additional studies will be needed to fully characterize the bioaerosol background at the Norwegian subway station, and likewise to define a complete set of bioaerosol background characteristics that may be considered truly representative of a "typical" subway station. Some potential next steps in terms of additional research efforts that could extend and improve the results obtained in the current study may involve:

- Efforts to validate the appropriateness of using the proposed airborne bacterial background characteristics, some of which were exclusively based on results from the Norwegian subway station, as representative airborne bacterial background characteristics for a "typical" subway station.
    - Can be achieved by validating, and updating if needed, the proposed airborne bacterial background characteristics by performing similar studies at other subway stations using the same, or at least well-defined and comparable, air sampling and analysis methods.
- Efforts to validate the appropriateness of using the proposed airborne fungal and total aerosol background characteristics, most of which were based on results from other subway stations, as representative background characteristics for the Norwegian subway station.
    - Can be achieved by validating, and updating if needed, the proposed airborne fungal and total aerosol background characteristics by performing additional studies at the Norwegian subway station using well-defined and comparable air sampling and analysis methods. In addition to the proposed characteristics, future studies could benefit from addressing bioaerosol constituents that have not yet been characterized at subway stations (e.g. pollen and viral aerosols).

While this PhD study established a broad range of different airborne bacterial background characteristics, several airborne bacterial characteristics that may be considered relevant in a BioDIM context was not obtained. One limitation could be proposed to be the study's reliance on the use of cultivation-based quantification and identification methods for airborne bacteria, which resulted in the characterization of CAB and not TAB. However, based on the fact that

previous airborne bacterial characterization studies at subway stations have almost exclusively investigated CAB, in combination with the various limitations and benefits that will be associated with both cultivation-based and cultivation-independent airborne bacterial analysis methods (described in section 1.7.1), it may be suggested that both CAB and TAB characteristics could be relevant in a BioDIM context. Although only CAB characteristics were reported from the Norwegian subway station as part of this study, several attempts were also made to obtain corresponding TAB characteristics. Unfortunately, most of these efforts had to be abandoned due to technical challenges.

4.2.1. Technical challenges associated with TAB quantification methods

FM combined with various staining techniques (e.g. SYBR green I, acridine orange, and BacLight Live/Dead staining) was attempted as a TAB quantification method as part of this PhD study. The FM method was used to analyze the subway station air samples describe in **Paper I**. Due to the presence of high amounts of particulate matter (Figure 13), and which also included a substantial amount of autofluorescent particulates, it was not possible to obtain accurate bacterial counts from the these air samples, and the FM method had to be abandoned. FCM combined with the same staining techniques was similarly attempted as a TAB quantification method. The FCM method was used to analyze the same air samples as the FM method, and had to be abandoned due to similar technical challenges. In a recent paper from the New York City subway [209] it was found that aerosolized iron in subway air may interfere with FM analyses. A procedure for removing magnetic iron from air samples using a magnet rack was proposed and enabled the investigators to successfully perform FM analyses on subway station air samples. It may therefore be reasonable to suggest that the use of such a procedure could have reduced the technical challenges that were observed in this PhD study.

Several of the technical challenges (e.g. autofluorescent particulates and unspecific staining) that were associated with both the FM and FCM methods appeared to have been caused by the high particulate matter background, and it may be proposed that a more-selective method, such as FISH, could have helped to reduce or even circumvent these challenges. Although it was not possible due to resource limitations to include FISH-based FM or FCM methods as

part of this study, such methods could represent an interesting and potentially promising alternative to traditional staining methods.

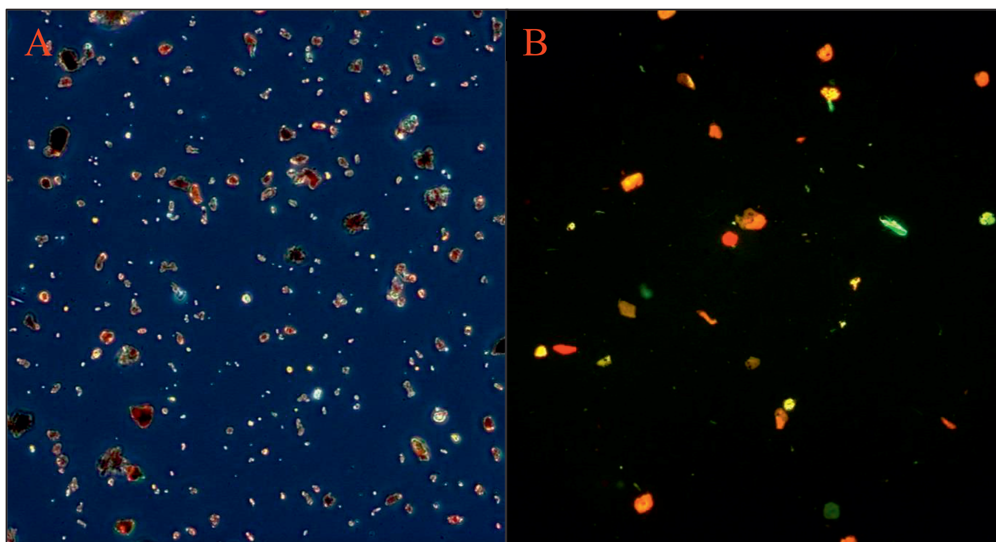

Figure 13. Microscopy analysis (AxioSkop 2, Carl Zeiss) performed on air samples from the Nationaltheatret subway station. Light microscopy image (A) showing high amounts of particulate matter. Epifluorescence microscopy image (B) showing high amounts of autofluorescent particulates in unstained samples.

Similar efforts were also made to implement qPCR as a TAB quantification method as part of this study. The tested qPCR assay targeted the bacterial 16S rRNA gene and was used on the same air samples as the FM and FCM methods. Nucleic acids were purified from the subway station air samples using a MoBio UltraClean Soil DNA isolation kit. An in-house inhibition control assay (lambda-DNA qPCR assay) was used to screen the samples for PCR inhibition by spiking them with an appropriate lambda-DNA standard. An appreciable amount of PCR inhibition was observed for the majority of the samples, and it was therefore necessary to abandon the qPCR-based TAB quantification method. However, the main reason nucleic acids were purified from the subway station air samples was actually their intended use in a PhyloChip microarray analysis [151] to study TAB diversity. After the qPCR method had been abandoned, several attempts were made to remove the PCR inhibition, and in the end it was found that the addition of bovine serum albumin could restore PCR amplification for both the inhibition control and 16S rRNA gene-based qPCR assays. However, when the inhibition issues were resolved the residual amount of purified nucleic acids from the subway station air samples was not sufficient to allow for both a qPCR and a PhyloChip microarray analysis,

and it was decided to prioritize the latter. The PhyloChip results are currently being processed for publication, and will not be further detailed in this PhD study.

4.2.2. MALDI-TOF MS as a cost-effective method for CAB identification

Our results (**Paper I+II**) demonstrated that the MALDI-TOF MS-based MALDI Biotyper could be a valuable method for identification of airborne bacteria and screening of airborne bacterial isolate collections. The classification results obtained with the MALDI Biotyper generally corresponded well with partial 16S rRNA gene sequencing-based results (**Paper I**). When compared to the sequencing approach which involved PCR amplification followed by amplicon clean-up and Sanger sequencing, the MALDI Biotyper offered several benefits in terms of speed and cost which may be particularly valuable during screening of large bacterial isolate collections. However, the performance of the MALDI Biotyper was shown to directly depend on the availability of representative library entries (at the corresponding species or genus level) in the reference database (Bruker Taxonomy), and an appreciable number of bacterial isolates derived from the subway station air could not be confidently classified because no representative entries were present in the reference database. The coverage of environmental bacteria in the Bruker Taxonomy database is gradually increasing, but there is still a bias towards bacterial taxa that are clinically relevant and highly studied (**Paper I**). Although the results obtained in **Paper I** and **Paper II** are not completely comparable due to differences in the isolate selection process, they could still be taken to suggest that the coverage of relevant environmental bacterial taxa in the Bruker Taxonomy database improved between the two studies (2011 and 2012, respectively). Approximately 36% of the obtained isolates during the first subway station study (**Paper I**) could not be reliably classified, while this number was reduced to 29% in the second study which employed an updated reference database (**Paper II**). As pointed out in **Paper I,** the MALDI Biotyper system allows the users to construct their own libraries. This could be an efficient way to increase the coverage of bacterial taxa that are not sufficiently represented in the reference database. Efforts to increase the coverage of relevant airborne bacterial taxa in the reference database are currently being planned, and may involve the use of the airborne bacterial isolate collections that were obtained in **Paper I** and **Paper II**. If successful, these efforts could increase the benefits that will be associated with the use of the MALDI Biotyper in airborne bacterial background

characterization studies, and similarly contribute to unleash the true potential of the MALDI Biotyper as a rapid and cost-effective screening tool for environmental bacterial isolates, which is currently being hampered by the limited coverage of environmental bacterial taxa in the reference database.

4.3. Future perspectives

Many of the topics that were addressed in this PhD study may clearly warrant further study. Several still remaining knowledge gaps have been identified and highlighted as part of the discussion, and suggested research efforts that may be used to address these knowledge gaps have been formulated. A selection of the suggested research efforts may be summarized in a non-prioritized order as follows:

- Efforts to obtain TAB characteristics from the Nationaltheatret subway station.
- Efforts to compare the bioaerosol background, including CAB and TAB characteristics, at different subway stations.
- Efforts to obtain background characteristics for potentially relevant bioaerosol classes (e.g. pollen and viruses) that have not yet been characterized at subway stations.
- Efforts to improve the MALDI Biotyper as an identification method for airborne bacteria by increasing the coverage of commonly observed airborne bacterial taxa in the reference database.
- Efforts to construct a synthetic subway station "core" background as a proof-of-principle.

In terms of future perspectives, it could also be interesting to briefly highlight two follow-up studies that have already been initiated:

- A TAB diversity study involving the use of PhyloChip microarrays to analyze the subway station air samples that were described in **Paper I**.
- An airborne bacterial background characterization study at an underground subway station in Prague, the Czech Republic, involving the use of air sampling and analysis schemes that correspond to those that were used in **Paper I+II**.

5. Conclusions

In order to generate new knowledge concerning the environmental background of bioaerosols that may be encountered by BioDIM systems while operating in a subway environment, the airborne bacterial background at the Nationaltheatret subway station in Oslo, Norway, was characterized as part of this PhD study (**Paper I+II**). The resulting scientific publications represent the first published accounts that describe the background of airborne bacteria or any other type of bioaerosol in a Norwegian subway environment. The overall aim was to establish a set of realistic subway station airborne bacterial background characteristics and to describe how this information may be applied in a BioDIM context to improve the operational performance of BioDIM systems and to develop an aerosol chamber-based T&E scheme that takes the real life operational bioaerosol background into account.

The conclusions from this work (**Paper I+II**) were:

- The average concentration level of airborne bacteria at the subway station was ~400 cfu m^{-3} during the daytime and ~20 cfu m^{-3} during the nighttime, and about 70 cfu m^{-3} during the daytime at an outdoor site located adjacent to the station's entrance.
- The airborne bacterial levels at the station ranged from not detected to 10^3 cfu m^{-3}, and typically showed increased levels during the daytime compared to the nighttime, as well as increase levels during rush hours compared to non-rush hours.
- The concentration level and diversity of airborne bacteria at the station was conserved between different days and yearly seasons, while the intraday variability was substantial although generally associated with a highly consistent diurnal pattern.
- The airborne bacterial level at the station displayed rapid temporal variation on some occasions (up to 270-fold within a 10-min period), which were both consistent and inconsistent with the typical diurnal profile.
- The majority of airborne bacteria at the station belonged to the genera *Micrococcus*, *Rhodococcus*, *Arthrobacter*, *Moraxella*, *Staphylococcus*, *Bacillus*, *Pseudomonas*, *Dermacoccus*, and *Kocuria*. A total of more than 37 different bacterial genera were successfully recovered from the subway station air.
- A higher relative abundance of Gram-negative bacteria was recovered from the station with the Andersen agar impactor (17%) compared to the SASS 3100 electret filter sampler (4%).

- Airborne bacterium-containing particles were distributed between different sizes for particles of >1.1 µm at the station, although ~50% were between 1.1-3.3 µm.
- Anthropogenic activities (mainly passengers) were demonstrated as a major source of airborne bacteria at the station, and shown to predominantly contribute to bacterium-containing particles between 1.1-3.3 µm.
- The airborne bacterial spore fraction was shown to be higher in outdoor air (~16%) than at the station (~2-3%).
- About 22% of the bacterial isolates derived from the station air showed hemolytic activity, and were predominantly distributed between the bacterial genera *Bacillus* (87%) and *Staphylococcus* (10%).
- About 27% of the bacterial isolates derived from the station air showed antibiotic resistance against at least two different antibiotics, while ~10% of the isolates did not display antibiotic resistance.
- About 48% of the bacterial isolates derived from the station air displayed visual colony pigmentation. The predominant pigmentation was various shades of yellow, although orange, pink, and red pigmentations were also commonly observed.
- A MALDI-TOF MS-based bacterial identification method (MALDI Biotyper) was successfully implemented as a rapid and cost-effective screening tool for airborne bacterial isolate collections. The MALDI Biotyper results corresponded well with 16S rRNA gene sequencing-based results. The coverage of environmental airborne bacterial taxa in the MALDI Biotyper reference database was however a limiting factor of the MALDI-TOF MS method, and it was concluded that efforts should be made to increase the coverage.

The use of air sampling equipment with well-defined performance criteria is important for any bioaerosol sampling application since it is the only way to ensure that the obtained results correctly describe the real life characteristics of the targeted bioaerosol. An air sampler's physical sampling efficiency is an important performance measurement for sampling of any bioaerosol regardless of downstream analysis method that is used. However, an air sampler's biological sampling efficiency is an important performance measurement if the downstream analysis methods depend on the biological state of the sampled material (e.g. cultivation-based methods). Changes in the biological state of bioaerosols as a result of the air sampling process are commonly attributed to sampling-associated stress (e.g. desiccation and shear forces), and an air sampler's propensity to inflict such stress must be carefully matched to the

stress-sensitivity of the targeted bioaerosol to ensure that the obtained results correctly describe the real life characteristics of the targeted bioaerosol. Both the design and collection principle of an air sampler may have an impact on its physical and biological sampling efficiency, and another important part of this PhD study therefore involved aerosol chamber-based comparative T&E of several different samplers based on a variety of collection principles and sampler designs.

The conclusions from this work (**Paper III**) were:

- Air samplers employing dry collection principles (e.g. filtration, dry impaction, and electrostatic precipitation) showed reduced biological sampling efficiencies for stress-sensitive bioaerosols compared to samplers employing wet collection principles (e.g. liquid impingement and wetted-wall cyclones).
- Filter-based air samplers had similar physical sampling efficiencies for 1 µm compared to 4 µm bioaerosols, while non-filter-based samplers had reduced physical sampling efficiencies for 1 µm compared to 4 µm bioaerosols.
- The end-to-end sampling efficiencies of several of the evaluated samplers showed that they will be prone to underestimate bioaerosol concentration levels (relative to the BioSampler). However, their end-to-end concentration factors showed that they generally will still be able to obtain samples with a higher concentration of sampled material than the BioSampler.
- Gram-negative vegetative bacteria (*Serratia marcescens*) were shown to be more stress-sensitive than viruses (bacteriophage MS2), Gram-positive vegetative bacteria (*Kocuria rhizophila*), and bacterial spores (*Bacillus atrophaeus*).

Suspicious powders containing *B. anthracis* spores were demonstrated as a serious bioterrorism threat during the 2001 "Amerithrax" incident in the US, and resulted in the loss of human lives and massive societal burdens. While the discovery of powders that actually contain *B. anthracis* spores may be considered as rare events, hoax incidents are much more common and represent a major societal burden since they must be handled as real threats until the presence of hazardous materials can be confidently ruled out. One way to collectively reduce the societal burdens associated with bioterrorism and hoax incidents may be to develop reliable analysis methods that are able to confirm or rule out the presence of *B. anthracis* spores with minimum processing time. BioDIM has been a common theme throughout this

PhD study, and the main BioDIM objective in this case was to develop and test a MALDI-TOF MS-based rapid identification method for *B. anthracis* spores in powder samples.

The conclusions from this work (**Paper IV**) were:

- A MALDI-TOF MS-based method for rapid and reliable screening of powder samples and identification of *B. anthracis* spores was successfully developed and tested.
- The recorded mass spectra based on MALDI-TOF MS measurements of acid-extracted proteins from *Bacillus* sp. spores revealed that the predominant biomarkers could be related to small acid-soluble proteins (SASPs), including α-, β-, and γ-SASPs.
- The method's limit of *B. anthracis* detection was determined to be 2.5 x 10^6 spores, equivalent to a 55-μg sample size of the crudest *B. anthracis*-containing powder discovered during the 2001 "Amerithrax" incident.
- The method was able to successfully discriminate among samples containing *B. anthracis* spores, closely related *Bacillus* sp. spores, and commonly encountered hoax materials.
- No false positive or negative classifications of *B. anthracis* spores were observed, even when the method was challenged to a wide range of other bacterial agents.
- The robustness of the method was demonstrated by successfully analyzing samples: i) at an external facility using a different MALDI-TOF MS instrument than the one that was used to develop the method, ii) using an untrained operator, and iii) containing mixtures of *Bacillus* sp. spores and commonly encountered hoax materials.
- The observed performance of the method demonstrated its potential applicability as a rapid, specific, sensitive, robust, and cost-effective analysis tool capable of resolving incidents involving suspicious powders in less than 30 min.

Several of the results that were obtained as part of this PhD study may also be relevant when viewed in the context of public and occupational health. However, the potential public and occupational health implications of the results were intentionally not addressed as part of this PhD study since the specified objective was to address and emphasize the implications and applications of the obtained results in a BioDIM context. Nevertheless, this PhD study has resulted in the publication of a substantial amount of detailed information concerning: i) the airborne bacterial background at a subway station (**Paper I+II**), ii) the performance of a wide selection of air samplers for a variety of bioaerosol sampling applications (**Paper III**), and iii)

the potential applicability of a novel MALDI-TOF MS-based rapid identification method for *B. anthracis* spores in powders (**Paper IV**). The results have thus been made available to the scientific community and may be evaluated and assessed by public and occupational health professionals with respect to their potential public and occupational health implications.

In summary, this PhD study contributes to a deeper understanding of the bioaerosol background, especially at subway stations. It similarly highlights the potential importance of the bioaerosol background in a BioDIM context, and emphasizes the need for increased research efforts to close existing knowledge gaps. It may also serve to highlight the research efforts that will be needed before real life bioaerosol background information may be fully exploited in a BioDIM context. This PhD study has brought forth important results with respect to the long term ambition of developing aerosol chamber-based simulated operational T&E schemes for BioDIM systems that combine the reproducibility and cost-effectiveness of aerosol chamber-based T&E with the operational realism of on-site T&E.

References

1. Green, M.S., et al., *Risk assessment and risk communication strategies in bioterrorism preparedness*. 2007: Springer.

2. Al-Agamy, H.M., *Tools of biological warfare.* Research Journal of Microbiology, 2011. **6**(3): p. 193-245.

3. Richardt, A. and F. Sabath, *A glance back - Myths and facts about CBRN incidents*, in *CBRN Protection: Managing the Threat of Chemical, Biological, Radioactive and Nuclear Weapons*, A. Richardt, et al., Editors. 2013, Wiley-VCH Verlag. p. 1-38.

4. Wheelis, M., L. Rózsa, and M. Dando, *Deadly cultures: biological weapons since 1945*. 2009: Harvard University Press.

5. Davis, C.J., *Nuclear blindness: An overview of the biological weapons programs of the former Soviet Union and Iraq.* Emerging Infectious Diseases, 1999. **5**(4): p. 509-512.

6. Meselson, M., et al., *The Sverdlovsk anthrax outbreak of 1979.* Science, 1994. **266**(5188): p. 1202-1208.

7. Frischknecht, F., *The history of biological warfare.* EMBO Rep, 2003. **4**(S): p. S47-S52.

8. Tucker, J.B., *The current bioweapons threat*, in *Biopreparedness and Public Health*, I. Hunger, et al., Editors. 2013, Springer. p. 7-16.

9. CDC. *Bioterrorism agents list*. Centers for Disease Control and Prevention, Atlanta, GA 2013 [cited 2013 September 21]; Available from: http://www.emergency.cdc.gov/agent/agentlist-category.asp.

10. Merriam-Webster Online Dictionary. 2013 [cited 2013 September 23]; Available from: http://www.merriam-webster.com/.

11. Hilleman, M.R., *Overview: cause and prevention in biowarfare and bioterrorism.* Vaccine, 2002. **20**(25): p. 3055-3067.

12. Gerstein, D.M., *National security and arms control in the age of biotechnology: the biological and toxin weapons convention*. 2013: Rowman & Littlefield Publishers.

13. Lindler, L., F. Lebeda, and G.W. Korch, *Biological weapons defense: Infectious diseases and counterbioterrorism*. 2005: Springer.

14. Salerno, R.M., et al., *A BW risk assessment: historical and technical perspectives.* The Nonproliferation Review, 2004. **11**(3): p. 25-55.

15. Takahashi, H., et al., *Bacillus anthracis bioterrorism incident, Kameido, Tokyo, 1993.* Emerging infectious diseases, 2004. **10**(1): p. 117-120.

16. Jernigan, D.B., et al., *Investigation of bioterrorism-related anthrax, United States, 2001: epidemiologic findings.* Emerging infectious diseases, 2002. **8**(10): p. 1019-1028.

17. Jernigan, J.A., et al., *Bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States.* Emerging infectious diseases, 2001. **7**(6): p. 933-944.

18. Zietz, B.P. and H. Dunkelberg, *The history of the plague and the research on the causative agent Yersinia pestis.* International journal of hygiene and environmental health, 2004. **207**(2): p. 165-178.

19. Fenner, F., et al., *Smallpox and its eradication*. Vol. 6. 1988: World Health Organization.

20. Reid, A.H., J.K. Taubenberger, and T.G. Fanning, *The 1918 Spanish influenza: integrating history and biology.* Microbes and infection, 2001. **3**(1): p. 81-87.

21. Riedel, S., *Biological warfare and bioterrorism: a historical review.* Proceedings (Baylor University Medical Center), 2004. **17**(4): p. 400-406.

22. Bartoszcze, M. and M. Niemciewicz, *Biological threats: aerosol, food, water, and unconventional*, in *Technology for Combating WMD Terrorism*, P.J. Stopa and Z. Orahovec, Editors. 2004, Springer. p. 5-10.

23. Ivnitski, D., D. Morrison, and D. O'neil, *Critical elements of biological sensor technology for deployment in an environmental network system*, in *Defense against Bioterror*, D. Morrison, et al., Editors. 2005, Springer. p. 207-220.

24. Hong, T., et al., *Prioritizing risks and uncertainties from intentional release of selected Category A pathogens.* PLoS ONE, 2012. **7**(3): p. e32732.

25. Casadevall, A., *The future of biological warfare.* Microbial Biotechnology, 2012. **5**(5): p. 584-587.

26. EU, *Communication from the Commission to the European Parliament and the Council on strengthening chemical, biological, radiological and nuclear security in the European Union - an EU CBRN action plan.* European Commission, COM 273, 2009.

27. NATO, *NATO's comprehensive, strategic-level policy for preventing the proliferation of weapons of mass destruction (WMD) and defending against chemical, biological, radiological and nuclear (CBRN) threats.* North Atlantic Treaty Organization, 2009.

28. Subcommittee on Standards, *A national strategy for CBRNE standards.* National Science and Technology Council, Committee on Homeland and National Security, 2011.

29. The White House, *Homeland security presidential directive (HSPD-10): biodefense for the 21st century.* The White House, 2004.

30. The White House, *Presidential policy directive 8 (PPD-8): national preparedness.* The White House, 2011.

31. UN, *The United Nations global counter-terrorism strategy.* General Assembly Resolution 60-288, United Nations, 2006.

32. NATO, *Allied joint doctrine for chemical, biological, radiological, and nuclear defence, AJP-3.8.* North Atlantic Treaty Organization, NATO Standardization Agency, 2012.

33. Franz, D.R., P.B. Jahrling, and A.M. Friedlander, *Clinical recognition and management of patients exposed to biological warfare agents.* JAMA, 1997. **278**(5): p. 399-411.

34. Hindson, B.J., et al., *APDS: the autonomous pathogen detection system.* Biosensors and Bioelectronics, 2005. **20**(10): p. 1925-1931.

35. EDA, *Concept for the detection, identification and monitoring (DIM) of chemical, biological, radiological and nuclear (CBRN) and toxic industrial hazards (TIH).* European Defence Agency, Report of the Project Team on CBRN Detection, Identification and Monitoring, 2006.

36. Fatah, A., et al., *Guide for the selection of biological agent detection equipment for emergency first responders - 101–06*. 2007: Office of Law Enforcement Standards, National Institute of Standards and Technology.

37. DOD, *JP 3-11: Operations in chemical, biological, radiological, and nuclear (CBRN) environments.* US Department of Defense, Joint Staff, 2008.

38. DOD, *JP 3-40: Combating weapons of mass destruction.* US Department of Defense, Joint Staff, 2008.

39. NATO, *NATO handbook for sampling and identification of biological, chemical and radiological agents (SIBCRA), AEP-66.* North Atlantic Treaty Organization, NATO Standardization Agency, 2013.

40. Baird, C., et al., *Biodetection technologies for first responders*. 2012: Pacific Northwest National Laboratory.

41. Emanuel, P. and M. Caples, *Chemical, biological, radiological technology survey*. 2011: Edgewood Chemical Biological Center.

42. Emanuel, P. and I.R. Fruchey, *Biological detectors: market survey*. 2007: Edgewood Chemical Biological Center.

43. Committee on Materials and Manufacturing Processes for Advanced Sensors, *Sensor systems for biological agent attacks: protecting buildings and military bases.* The National Research Council of the National Academies. The National Academies Press, 2005.

44. Brown, K., *Up in the air.* Science, 2004. **305**(5688): p. 1228-1229.

45. Kaufmann, A.F., M.I. Meltzer, and G.P. Schmid, *The economic impact of a bioterrorist attack: are prevention and postattack intervention programs justifiable?* Emerging infectious diseases, 1997. **3**(2): p. 83-94.

46. Greenwood, D.P., et al., *Optical techniques for detecting and identifying biological-warfare agents.* Proceedings of the IEEE, 2009. **97**(6): p. 971-989.

47. Jeys, T.H., et al., *Advanced trigger development.* Lincoln Laboratory Journal, 2007. **17**(1): p. 29-62.

48. Ho, J., *Future of biological aerosol detection.* Analytica Chimica Acta, 2002. **457**(1): p. 125-148.

49. Ho, J., *History of biological detection.* Defence Research and Development Canada, DRDC, Technical Report TR2009-180, 2009.

50. Gard, E., et al., *Real-time analysis of individual atmospheric aerosol particles: design and performance of a portable ATOFMS.* Analytical Chemistry, 1997. **69**(20): p. 4083-4091.

51. Steele, P.T., et al., *Autonomous, broad-spectrum detection of hazardous aerosols in seconds.* Analytical Chemistry, 2008. **80**(12): p. 4583-4589.

52. van Wuijckhuijse, A.L., et al., *Matrix-assisted laser desorption/ionisation aerosol time-of-flight mass spectrometry for the analysis of bioaerosols: development of a fast detector for airborne biological pathogens.* Journal of Aerosol Science, 2005. **36**(5–6): p. 677-687.

53. Holman, C. and R.L. Berkowitz, *Chemical and biological defense test and evaluation - A hallmark of integrating DT and OT.* ITEA Journal, 2009. **30**(3): p. 381-385.

54. Committee on Determining a Standard Unit of Measure for Biological Aerosols, *A framework for assessing the health hazard posed by bioaerosols.* The National Research Council of the National Academies. The National Academies Press, 2008.

55. Samuels, A.C., et al., *Test methodology development for biological agent detection systems.* Proc. of SPIE, 2006. **6378**(637802).

56. Committee on the Review of Testing and Evaluation Methodology for Biological Point Detectors, *Review of testing and evaluation methodology for biological point detectors.* The National Research Council of the National Academies. The National Academies Press, 2005.

57. Valdes, J.J., et al., *Total agent per liter of air with particle size distribution (TALAp): a new unit of measure for the test and evaluation of biodetectors.* ITEA Journal, 2010. **31**(3): p. 417-425.

58. Birenzvige, A. and C. Wick, *Background aerosols and BW detection: problems and suggested solutions.* Reasearch and Technology Directorate, Edgewood Chemical Biological Center (ECBC-TR-255), 2002.

59. Ratnesar-Shumate, S., et al., *Improved method for the evaluation of real-time biological aerosol detection technologies.* Aerosol Science and Technology, 2011. **45**(5): p. 635-644.

60. Carrano, J., *Chemical and biological sensor standards study.* Defense Advanced Research Projects Agency, DARPA, 2005.

61. Kulkarni, P., P.A. Baron, and K. Willeke, *Aerosol measurement: principles, techniques, and applications*. 2011: John Wiley & Sons.

62. Hinds, W.C., *Aerosol technology: properties, behavior, and measurement of airborne particles*. 1999: John Wiley & Sons.

63. Ruzer, L.S. and N.H. Harley, *Aerosols handbook: measurement, dosimetry & health effects*. 2004: CRC Press.

64. Spurnây, K. and J. Marijnissen, *Nicolai Albertowich Fuchs: The pioneer of aerosol science: biography*. 1998: Delft University Press.

65. Fuchs, N.A., *The mechanics of aerosols*. 1964: Dover Publications.

66. Hidy, G.M., *Aerosols, an industrial and environmental science*. 1984: Academic Press.

67. Grinshpun, S.A. and J.M. Clark, *Measurement and characterization of bioaerosols.* Journal of Aerosol Science, 2005. **36**(5): p. 553-555.

68. Reponen, T., et al., *Biological particle sampling*, in *Aerosol measurement: principles, techniques, and applications*, P. Kulkarni, P.A. Baron, and K. Willeke, Editors. 2011, John Wiley & Sons. p. 549-570.

69. Cox, C.S. and C.M. Wathes, eds. *Bioaerosols handbook*. 1995, Lewis Publishers.

70. Jaenicke, R., *Abundance of cellular material and proteins in the atmosphere.* Science, 2005. **308**(5718): p. 73.

71. Grinshpun, S.A., *Biological aerosols*, in *Aerosols - Science and Technology*, I. Agranovski, Editor. 2010, Wiley-VCH Verlag. p. 379-406.

72. Blatny, J.M., et al., *Tracking airborne Legionella and Legionella pneumophila at a biological treatment plant.* Environmental science & technology, 2008. **42**(19): p. 7360-7367.

73. Uhrbrand, K., A.C. Schultz, and A.M. Madsen, *Exposure to airborne noroviruses and other bioaerosol components at a wastewater treatment plant in Denmark.* Food and Environmental Virology, 2011. **3**(3-4): p. 130-137.

74. Haas, D., et al., *Exposure to bioaerosol from sewage systems.* Water, Air, and Soil Pollution, 2010. **207**(1-4): p. 49-56.

75. Chiang, C.-F., H.-H. Yang, and T.-W. Chi, *Monitoring of bioaerosol emission from sludge composting facility.* Int J Appl Sci Eng, 2003. **1**(2): p. 148-159.

76. Brooks, J., et al., *Bioaerosols from the land application of biosolids in the desert southwest USA.* Water Science & Technology, 2004. **50**(1): p. 7-12.

77. Gilbert, Y. and C. Duchaine, *Bioaerosols in industrial environments: a review.* Canadian Journal of Civil Engineering, 2009. **36**(12): p. 1873-1886.

78. Millner, P.D., *Bioaerosols associated with animal production operations.* Bioresource Technology, 2009. **100**(22): p. 5379-5385.

79. Mandal, J. and H. Brandl, *Bioaerosols in indoor environment - a review with special reference to residential and occupational locations.* Open Environmental and Biological Monitoring Journal, 2011. **4**(0): p. 83-96.

80. Xu, Z., et al., *Bioaerosol science, technology, and engineering: past, present, and future.* Aerosol Science and Technology, 2011. **45**(11): p. 1337-1349.

81. Després, V.R., et al., *Primary biological aerosol particles in the atmosphere: a review.* Tellus B, 2012. **64**: p. 1-58.

82. Douwes, J., et al., *Bioaerosol health effects and exposure assessment: progress and prospects.* Annals of Occupational Hygiene, 2003. **47**(3): p. 187-200.

83. Stahlhofen, W., J. Gebhart, and J. Heyder, *Experimental determination of the regional deposition of aerosol particles in the human respiratory tract.* The American Industrial Hygiene Association Journal, 1980. **41**(6): p. 385-398.

84. Mohr, A.J., *Fate and transport of microorganisms in air*, in *Manual of environmental microbiology, 3rd Edition*, C.J. Hurst, et al., Editors. 2007, ASM Press. p. 961-971.

85. Ho, J. and W. Griffiths, *Edited special issue: Bioaerosols.* Journal of Aerosol Science, 1994. **25**(8): p. 1369-1613.

86. Lacey, J., *Edited special issue: Sampling and rapid assay of bioaerosols.* Journal of Aerosol Science, 1997. **28**(3): p. 345-538.

87. Grinshpun, S.A. and J.M. Clark, *Edited special issue: Measurement and characterization of bioaerosols.* Journal of Aerosol Science, 2005. **36**(5): p. 553-812.

88. Grinshpun, S.A. and I.E. Agranovski, *Edited special issue: Bioaerosol research.* CLEAN - Soil, Air, Water, 2008. **36**(7): p. 535-621.

89. Burge, H.A., ed. *Bioaerosols*. Vol. 2. 1995, CRC Press.

90. Macher, J., ed. *Bioaerosols: assessment and control*. 1999, American Conference of Governmental Industrial Hygienists, ACGIH.

91. Dimmick, R.L. and A.B. Akers, eds. *An introduction to experimental aerobiology*. 1969, Wiley Interscience.

92. Cruz, P. and M. Buttner, *Analysis of bioaerosol samples*, in *Manual of environmental microbiology, 3rd Edition*, C. Hurst, et al., Editors. 2007, ASM Press. p. 952-960.

93. Grinshpun, S., M. Buttner, and K. Willeke, *Sampling for airborne microorganisms*, in *Manual of environmental microbiology, 3rd Edition*, C.J. Hurst, et al., Editors. 2007, ASM Press. p. 939-951.

94. Stetzenbach, L., *Introduction to aerobiology*, in *Manual of environmental microbiology, 3rd Edition*, C.J. Hurst, et al., Editors. 2007, ASM Press. p. 925-938.

95. Salem, H. and D. Gardner, *Health aspects of bioaerosols*, in *Atmospheric Microbial Aerosols*, B. Lighthart and A.J. Mohr, Editors. 1994, Springer. p. 304-330.

96. Cole, E.C. and C.E. Cook, *Characterization of infectious aerosols in health care facilities: an aid to effective engineering controls and preventive strategies.* American journal of infection control, 1998. **26**(4): p. 453-464.

97. Kuske, C.R., *Current and emerging technologies for the study of bacteria in the outdoor air.* Current Opinion in Biotechnology, 2006. **17**(3): p. 291-296.

98. Omenn, G.S., *Evolution and public health.* Proceedings of the National Academy of Sciences, 2010. **107**(suppl 1): p. 1702-1709.

99. Brooke, J.S., *Stenotrophomonas maltophilia: an emerging global opportunistic pathogen.* Clinical microbiology reviews, 2012. **25**(1): p. 2-41.

100. Chambers, H.F. and F.R. DeLeo, *Waves of resistance: Staphylococcus aureus in the antibiotic era.* Nature Reviews Microbiology, 2009. **7**(9): p. 629-641.

101. Arias, C.A. and B.E. Murray, *The rise of the Enterococcus: beyond vancomycin resistance.* Nat Rev Micro, 2012. **10**(4): p. 266-278.

102. Zumla, A., et al., *Drug-resistant tuberculosis - current dilemmas, unanswered questions, challenges, and priority needs.* Journal of Infectious Diseases, 2012. **205**(suppl 2): p. S228-S240.

103. Griffiths, W. and G. DeCosemo, *The assessment of bioaerosols: a critical review.* Journal of Aerosol Science, 1994. **25**(8): p. 1425-1458.

104. Cox, C., *The aerobiological pathway of microorganisms*. 1987: John Wiley & Sons.

105. Roszak, D. and R. Colwell, *Survival strategies of bacteria in the natural environment.* Microbiological reviews, 1987. **51**(3): p. 365-379.

106. Agranovski, I.E., et al., *Development and evaluation of a new personal sampler for culturable airborne microorganisms.* Atmospheric Environment, 2002. **36**(5): p. 889-898.

107. Cox, C.S., *Airborne bacteria and viruses.* Science progress, 1989. **73**(292): p. 469-499.

108. Alvarez, A.J., M.P. Buttner, and L.D. Stetzenbach, *PCR for bioaerosol monitoring: sensitivity and environmental interference.* Applied and Environmental Microbiology, 1995. **61**(10): p. 3639-3644.

109. Andersen, A.A., *New sampler for the collection, sizing, and enumeration of viable airborne particles.* Journal of Bacteriology, 1958. **76**(5): p. 471-484.

110. Bourdillon, R., O. Lidwell, and J.C. Thomas, *A slit sampler for collecting and counting air-borne bacteria.* Journal of Hygiene, 1941. **41**(2): p. 197-224.

111. May, K. and G. Harper, *The efficiency of various liquid impinger samplers in bacterial aerosols.* British Journal of Industrial Medicine, 1957. **14**(4): p. 287-297.

112. Willeke, K., X. Lin, and S.A. Grinshpun, *Improved aerosol collection by combined impaction and centrifugal motion.* Aerosol Science and Technology, 1998. **28**(5): p. 439-456.

113. An, H.R., G. Mainelis, and M. Yao, *Evaluation of a high-volume portable bioaerosol sampler in laboratory and field environments.* Indoor Air, 2004. **14**(6): p. 385-393.

114. Bergman, W., et al., *High volume, low pressure drop, bioaerosol collector using a multi-slit virtual impactor.* Journal of Aerosol Science, 2004. **36**(5-6): p. 619-638.

115. Buttner, M.P. and L.D. Stetzenbach, *Evaluation of four aerobiological sampling methods for the retrieval of aerosolized Pseudomonas syringae.* Applied and Environmental Microbiology, 1991. **57**(4): p. 1268-1270.

116. Carvalho, E., et al., *Performance of the Coriolis air sampler, a high-volume aerosol-collection system for quantification of airborne spores and pollen grains.* Aerobiologia, 2008. **24**(4): p. 191-201.

117. Kesavan, J., J.R. Bottiger, and A.R. McFarland, *Bioaerosol concentrator performance: comparative tests with viable and with solid and liquid nonviable particles.* Journal of Applied Microbiology, 2008. **104**(1): p. 285-295.

118. Kesavan, J., D. Schepers, and A.R. McFarland, *Sampling and retention efficiencies of batch-type liquid-based bioaerosol samplers.* Aerosol Science and Technology, 2010. **44**(10): p. 817-829.

119. Li, C.-S., *Evaluation of microbial samplers for bacterial microorganisms.* Aerosol Science and Technology, 1999. **30**(2): p. 100 - 108.

120. McFarland, A.R., et al., *Wetted wall cyclones for bioaerosol sampling.* Aerosol Science and Technology, 2010. **44**(4): p. 241-252.

121. Radosevich, J.L., et al., *Development of a high-volume aerosol collection system for the identification of air-borne micro-organisms.* Letters in Applied Microbiology, 2002. **34**(3): p. 162-167.

122. Yao, M. and G. Mainelis, *Analysis of portable impactor performance for enumeration of viable bioaerosols.* Journal of Occupational and Environmental Hygiene, 2007. **4**(7): p. 514 - 524.

123. Macher, J.M. and K. Willeke, *Performance criteria for bioaerosol samplers.* Journal of Aerosol Science, 1992. **23**(Supplement 1): p. 647-650.

124. Nevalainen, A., et al., *Performance of bioaerosol samplers: collection characteristics and sampler design considerations.* Atmospheric Environment. Part A. General Topics, 1992. **26**(4): p. 531-540.

125. Lighthart, B. and B.T. Shaffer, *Airborne bacteria in the atmospheric surface layer: temporal distribution above a grass seed field.* Applied and Environmental Microbiology, 1995. **61**(4): p. 1492-1496.

126. Shaffer, B.T. and B. Lighthart, *Survey of culturable airborne bacteria at four diverse locations in Oregon: urban, rural, forest, and coastal.* Microbial Ecology, 1997. **34**(3): p. 167-177.

127. Titball, R. and P. GS, *BWC verification measures: technologies for the identification of biological warefare agents.* Polit. Life Sci., 1993. **12**(2): p. 255-263.

128. Gandolfi, I., et al., *Unravelling the bacterial diversity in the atmosphere.* Applied microbiology and biotechnology, 2013. **97**(11): p. 4727-4736.

129. Henningson, E.W., et al., *A comparative study of different methods to determine the total number and the survival ratio of bacteria in aerobiological samples.* Journal of Aerosol Science, 1997. **28**(3): p. 459-469.

130. Walter, M.V., et al., *Effect of aerosolization on subsequent bacterial survival.* Applied and Environmental Microbiology, 1990. **56**(11): p. 3468-3472.

131. Heidelberg, J., et al., *Effect of aerosolization on culturability and viability of gram-negative bacteria.* Applied and Environmental Microbiology, 1997. **63**(9): p. 3585-3588.

132. Amann, R., W. Ludwig, and K. Schleifer, *Phylogenetic identification and in situ detection of individual microbial cells without cultivation.* Microbiol. Rev., 1995. **59**(1): p. 143-169.

133. Lighthart, B., *The ecology of bacteria in the alfresco atmosphere.* FEMS Microbiology Ecology, 1997. **23**(4): p. 263-274.

134. Lighthart, B., *Mini-review of the concentration variations found inthe alfresco atmospheric bacterial populations.* Aerobiologia, 2000. **16**(1): p. 7-16.

135. Fahlgren, C., et al., *Annual variations in the diversity, viability, and origin of airborne bacteria.* Applied and environmental microbiology, 2010. **76**(9): p. 3015-3025.

136. Urbano, R., et al., *Detection and phylogenetic analysis of coastal bioaerosols using culture dependent and independent techniques.* Biogeosciences, 2011. **8**(2): p. 301-309.

137. Tong, Y., *Diurnal distribution of total and culturable atmospheric bacteria at a rural site.* Aerosol Science & Technology, 1999. **30**(2): p. 246-254.

138. Tong, Y. and B. Lighthart, *The annual bacterial particle concentration and size distribution in the ambient atmosphere in a rural area of the Willamette Valley, Oregon.* Aerosol Science & Technology, 2000. **32**(5): p. 393-403.

139. Lange, J.L., P.S. Thorne, and N. Lynch, *Application of flow cytometry and fluorescent in situ hybridization for assessment of exposures to airborne bacteria.* Applied and environmental microbiology, 1997. **63**(4): p. 1557-1563.

140. Peccia, J. and M. Hernandez, *Incorporating polymerase chain reaction-based identification, population characterization, and quantification of microorganisms into aerosol science: A review.* Atmospheric Environment, 2006. **40**(21): p. 3941-3961.

141. An, H.R., G. Mainelis, and L. White, *Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples.* Atmospheric Environment, 2006. **40**(40): p. 7924-7939.

142. Stetzenbach, L.D., M.P. Buttner, and P. Cruz, *Detection and enumeration of airborne biocontaminants.* Current Opinion in Biotechnology, 2004. **15**(3): p. 170-174.

143. Nehme, B., et al., *Culture-independent approach of the bacterial bioaerosol diversity in the standard swine confinement buildings, and assessment of the seasonal effect.* Environmental Microbiology, 2008. **10**(3): p. 665-675.

144. Jeon, E.M., et al., *Impact of Asian dust events on airborne bacterial community assessed by molecular analyses.* Atmospheric Environment, 2011. **45**(25): p. 4313-4321.

145. Franzetti, A., et al., *Seasonal variability of bacteria in fine and coarse urban air particulate matter.* Applied microbiology and biotechnology, 2011. **90**(2): p. 745-753.

146. Bowers, R.M., et al., *Characterization of airborne microbial communities at a high-elevation site and their potential to act as atmospheric ice nuclei.* Applied and Environmental Microbiology, 2009. **75**(15): p. 5121-5130.

147. Bowers, R.M., et al., *Seasonal variability in airborne bacterial communities at a high-elevation site.* Atmospheric Environment, 2012. **50**: p. 41-49.

148. Bowers, R.M., et al., *Spatial variability in airborne bacterial communities across land-use types and their relationship to the bacterial communities of potential source environments.* The ISME journal, 2010. **5**(4): p. 601-612.

149. Bowers, R.M., et al., *Sources of bacteria in outdoor air across cities in the midwestern United States.* Applied and environmental microbiology, 2011. **77**(18): p. 6350-6356.

150. Hospodsky, D., et al., *Human occupancy as a source of indoor airborne bacteria.* PloS one, 2012. **7**(4): p. e34867.

151. Brodie, E.L., et al., *Urban aerosols harbor diverse and dynamic bacterial populations.* Proceedings of the National Academy of Sciences, 2007. **104**(1): p. 299-304.

152. Smith, D.J., et al., *Intercontinental dispersal of bacteria and archaea by transpacific winds.* Applied and environmental microbiology, 2013. **79**(4): p. 1134-1139.

153. Georgakopoulos, D.G., et al., *Microbiology and atmospheric processes: biological, physical and chemical characterization of aerosol particles.* Biogeosciences, 2009. **6**(4): p. 721-737.

154. Hospodsky, D., N. Yamamoto, and J. Peccia, *Accuracy, precision, and method detection limits of quantitative PCR for airborne bacteria and fungi.* Applied and Environmental Microbiology, 2010. **76**(21): p. 7004-7012.

155. Just, N., et al., *Bacterial diversity characterization of bioaerosols from cage-housed and floor-housed poultry operations.* Environmental Research, 2011. **111**(4): p. 492-498.

156. Oppliger, A., F.G. Masclaux, and H. Niculita-Hirzel, *Assessment of airborne microorganisms by real-time PCR: optimistic findings and research challenges.* Front Biosci, 2011. **3**(1): p. 445-453.

157. Rinsoz, T., et al., *Application of real-time PCR for total airborne bacterial assessment: comparison with epifluorescence microscopy and culture-dependent methods.* Atmospheric Environment, 2008. **42**(28): p. 6767-6774.

158. Chang, C.-W. and F.-C. Chou, *Assessment of bioaerosol sampling techniques for viable Legionella pneumophila by ethidium monoazide quantitative PCR.* Aerosol Science and Technology, 2011. **45**(3): p. 343-351.

159. Hara, K. and D. Zhang, *Bacterial abundance and viability in long-range transported dust.* Atmospheric Environment, 2012. **47**(0): p. 20-25.

160. Buttner, M., K. Willeke, and S. Grinshpun, *Sampling and analysis of airborne microorganisms*, in *Manual of Environmental Microbiolgy, 2 edition*, C.J. Hurst, et al., Editors. 2002, ASM Press.

161. Eduard, W. and D. Heederik, *Methods for quantitative assessment of airborne levels of noninfectious microorganisms in highly contaminated work environments.* American Industrial Hygiene Association Journal, 1998. **59**(2): p. 113-127.

162. Griffin, D.W., et al., *Observations on the use of membrane filtration and liquid impingement to collect airborne microorganisms in various atmospheric environments.* Aerobiologia, 2011. **27**(1): p. 25-35.

163. Shahamat, M., et al., *Evaluation of media for recovery of aerosolized bacteria.* Aerobiologia, 1997. **13**(4): p. 219-226.

164. Morris, C., et al., *Microbiology and atmospheric processes: research challenges concerning the impact of airborne micro-organisms on the atmosphere and climate.* Biogeosciences, 2011. **8**(1): p. 17-25.

165. Womack, A.M., B.J. Bohannan, and J.L. Green, *Biodiversity and biogeography of the atmosphere.* Philosophical Transactions of the Royal Society B: Biological Sciences, 2010. **365**(1558): p. 3645-3653.

166. Burrows, S.M., et al., *Bacteria in the global atmosphere - Part 1: Review and synthesis of literature data for different ecosystems.* Atmos. Chem. Phys. Discuss., 2009. **9**(3): p. 10777-10827.

167. Pearce, D.A., et al., *Microorganisms in the atmosphere over Antarctica.* FEMS microbiology ecology, 2009. **69**(2): p. 143-157.

168. Srikanth, P., S. Sudharsanam, and R. Steinberg, *Bio-aerosols in indoor environment: composition, health effects and analysis.* Indian Journal of Medical Microbiology, 2008. **26**(4): p. 302-312.

169. Griffin, D.W., *Atmospheric movement of microorganisms in clouds of desert dust and implications for human health.* Clinical Microbiology Reviews, 2007. **20**(3): p. 459-477.

170. Kellogg, C.A. and D.W. Griffin, *Aerobiology and the global transport of desert dust.* Trends in Ecology & Evolution, 2006. **21**(11): p. 638-644.

171. Jones, A.M. and R.M. Harrison, *The effects of meteorological factors on atmospheric bioaerosol concentrations - a review.* Science of the total environment, 2004. **326**(1): p. 151-180.

172. Górny, R.L. and J. Dutkiewicz, *Bacterial and fungal aerosols in indoor environment in central and eastern European countries.* Annals of agricultural and environmental medicine, 2002. **9**(1): p. 17-23.

173. Fang, Z., et al., *Culturable airborne bacteria in outdoor environments in Beijing, China.* Microbial Ecology, 2007. **54**(3): p. 487-496.

174. Lighthart, B. and B.T. Shaffer, *Viable bacterial aerosol particle size distributions in the midsummer atmosphere at an isolated location in the high desert chaparral.* Aerobiologia, 1995. **11**(1): p. 19-25.

175. Harrison, R.M., et al., *Climate factors influencing bacterial count in background air samples.* International Journal of Biometeorology, 2005. **49**(3): p. 167-178.

176. Griffin, D.W., D.L. Westphal, and M.A. Gray, *Airborne microorganisms in the African desert dust corridor over the mid-Atlantic ridge, Ocean drilling program, Leg 209.* Aerobiologia, 2006. **22**(3): p. 211-226.

177. Marks, R., et al., *Bacteria and fungi in air over the gulf of Gdańsk and Baltic sea.* Journal of aerosol science, 2001. **32**(2): p. 237-250.

178. Aller, J.Y., et al., *The sea surface microlayer as a source of viral and bacterial enrichment in marine aerosols.* Journal of Aerosol Science, 2005. **36**(5–6): p. 801-812.

179. Hughes, K., et al., *A preliminary study of airborne microbial biodiversity over peninsular Antarctica.* Cellular and molecular biology, 2004. **50**(5): p. 537-542.

180. Cameron, R., F. Morelli, and R. Honour, *Aerobiological monitoring of dry valley drilling sites.* Antarctic Journal of the US, 1973. **8**(4): p. 2ll-2l3.

181. Pearce, D.A., et al., *Biodiversity of air-borne microorganisms at Halley station, Antarctica.* Extremophiles, 2010. **14**(2): p. 145-159.

182. Safatov, A.S., et al., *To what extent can viable bacteria in atmospheric aerosols be dangerous for humans?* CLEAN–Soil, Air, Water, 2008. **36**(7): p. 564-571.

183. Zweifel, U.L., et al., *High bacterial 16S rRNA gene diversity above the atmospheric boundary layer.* Aerobiologia, 2012. **28**(4): p. 481-498.

184. Wainwright, M., et al., *Microorganisms cultured from stratospheric air samples obtained at 41 km.* FEMS microbiology letters, 2003. **218**(1): p. 161-165.

185. Imshenetsky, A., S. Lysenko, and G. Kazakov, *Upper boundary of the biosphere.* Applied and environmental microbiology, 1978. **35**(1): p. 1-5.

186. Park, D.-U., et al., *Assessment of the levels of airborne bacteria, Gram-negative bacteria, and fungi in hospital lobbies.* International journal of environmental research and public health, 2013. **10**(2): p. 541-555.

187. Gilbert, Y., M. Veillette, and C. Duchaine, *Airborne bacteria and antibiotic resistance genes in hospital rooms.* Aerobiologia, 2010. **26**(3): p. 185-194.

188. Ortiz, G., et al., *A study of air microbe levels in different areas of a hospital.* Current microbiology, 2009. **59**(1): p. 53-58.

189. Brandl, H., et al., *Short-term dynamic patterns of bioaerosol generation and displacement in an indoor environment.* Aerobiologia, 2008. **24**(4): p. 203-209.

190. Jo, W.-K. and Y.-J. Seo, *Indoor and outdoor bioaerosol levels at recreation facilities, elementary schools, and homes.* Chemosphere, 2005. **61**(11): p. 1570-1579.

191. Goh, I., et al., *Airborne bacteria and fungal spores in the indoor environment. A case study in Singapore.* Acta Biotechnologica, 2000. **20**(1): p. 67-73.

192. Tringe, S.G., et al., *The airborne metagenome in an indoor urban environment.* PLoS One, 2008. **3**(4): p. e1862.

193. Gaüzère, C., et al., *Reliable procedure for molecular analysis of airborne microflora in three indoor environments: an office and two different museum contexts.* CLEAN–Soil, Air, Water, 2013. **43**(3): p. 226-234.

194. Chen, Y.-P., Y. Cui, and J.-G. Dong, *Variation of airborne bacteria and fungi at Emperor Qin's Terra-Cotta museum, Xi'an, China, during the "Oct. 1" Gold Week period of 2006.* Environmental Science and Pollution Research, 2010. **17**(2): p. 478-485.

195. Petushkova, J. and P. Kandyba, *Aeromicrobiological studies in the Moscow cathedrals.* Aerobiologia, 1999. **15**(3): p. 193-201.

196. Tsai, F. and J. Macher, *Concentrations of airborne culturable bacteria in 100 large US office buildings from the BASE study.* Indoor Air, 2005. **15**(Supplement 9): p. 71-81.

197. Zhu, H., et al., *Characterizations and relationships between outdoor and indoor bioaerosols in an office building.* China Particuology, 2003. **1**(3): p. 119-123.

198. Chen, Q. and L.M. Hildemann, *Size-resolved concentrations of particulate matter and bioaerosols inside versus outside of homes.* Aerosol Science and Technology, 2009. **43**(7): p. 699-713.

199. Fabian, M., et al., *Ambient bioaerosol indices for indoor air quality assessments of flood reclamation.* Journal of Aerosol Science, 2005. **36**(5): p. 763-783.

200. Lee, J.-H. and W.-K. Jo, *Characteristics of indoor and outdoor bioaerosols at Korean high-rise apartment buildings.* Environmental Research, 2006. **101**(1): p. 11-17.

201. Awad, A.H.A., *Environmental study in subway metro stations in Cairo, Egypt.* Journal of Occupational Health, 2002. **44**(2): p. 112-118.

202. Birenzvige, A., et al., *Aerosol characteristics in a subway environment.* Aerosol Science & Technology, 2003. **37**(3): p. 210-220.

203. Gilleberg, S., J. Faull, and K. Graeme-Cook, *A preliminary survey of aerial biocontaminants at six London underground stations.* International biodeterioration & biodegradation, 1998. **41**(2): p. 149-152.

204. Hwang, S.H., et al., *Assessment of airborne environmental bacteria and related factors in 25 underground railway stations in Seoul, Korea.* Atmospheric Environment, 2010. **44**(13): p. 1658-1662.

205. Forbes, J.G., *The atmosphere of the underground electric railways of London: a study of its bacterial content in 1920.* Journal of Hygiene, 1923. **22**(02): p. 123-155.

206. Kim, K., et al., *Exposure level and distribution characteristics of airborne bacteria and fungi in Seoul metropolitan subway stations.* Industrial health, 2011. **49**(2): p. 242-248.

207. Lee, C., et al., *Characterization of airborne bioaerosol concentration in public facilities.* J. Environ. Sci, 2004. **13**(0): p. 215-222.

208. Naddafi, K., et al., *Investigation of indoor and outdoor air bacterial density in Tehran subway system.* Iranian Journal of Environmental Health Science and Engineering, 2011. **8**(4): p. 381-386.

209. Robertson, C.E., et al., *Culture-independent analysis of aerosol microbiology in a metropolitan subway system.* Applied and environmental microbiology, 2013. **79**(11): p. 3485-3493.

210. Zhou, F. and Y. Wang, *Characteristics of antibiotic resistance of airborne Staphylococcus isolated from metro stations.* International journal of environmental research and public health, 2013. **10**(6): p. 2412-2426.

211. Szam, L., et al., *Airborne microflora found in some stations of the metro in the Hungarian capital of Budapest (author's translation).* Zentralblatt für Bakteriologie. 1. Abt. Originale B, Hygiene, Krankenhaushygiene, Betriebshygiene, präventive Medizin, 1980. **170**(1-2): p. 199-208.

212. Szam, L., et al., *Further microbiological studies of the air in a newly built (under the pavement) section of the underground railway in Budapest (author's translation).* Zentralblatt fur Bakteriologie, Mikrobiologie und Hygiene. 1. Abt. Originale B, Hygiene, 1983. **177**(3-4): p. 312-318.

213. Seino, K., et al., *An evidential example of airborne bacteria in a crowded, underground public concourse in Tokyo.* Atmospheric Environment, 2005. **39**(2): p. 337-341.

214. Osman, S., et al., *Microbial burden and diversity of commercial airline cabin air during short and long durations of travel.* The ISME journal, 2008. **2**(5): p. 482-497.

215. Korves, T.M., et al., *Bacterial communities in commercial aircraft high-efficiency particulate air (HEPA) filters assessed by PhyloChip analysis.* Indoor Air, 2013. **23**(1): p. 50-61.

216. Novikova, N., et al., *Survey of environmental biocontamination on board the International Space Station.* Research in microbiology, 2006. **157**(1): p. 5-12.

217. Novikova, N.D., *Review of the knowledge of microbial contamination of the Russian Manned Spacecraft.* Microbial Ecology, 2004. **47**(2): p. 127-132.

218. Newcombe, D.A., et al., *Impact of assembly, testing and launch operations on the airborne bacterial diversity within a spacecraft assembly facility clean-room.* International Journal of Astrobiology, 2008. **7**(3-4): p. 223-236.

219. Li, C.-S. and P.-A. Hou, *Bioaerosol characteristics in hospital clean rooms.* Science of The Total Environment, 2003. **305**(1–3): p. 169-176.

220. Dungan, R.S., et al., *Assessment of bioaerosols at a concentrated dairy operation.* Aerobiologia, 2010. **26**(3): p. 171-184.

221. Liang, R., et al., *Culturable airborne bacteria in outdoor poultry-slaughtering facility.* Microbes and environments, 2013. **28**(2): p. 251-256.

222. Sánchez-Monedero, M.A. and E.I. Stentiford, *Generation and dispersion of airborne microorganisms from composting facilities.* Process Safety and Environmental Protection, 2003. **81**(3): p. 166-170.

223. Albrecht, A., et al., *Detection of airborne microbes in a composting facility by cultivation based and cultivation-independent methods.* Annals of Agricultural and Environmental Medicine, 2007. **14**(1): p. 81-85.

224. Karra, S. and E. Katsivela, *Microorganisms in bioaerosol emissions from wastewater treatment plants during summer at a Mediterranean site.* Water Research, 2007. **41**(6): p. 1355-1365.

225. Blatny, J.M., et al., *Airborne Legionella bacteria from pulp waste treatment plant: aerosol particles characterized as aggregates and their potential hazard.* Aerobiologia, 2011. **27**(2): p. 147-162.

226. Tanner, B.D., et al., *Estimated occupational risk from bioaerosols generated during land application of class B biosolids.* Journal of environmental quality, 2008. **37**(6): p. 2311-2321.

227. Carducci, A., et al., *Assessing airborne biological hazard from urban wastewater treatment.* Water Research, 2000. **34**(4): p. 1173-1178.

228. Schulz, J., et al., *Detection of Coxiella burnetii in the air of a sheep barn during shearing.* Deutsche tierärztliche Wochenschrift, 2005. **112**(12): p. 470-472.

229. Angenent, L.T., et al., *Molecular identification of potential pathogens in water and air of a hospital therapy pool.* Proceedings of the National Academy of Sciences, 2005. **102**(13): p. 4860-4865.

230. Allen, K.D. and H.T. Green, *Hospital outbreak of multi-resistant Acinetobacter anitratus: an airborne mode of spread?* Journal of Hospital Infection, 1987. **9**(2): p. 110-119.

231. Pratt, K.A., et al., *In situ detection of biological particles in cloud ice-crystals.* Nature Geoscience, 2009. **2**(6): p. 398-401.

232. Ariya, P., et al., *Physical and chemical characterization of bioaerosols - Implications for nucleation processes.* International Reviews in Physical Chemistry, 2009. **28**(1): p. 1-32.

233. Huffman, J.A., et al., *High concentrations of biological aerosol particles and ice nuclei during and after rain.* Atmospheric Chemistry and Physics Discussions, 2013. **13**(1): p. 1767-1793.

234. Forest, J.J., *Homeland security: critical infrastructure*. Vol. 3. 2006: Greenwood Publishing Group.

235. Bonnett, C.F., *Practical railway engineering*. 2005: London Imperial College Press.

236. Okumura, T., et al., *Report on 640 victims of the Tokyo subway sarin attack.* Annals of Emergency Medicine, 1996. **28**(2): p. 129-135.

237. Dong, S. and M. Yao, *Exposure assessment in Beijing, China: biological agents, ultrafine particles, and lead.* Environmental monitoring and assessment, 2010. **170**(1-4): p. 331-343.

238. Bogomolova, E. and I. Kirtsideli, *Airborne fungi in four stations of the St. Petersburg underground railway system.* International Biodeterioration & Biodegradation, 2009. **63**(2): p. 156-160.

239. Hoseini, M., et al., *Concentration and distribution characteristics of airborne fungi in indoor and outdoor air of Tehran subway stations.* Aerobiologia, 2012: p. 1-9.

240. Ghosh, D., et al., *Identification and distribution of aeromycoflora in the indoor environment of Shyambazar metro-railway station, Kolkata, India.* African Journal of Microbiology Research, 2011. **5**(31): p. 5569-5574.

241. Cho, J.H., K. Hee Min, and N.W. Paik, *Temporal variation of airborne fungi concentrations and related factors in subway stations in Seoul, Korea.* International Journal of Hygiene and Environmental Health, 2006. **209**(3): p. 249-255.

242. Kawasaki, T., et al., *Distribution and identification of airborne fungi in railway stations in Tokyo, Japan.* Journal of occupational health, 2010. **52**(3): p. 186-193.

243. Picco, A.M. and M. Rodolfi, *Airborne fungi as biocontaminants at two Milan underground stations.* International biodeterioration & biodegradation, 2000. **45**(1): p. 43-47.

244. Fujii, R.K., et al., *Air pollution levels in two São Paulo subway stations*, in *Highway and Urban Environment*, G.M. Morrison and S. Rauch, Editors. 2007, Springer. p. 181-190.

245. Aarnio, P., et al., *The concentrations and composition of and exposure to fine particles (PM2.5) in the Helsinki subway system.* Atmospheric Environment, 2005. **39**(28): p. 5059-5066.

246. Boman, J., et al., *Elemental content of aerosol particles in an underground tram station.* X-Ray Spectrometry, 2009. **38**(4): p. 322-326.

247. Johansson, C. and P.-Å. Johansson, *Particulate matter in the underground of Stockholm.* Atmospheric Environment, 2003. **37**(1): p. 3-9.

248. Midander, K., et al., *Characterisation of nano- and micron-sized airborne and collected subway particles, a multi-analytical approach.* Science of The Total Environment, 2012. **427–428**(0): p. 390-400.

249. Braniš, M., *The contribution of ambient sources to particulate pollution in spaces and trains of the Prague underground transport system.* Atmospheric Environment, 2006. **40**(2): p. 348-356.

250. Cheng, Y.-H. and Y.-L. Lin, *Measurement of particle mass concentrations and size distributions in an underground station.* Aerosol Air Qual. Res, 2010. **10**(0): p. 22-29.

251. Cheng, Y.-H., Y.-L. Lin, and C.-C. Liu, *Levels of PM10 and PM2.5 in Taipei rapid transit system.* Atmospheric Environment, 2008. **42**(31): p. 7242-7249.

252. Cheng, Y.-H. and J.-W. Yan, *Comparisons of particulate matter, CO, and CO2 levels in underground and ground-level stations in the Taipei mass rapid transit system.* Atmospheric Environment, 2011. **45**(28): p. 4882-4891.

253. Cheng, Y.-H., Z.-S. Liu, and J. Ya, *Comparisons of PM10, PM2. 5, particle number, and CO2 levels inside metro trains traveling in underground tunnels and on elevated tracks.* Aerosol Air Qual. Res, 2012. **12**: p. 879-891.

254. Furuya, K., et al., *Seasonal variation and their characterization of suspended particulate matter in the air of subway stations.* Journal of Trace and Microprobe Techniques, 2001. **19**(4): p. 469-485.

255. Jung, H.-J., et al., *Source identification of particulate matter collected at underground subway stations in Seoul, Korea using quantitative single-particle analysis.* Atmospheric Environment, 2010. **44**(19): p. 2287-2293.

256. Kim, K.Y., et al., *Spatial distribution of particulate matter (PM10 and PM2.5) in Seoul metropolitan subway stations.* Journal of Hazardous Materials, 2008. **154**(1–3): p. 440-443.

257. Kim, K.-H., et al., *A noticeable shift in particulate matter levels after platform screen door installation in a Korean subway station.* Atmospheric Environment, 2012. **49**(0): p. 219-223.

258. Park, D.-U. and K.-C. Ha, *Characteristics of PM10, PM2.5, CO2 and CO monitored in interiors and platforms of subway train in Seoul, Korea.* Environment International, 2008. **34**(5): p. 629-634.

259. Park, J.H., H.Y. Woo, and J.C. Park, *Major factors affecting the aerosol particulate concentration in the underground stations.* Indoor and Built Environment, 2012. **November 23, 2012**.

260. Kam, W., et al., *Particulate matter (PM) concentrations in underground and ground-level rail systems of the Los Angeles Metro.* Atmospheric Environment, 2011. **45**(8): p. 1506-1516.

261. Chillrud, S.N., et al., *Elevated airborne exposures of teenagers to manganese, chromium, and iron from steel dust and New York City's subway system.* Environmental Science & Technology, 2003. **38**(3): p. 732-737.

262. Mugica-Álvarez, V., et al., *Concentrations and properties of airborne particles in the Mexico City subway system.* Atmospheric Environment, 2012. **49**(0): p. 284-293.

263. Gómez-Perales, J.E., et al., *Commuters' exposure to PM2.5, CO, and benzene in public transport in the metropolitan area of Mexico City.* Atmospheric Environment, 2004. **38**(8): p. 1219-1229.

264. Murruni, L.G., et al., *Concentrations and elemental composition of particulate matter in the Buenos Aires underground system.* Atmospheric Environment, 2009. **43**(30): p. 4577-4583.

265. Onat, B. and B. Stakeeva, *Assessment of fine particulate matters in the subway system of Istanbul.* Indoor and Built Environment, 2012. **October 18, 2012**.

266. Raut, J.C., P. Chazette, and A. Fortain, *Link between aerosol optical, microphysical and chemical measurements in an underground railway station in Paris.* Atmospheric Environment, 2009. **43**(4): p. 860-868.

267. Salma, I., T. Weidinger, and W. Maenhaut, *Time-resolved mass concentration, composition and sources of aerosol particles in a metropolitan underground railway station.* Atmospheric Environment, 2007. **41**(37): p. 8391-8405.

268. Ye, X., et al., *Investigation of indoor environmental quality in Shanghai metro stations, China.* Environmental Monitoring and Assessment, 2010. **167**(1-4): p. 643-651.

269. Ripanucci, G., et al., *Dust in the underground railway tunnels of an Italian town.* Journal of Occupational and Environmental Hygiene, 2006. **3**(1): p. 16-25.

270. Seaton, A., et al., *The London underground: dust and hazards to health.* Occupational and Environmental Medicine, 2005. **62**(6): p. 355-362.

271. Gorbunov, B., N. Priest, and G. Burns, *London underground aerosol (modelling and measurements).* Journal of Aerosol Science, 1999. **30**: p. S525-S526.

272. Pfeifer, G.D., R.M. Harrison, and D.R. Lynam, *Personal exposures to airborne metals in London taxi drivers and office workers in 1995 and 1996.* Science of The Total Environment, 1999. **235**(1–3): p. 253-260.

273. Nieuwenhuijsen, M.J., J.E. Gómez-Perales, and R.N. Colvile, *Levels of particulate air pollution, its elemental composition, determinants and health effects in metro systems.* Atmospheric Environment, 2007. **41**(37): p. 7995-8006.

274. Anhalt, J.P. and C. Fenselau, *Identification of bacteria using mass spectrometry.* Analytical Chemistry, 1975. **47**(2): p. 219-225.

275. Demirev, P.A. and C. Fenselau, *Mass spectrometry for rapid characterization of microorganisms.* Annu. Rev. Anal. Chem., 2008. **1**: p. 71-93.

276. Demirev, P.A. and C. Fenselau, *Mass spectrometry in biodefense.* Journal of Mass Spectrometry, 2008. **43**(11): p. 1441-1457.

277. Fox, A., *Mass spectrometry for species or strain identification after culture or without culture: past, present, and future.* Journal of clinical microbiology, 2006. **44**(8): p. 2677-2680.

278. Ho, Y.-P. and P.M. Reddy, *Identification of pathogens by mass spectrometry.* Clinical chemistry, 2010. **56**(4): p. 525-536.

279. Carbonnelle, E., et al., *Robustness of two MALDI-TOF mass spectrometry systems for bacterial identification.* Journal of microbiological methods, 2012. **89**(2): p. 133-136.

280. Carbonnelle, E., et al., *MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory.* Clinical biochemistry, 2011. **44**(1): p. 104-109.

281. Mellmann, A., et al., *High interlaboratory reproducibility of matrix-assisted laser desorption ionization-time of flight mass spectrometry-based species identification of nonfermenting bacteria.* Journal of clinical microbiology, 2009. **47**(11): p. 3732-3734.

282. Seng, P., et al., *Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.* Clinical Infectious Diseases, 2009. **49**(4): p. 543-551.

283. Van Veen, S., E. Claas, and E.J. Kuijper, *High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories.* Journal of clinical microbiology, 2010. **48**(3): p. 900-907.

284. Stafsnes, M.H., et al., *Large scale MALDI-TOF MS based taxa identification to identify novel pigment producers in a marine bacterial culture collection.* Antonie van Leeuwenhoek, 2013. **103**(3): p. 603-615.

285. Castanha, E.R., A. Fox, and K.F. Fox, *Rapid discrimination of Bacillus anthracis from other members of the B. cereus group by mass and sequence of "intact" small acid soluble proteins (SASPs) using mass spectrometry.* Journal of microbiological methods, 2006. **67**(2): p. 230-240.

286. Dickinson, D.N., et al., *Species differentiation of a diverse suite of Bacillus spores by mass spectrometry-based protein profiling.* Applied and environmental microbiology, 2004. **70**(1): p. 475-482.

287. Elhanany, E., et al., *Detection of specific Bacillus anthracis spore biomarkers by matrix‐assisted laser desorption/ionization time‐of‐flight mass spectrometry.* Rapid Communications in Mass Spectrometry, 2001. **15**(22): p. 2110-2116.

288. Hathout, Y., et al., *Identification of Bacillus spores by matrix-assisted laser desorption ionization–mass spectrometry.* Applied and environmental microbiology, 1999. **65**(10): p. 4313-4319.

289. Hathout, Y., et al., *Small, acid-soluble proteins as biomarkers in mass spectrometry analysis of Bacillus spores.* Applied and environmental microbiology, 2003. **69**(2): p. 1100-1107.

290. Ryzhov, V., Y. Hathout, and C. Fenselau, *Rapid characterization of spores of Bacillus cereus group bacteria by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry.* Applied and environmental microbiology, 2000. **66**(9): p. 3828-3834.

291. NATO, *Biological aerosol background in various environments and its relevance to detection of biological agents, TR-SET-162.* North Atlantic Treaty Organization, NATO Science and Technology Organization, Final Report of Task Group SET-162, 2013.

292. Paterno, D., *Ambient background particulate composition - outdoor natural background: interferents/clutter.* Reasearch and Technology Directorate, Edgewood Chemical Biological Center (ECBC-TR-882), 2011.

293. Hodson, H., *Detecting a subway bioterror attack terror in the underground.* New Scientist, 2012. **215**(2882): p. 15.

294. Muller, J., *Form and function in angiosperm pollen.* Annals of the Missouri Botanical Garden, 1979: p. 593-632.

295. La Rosa, G., et al., *Viral infections acquired indoors through airborne, droplet or contact transmission.* Annali dell'Istituto superiore di sanita, 2012. **49**(2): p. 124-132.

Papers I-IV

The following section contains the peer-reviewed publications (**Paper I-IV**) that formed the basis for this thesis.

Reprint permissions were obtained from the respective publishers: American Society for Microbiology (**Paper I+II+IV**) and Taylor & Francis (**Paper III**)

Paper I

Characterization of Airborne Bacteria at an Underground Subway Station

Dybwad, M, PE Granum, P Bruheim, and JM Blatny

Applied and Environmental Microbiology, 2012, 78(6):1917-1929

AEM
Journals.ASM.org

Characterization of Airborne Bacteria at an Underground Subway Station

Marius Dybwad,[a,c] Per Einar Granum,[a,b] Per Bruheim,[c] and Janet Martha Blatny[a]

Norwegian Defence Research Establishment, Kjeller, Norway[a]; Norwegian School of Veterinary Science, Oslo, Norway[b]; and Norwegian University of Science and Technology, Department of Biotechnology, Trondheim, Norway[c]

The reliable detection of airborne biological threat agents depends on several factors, including the performance criteria of the detector and its operational environment. One step in improving the detector's performance is to increase our knowledge of the biological aerosol background in potential operational environments. Subway stations are enclosed public environments, which may be regarded as potential targets for incidents involving biological threat agents. In this study, the airborne bacterial community at a subway station in Norway was characterized (concentration level, diversity, and virulence- and survival-associated properties). In addition, a SASS 3100 high-volume air sampler and a matrix-assisted laser desorption ionization–time of flight mass spectrometry-based isolate screening procedure was used for these studies. The daytime level of airborne bacteria at the station was higher than the nighttime and outdoor levels, and the relative bacterial spore number was higher in outdoor air than at the station. The bacterial content, particle concentration, and size distribution were stable within each environment throughout the study (May to September 2010). The majority of the airborne bacteria belonged to the genera *Bacillus*, *Micrococcus*, and *Staphylococcus*, but a total of 37 different genera were identified in the air. These results suggest that anthropogenic sources are major contributors to airborne bacteria at subway stations and that such airborne communities could harbor virulence- and survival-associated properties of potential relevance for biological detection and surveillance, as well as for public health. Our findings also contribute to the development of realistic testing and evaluation schemes for biological detection/surveillance systems by providing information that can be used to mimic real-life operational airborne environments in controlled aerosol test chambers.

As of 2011, more than 120 cities worldwide have underground railway transportation systems (subways), which transport about 200 million people daily. Public places, and especially locations where people are confined in enclosed spaces, such as subway stations, may be regarded as potential targets for the dispersion of biological threat agents in air. Even though no successful deliberate dispersion of biological threat agents in subway environments has been previously reported, such incidents cannot be ruled out. The dispersion of sarin nerve gas (a chemical threat agent) by the Japanese religious cult, Aum Shinrikyo, killed 11 and injured over 5,000 persons in the Tokyo subway in 1995 (50). Aum Shinrikyo failed in causing anthrax infection when aerosolizing *B. anthracis* in Tokyo 2 years earlier, due to the use of a low-virulence vaccine strain (68).

Harmful concentrations of pathogenic microorganisms or their toxins in airborne environments could occur following a deliberate dispersion of biological threat agents but might also be a result of an unintentional release from natural sources. Most biological threat agents will not induce any immediate effects in humans even after fatal exposures, and in the absence of a reliable surveillance system, the public would most likely be unaware of an incident involving biological threat agents until exposed individuals seek medical assistance up to several days later. An early warning, detection, and response scheme could contribute to minimizing the consequences of such incidents (49). Both military and civilian societies/authorities have expressed an urgent need for detection/surveillance systems, and a lot of effort is being put into the development and "testing and evaluation" (T&E) of biological detectors. Biological detectors need to fulfill stringent requirements before they can be deployed for reliable surveillance purposes. The detectors' performance criteria, such as sensitivity and specificity, will be challenged during operation in complex airborne environments, which could lead to false-positive or -negative detection events reducing the detection equipment's credibility. Currently, few if any, available biological detectors have been able to meet the users' requirements regarding reliable sensitive and specific real-time monitoring of biological threat agents in different operational environments (49). This is partly due to the complex nature of microorganisms, and the natural occurrence of similar but nonpathogenic environmental relatives of the biological threat agents. T&E of biological detectors is in general performed in aerosol test chambers, where they are challenged with known amounts of live, attenuated, or killed biological threat agents, or their appropriate simulants, to determine important detector properties such as detection limits. However, it is also necessary to test biological detectors in realistic operational environments since there are several major differences between these environments and aerosol test chambers. In real-life environments, the biological detector will continuously be challenged with a complex and dynamic mixture of both biological and nonbiological airborne material, which should not interfere with its performance in detecting the biological threat agents of concern. Research has shown that several biological detectors do not per-

Received 16 October 2011 Accepted 22 December 2011
Published ahead of print 13 January 2012
Address correspondence to Janet Martha Blatny, janet-martha.blatny@ffi.no.
Supplemental material for this article may be found at http://aem.asm.org/.
Copyright © 2012, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AEM.07212-11

TABLE 1 Overview of airborne bacterial concentrations and meteorological parameters

		Mean ± SD		CFU m of air^{-3} ± SD[b]					
Sample[a]	Cloud cover	Temp (°C)	Humidity (% RH)	TSA	R2A	TSA*	R2A*	TSA†	R2A†
180510D		17.5 ± 0.4	69.1 ± 3.8	480 ± 26	517 ± 22	<	<	<	<
140610D		18.3 ± 0.3	62.9 ± 6.4	461 ± 82	454 ± 74	11 ± 0	17 ± 10	<	<
280610D		19.6 ± 0.4	69.8 ± 3.3	352 ± 69	344 ± 20	15 ± 8	7 ± 8	<	<
280610N		18.9 ± 0.5	63.3 ± 2.0	13 ± 18	30 ± 13	<	<	<	<
260710D		19.4 ± 0.5	75.6 ± 0.5	222 ± 29	289 ± 67	<	<	<	<
260710N		20.4 ± 0.3	71.6 ± 1.5	17 ± 6	30 ± 14	<	<	<	<
260710R	Overcast	20.2 ± 1.2	65.2 ± 4.4	35 ± 17	76 ± 55	22 ± 24	76 ± 63	<	30 ± 14
160810D		21.7 ± 0.5	69.5 ± 5.9	461 ± 212	276 ± 12	<	<	<	<
160810N		19.9 ± 0.2	74.7 ± 3.4	31 ± 3	30 ± 3	<	<	<	<
160810R	Overcast	19.0 ± 0.6	56.2 ± 3.3	181 ± 25	104 ± 33	20 ± 12	11 ± 10	6 ± 0	<
300810D		19.3 ± 0.3	66.3 ± 6.9	441 ± 50	444 ± 112	11 ± 10	6 ± 6	<	<
300810N		18.8 ± 0.3	61.1 ± 1.4	43 ± 20	28 ± 17	<	<	<	<
300810R	Sunny	17.7 ± 1.1	46.0 ± 3.7	161 ± 19	228 ± 124	13 ± 3	6 ± 6	<	<
130910D		19.3 ± 0.5	69.8 ± 5.0	469 ± 143	493 ± 43	65 ± 98	11 ± 6	<	<
130910N		18.3 ± 0.5	67.1 ± 1.1	6 ± 6	7 ± 3	<	<	<	<
130910R	Sunny	15.9 ± 1.7	67.4 ± 8.3	57 ± 26	137 ± 18	7 ± 8	11 ± 11	<	<
270910D		16.6 ± 0.3	62.4 ± 7.4	341 ± 46	350 ± 47	26 ± 18	7 ± 3	22 ± 11	9 ± 6
270910N		14.9 ± 0.4	47.6 ± 0.8	7 ± 8	<	<	<	<	<
270910R	Sunny	12.6 ± 1.0	47.6 ± 3.2	70 ± 35	94 ± 15	7 ± 3	9 ± 12	9 ± 8	<

[a] D, daytime station; N, nighttime station; R, daytime outdoor reference. N and R samples were not collected on the first sampling dates because the required permissions were not yet available.
[b] Airborne bacterial concentrations are reported as the averages of the triplicate cultivation plates. *, Spore-specific cultivation (aerobic); †, spore-specific cultivation (anaerobic). <, below the limit of detection (5.5 CFU m^{-3}).

form optimally when tested in operational environments (20, 49). A lot of effort has been put into improving T&E methodologies for biological detectors by establishing aerosol test chamber systems that can mimic real-life environmental backgrounds (55, 59, 74), thus enabling operational testing of biological detectors by challenging them with biological threat agents in the presence of a more realistic background. The ability to mimic real-life conditions depends on the availability of detailed information about the environments that are to be mimicked. Such information is currently very limited, and further investigation of naturally occurring airborne microorganisms in potential target environments is therefore important. Information about naturally occurring airborne microorganisms in crowded public environments may also be important from a public health perspective, since elevated levels of airborne microorganisms are considered an important factor that affects indoor air quality and have been linked to adverse human health effects due to their potential toxigenic, allergenic, and infectious nature (19).

For the present study, we performed a detailed characterization of the cultivable airborne bacterial environment at the largest underground subway station in Oslo, Norway, from May to September of 2010. Daytime, nighttime, and outdoor reference samples were collected using a high-volume open-faced electret filter-based air sampler (SASS 3100) and analyzed by cultivation to enumerate total and spore-specific airborne bacterial concentrations. Microbiological, biochemical, and molecular methods were used to taxonomically classify the obtained bacterial isolates and to investigate virulence- and survival-associated properties, such as antibiotic resistance, hemolytic activity, and pigmentation. The results described here regarding the natural concentration level, composition, and variability of airborne bacteria in a subway station increase our knowledge about potential target airborne environments that biological detectors may be subjected to and provide information about the naturally occurring biological aerosols (bioaerosols) that biological detectors should be tested against during T&E in aerosol test chambers. Also, our work generates relevant baseline data that could be used when assessing human exposure to airborne bacteria in subway environments. In addition, we describe new methodologies for air quality analysis that might be of interest and applied in public health studies and safety assessments.

MATERIALS AND METHODS

Study location. The study was conducted at the underground subway station Nationaltheatret, Oslo (ca. 600,000 inhabitants), Norway. On average one train departs every minute during the operating hours of the station, and the subway network yearly transports over 70 million people. The station is nonoperative between 1 and 5 a.m., except for maintenance activities. No heating, ventilation, and air conditioning system is installed at the station. Air samples were collected during a 5-month period, from May through September of 2010, at 2- to 3-week intervals (Table 1). Samples were collected at one location inside the subway station and at one outdoor location (reference). The indoor sampling was performed at the westbound concourse during the day (11 a.m. to 1 p.m.) and night (2 to 4 a.m.), and the outdoor reference sampling was performed during the day (9 to 11 a.m.) at a square adjacent to the stations entrance. Nighttime outdoor reference samples were not collected because of practical and security-related issues. Nighttime station samples and daytime outdoor samples were not collected on the first two and three sampling dates, respectively, since permissions to do such sampling were not available at these initial dates.

Bioaerosol collection. Air samples were collected using a high-volume air sampler, SASS 3100 (Research International, Monroe, WA), using filter-based electret capture technology. The SASS 3100 instrument offers an user-adjustable airflow of between 50 and 360 liters per min (lpm), and the particle collection efficiencies are ca. 92% for particles in

the 0.5- to 5.0-μm size range when sampling at 120 lpm and 78 to 79% for similar-sized particles at 320 lpm. The electret filter is composed of an injection-molded frame with an acoustically welded 44-mm diameter microfibrous capture disc, where each fiber has an electric field frozen into it. These fields will induce a charge in aerosols passing through the filter and provide an electret capture mechanism. The capture disc has a void volume of ca. 96% and an effective airflow velocity at the filter face of ~3.5 m per s when sampling at 320 lpm. These properties translate into substantially lower particle impact speeds and pressure drops compared to most traditional dry filter collection methods, and the employed electret filter technology should therefore offer relatively benign capture conditions for delicate microorganisms even at high airflow rates (56). The instrument was mounted on a tripod with the filter at a height of 1.5 m above the ground, facing the tracks at a 45° downward angle from the horizontal position. The downward-facing angle was selected to avoid direct deposition of large particulates (>100 μm) that originated from sources immediately above or close to the sampler, since such particulates would not represent true aerosols due to their limited residence time in air (6). The tripod was positioned in the middle of the westbound concourse about 4 m from the train tracks. The airflow was 300 lpm, and the sampling period lasted for 2 h, corresponding to a total air sample of 36 m^3. Sampled filters were placed back into their original sterile packaging, transported directly to the laboratory at room temperature, and processed within 2 h after sampling. The open-faced filter holder on the SASS 3100 instrument was disinfected with ethanol (70%) between samples to avoid cross-contamination. Field blanks were generated by mounting filters on the SASS 3100 instrument without drawing air on a few occasions and subjecting them to the same downstream procedures as the sampled filters. The collected particles were extracted from the filters into liquid using an extraction buffer (phosphate-buffered saline with 0.05% Triton X-100 [pH 7.4]) and the SASS 3010 extractor instrument (Research International) according to the manufacturer's standard instructions. The extractor instrument was disinfected with hydrogen peroxide (35%) or sodium hypochlorite solution (5,000 ppm free available chlorine), followed by multiple flushes with the extraction buffer, as per the manufacturer's recommendations, to avoid cross-contamination.

Particle and meteorological data collection. Particle concentrations and size distribution data were measured with an optical particle counter (Aerotrak 8220; TSI, Shoreview, MN) mounted on a tripod, with the inlet pointing in the vertical direction at the same height as the SASS 3100. The instrument was equipped with an external temperature and humidity probe. Particle data were binned into size intervals corresponding to 0.5 to 1.0 μm, 1.0 to 2.0 μm, 2.0 to 3.0 μm, 3.0 to 4.0 μm, 4.0 to 5.0 μm, and >5.0 μm. Particle and meteorological data were collected simultaneously with the bioaerosol sampling and averaged over the entire 2-h sampling period.

Bacterial cultivation. To enumerate total airborne cultivable bacteria, filter extracts (100 μl) were plated in triplicate using a standard spread plate method onto Trypticase soy agar (TSA; Merck, Darmstadt, Germany) and Reasoner's 2a (R2A) (Oxoid, Cambridge, United Kingdom) plates, supplemented with 100 μg of cycloheximide (Sigma-Aldrich, St. Louis, MO) ml^{-1} to avoid fungal growth (60). Incubation was performed at 30°C for 48 h before colony counting. To exclusively enumerate cultivable anaerobic and aerobic bacterial spores, filter extracts were heat shocked (75°C, 20 min) and then plated out and incubated as for vegetative bacteria (5). Anaerobic cultivation jars, AnaeroGen packs, and anaerobic indicators (Oxoid) were used to generate and verify anaerobic growth conditions. Airborne cultivable bacterial concentrations are presented as the average of the triplicate cultivation plates and expressed as CFU per cubic meter of air (CFU m^{-3}) $\pm$ the standard deviation. The limit of detection (LOD) was calculated to be 5.5 CFU m^{-3} for the cultivation assay used, corresponding to the observation of at least one CFU on each of the triplicate plates. A representative selection of morphologically distinct colonies was isolated from the primary cultivation plates to obtain pure isolates for further characterization. Selected colonies were transferred to new TSA plates and incubated at 30°C for 48 h. The process was repeated until pure isolates were obtained. Long-time storage was done at −80°C in brain heart infusion broth (Oxoid) supplemented with 18% glycerol (Merck).

Partial 16S rRNA gene sequencing. A single colony from each isolate was transferred to a polypropylene tube (1.5 ml; Axygen, Union City, CA) filled with PCR-grade H_2O (100 μl; Ambion, Austin, TX) and submitted to five freeze-thaw cycles before centrifugation (10,000 $\times$ *g*, 2 min). Each cycle consisted of submersion in liquid nitrogen (1 min), followed by submersion in boiling water (1 min) and a brief vortex mixing. Real-time PCR was performed using the supernatant (2 μl) as a template in white 96-well PCR plates (Roche Diagnostics, Indianapolis, IN) on a LightCycler 480 instrument (Roche Diagnostics). Briefly, each reaction (30 μl) consisted of 2$\times$ SYBR green master mix (15 μl; Roche Diagnostics), PCR-grade H_2O (9 μl), 10 μM forward and reverse primers (2 μl each), and sample (2 μl). Universal *Bacteria* 16S rRNA gene primers were used, including the forward primer 27F (5′-GAGTTTGATCMTGGCTCAG-3′) and the reverse primers 519R (5′-GWATTACCGCGGCKGCTG-3′) (40). The PCR program consisted of an initial denaturation (95°C, 5 min), 35 cycles of denaturation (95°C, 20 s), annealing (55°C, 10 s), and extension (72°C, 90 s), and finally a terminal extension (72°C, 5 min). Negative amplification controls were included on each PCR plate to verify the absence of contaminating DNA in the PCR reagents. Standard melting-curve and gel analyses were performed to verify amplification of specific PCR products. Purification and bidirectional sequencing using the primers 27F and 519R were performed at a commercial sequencing facility (Eurofins MWG Operon, Ebersberg, Germany). The sequence trace files were trimmed, aligned, and manually checked using BioNumerics 6.0 (Applied Maths, Sint-Martens-Latem, Belgium), and only sequences with read lengths greater than 400 bp and less than 1% ambiguous base calls were approved.

16S rRNA gene-based taxonomical classification. Isolates were classified by submitting their 16S rRNA gene sequence to the Classifier and SeqMatch tools at the Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu, release 10, update 22) (75), yielding classification down to the genus level and best hits against the RDP database, respectively. In addition, a phylogenetic cluster analysis based on the 16S rRNA gene sequences was performed in BioNumerics 6.0 (Applied Maths) using the software's standard settings to allow selection of a single isolate to represent each observed environmental phylotype during further isolate characterization. An unweighted pair group method with arithmetic mean (UPGMA) algorithm was used for pairwise alignment, followed by construction and manual editing of a multiple alignment, and hierarchical clustering using a complete linkage algorithm. A 97% similarity cutoff was used to separate the isolates into operational taxonomic units (OTU) before selecting the isolate with the highest average similarity to the other isolates in the respective OTU.

MALDI-TOF MS-based taxonomical classification. The representative isolate from each 16S rRNA gene-based OTU was classified using the Biotyper 2.0 microbial identification platform (Bruker Daltonics, Bremen, Germany) coupled to the MicroFlex matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) instrument (Bruker Daltonics). Pure bacterial colonies were prepared and analyzed according to the standard direct transfer method recommended by the manufacturer. Bacterial isolates were streaked for isolation on TSA plates and incubated at 30°C for 48 h before transferring a single colony onto a MSP 96 ground steel target (Bruker Daltonics) as triplicates. The α-cyano-4-hydroxycinnamic acid (HCCA) matrix (Bruker Daltonics) was prepared in accordance with the manufacturer's recommendations and overlaid each target spot (1 μl) immediately after the bacterial smear had dried. The target was loaded into the MicroFlex MALDI-TOF MS instrument immediately after the HCCA matrix had dried. The Biotyper 2.0 system was run in automatic classification mode, and the reference database used was the Bruker Taxonomy database (v3.1.1.0, containing 3,740 library entries).

Antibiotic resistance. All isolates were assayed for resistance against various antibiotics by cultivation on TSA plates supplemented with nalidixic acid (NAL; 20 μg ml^{-1}), ampicillin (AMP; 50 μg ml^{-1}), tetracycline (TET; 10 μg ml^{-1}), streptomycin (STR; 50 μg ml^{-1}), or chloramphenicol (CHL; 25 μg ml^{-1}). The antibiotics used were supplied by Sigma-Aldrich. Antibiotic resistance was assessed qualitatively by designating isolates as being resistant or sensitive based on the presence or absence of colony growth after incubation at 30°C for 48 h.

Hemolytic activity. All isolates were initially screened for hemolytic activity by cultivation on Colombia agar plates supplemented with 5% sheep blood (Oxoid) at 30°C for 24 to 48 h. Isolates classified into the bacterial genus *Bacillus* that showed hemolytic activity were subsequently analyzed further to investigate virulence-associated factors. The hemolytic *Bacillus* spp. isolates were streaked out on Colombia agar plates supplemented with 5% bovine blood and incubated at 37°C. The plates were incubated until the clearing zone around the colonies was fully developed (24 to 72 h). The hemolytic activity was assessed as in the initial screening, but special attention was directed toward identifying hemolysis induced by small cyclic lipopeptide toxins (SCLPT), such as the surfactin-like peptides (surfactins, pumilacidins, and lichenysins), often characterized by an opaque and slowly growing clearing zone.

Nonribosomal peptide synthetases. A universal PCR assay targeting the surfactin class of nonribosomal peptide synthetases (NRPS), developed by Tapi et al. (69), was performed to elucidate the nonribosomal peptide synthesis capabilities of the obtained hemolytic *Bacillus* spp. isolates. The PCR assay was performed according to the original publication and used the forward primer NRPS-F (5′-CGCGGMTACCGVATYGAGC-3′) and the reverse primer NRPS-R (5′-ATBCCTTTBTWDGAATGTCC GC-3′) that produced a PCR product between 419 and 431 bp for different types of NRPS within the surfactin class. The surfactin-producing strain *Bacillus mojavensis* B31 isolated from imported basil spices of unknown origin described by From et al. (24) was used as a positive control. A specific PCR assay for the cereulide synthase gene (*ces*), the NRPS that produces cereulide (*B. cereus* emetic toxin), developed by Fricker et al. (21), was also performed on the obtained hemolytic *Bacillus* spp. isolates. The PCR assay was performed according to the original publication and used the forward primer *ces*F (5′-GGTGACACATTATCATATAAGGTG-3′) and the reverse primer *ces*R (5′-ATBCCTTTBTWDGAATGTCCGC-3′) that produced a PCR product of 1,271 bp. DNA from a *ces*-positive strain (NVH 0137/09) and a *ces*-negative strain (NVH 1230/88), both isolated from commercial food products at the Norwegian School of Veterinary Science, was used as a positive and a negative control, respectively. Both PCR assays (NRPS and *ces*) were performed in a PTC-100 Peltier thermal cycler (MJ Research, Waltham, MA). Each reaction consisted of template DNA (1 μl), DyNAzyme II DNA polymerase/deoxynucleoside triphosphate mix (Finnzymes, Espoo, Finland), and a final primer concentration of 1 μM.

Phylogenetic clustering of hemolytic *Bacillus* spp. isolates. The phylogenetic relationship between the hemolytic *Bacillus* spp. isolates obtained in the present study and closely related *Bacillus* spp., including species known to harbor NRPS-produced small cyclic peptides (SCPs), was investigated by hierarchical clustering based on their 16S rRNA gene sequences. Type strains of *Bacillus* spp. that were closely related to isolates obtained here were identified from the RDP SeqMatch results, and their 16S rRNA gene sequences were downloaded from the RDP database. To avoid large dendrograms with many closely related environmental isolates, Bionumerics 6.0 (Applied Maths) was used to cluster the hemolytic *Bacillus* spp. isolates obtained here into OTUs and select a single representative isolate from each OTU. The alignment and clustering methods were the same as those described for selecting OTU representatives from all of the obtained bacterial isolates, except that a 99% similarity cutoff was used. The partial 16S rRNA gene sequences from the OTU representative hemolytic *Bacillus* spp. isolates were then aligned with the RDP-derived full-length 16S rRNA gene sequences from closely related *Bacillus* spp. using the same alignment and clustering method, but without using any similarity cutoff. The RDP-derived 16S rRNA gene sequence from a type strain of *B. megaterium* (IAM 13418) and the partial 16S rRNA gene sequence from a closely related nonhemolytic isolate obtained here were included in the analysis. The RDP-derived 16S rRNA gene sequence from a type strain of *E. coli* (ATCC 11775T) was included in the analysis as an outgroup.

Cereulide and surfactin-like peptides. A high-performance liquid chromatography-mass spectrometry assay targeting cereulide was performed on the hemolytic *Bacillus* spp. isolates that were positive for NRPS, as described elsewhere (31). Surfactin-like peptides, such as surfactins, pumilacidins, and lichenysins, were detected by a liquid chromatography mass spectrometry (LC-MS) assay, as described by From et al. (23), with minor modifications. Briefly, the hemolytic *Bacillus* spp. isolates positive for NRPS were grown on Colombia agar plates supplemented with 5% bovine blood at room temperature (~22°C) for 72 h. Three colonies were collected and resuspended in ultrapure (18.2 mΩ cm^1) H_2O (500 μl), vortexed (10 s) and transferred to glass vials (10 ml). Acetone (3 ml) and chloroform (4 ml) were added, and the mixture was vigorously shaken (10 s) and centrifuged (1,600 × *g*, 3 min). The organic phase was transferred to another glass vial and evaporated to dryness at 60°C under a stream of air. The dry residue was dissolved in methanol (100 μl), followed by the addition of methanol-water (60:40, 300 μl), and mixed. The mixture was centrifuged (1,600 × *g*, 3 min), and the organic phase was recovered and centrifuged (5,600 × *g*, 2 min) through a Costar Spin-X centrifuge filter (0.22-μm-pore-size nylon; Corning, Corning, NY). Aliquots (25 μl) were injected into the LC-MS apparatus at intervals of 7 min. A Zorbax SB-C_{18} Rapid Resolution HT analytical column (2.1 mm by 5 mm 1.8 μm; Agilent Technologies, Santa Clara, CA) was used and operated at a constant temperature of 30°C. The mobile phase was a mixture of methanol-water (92:8) containing 0.1% formic acid. The pump was operated isocratically at a constant flow rate (300 μl min^{-1}). The LC-MS instrumentation consisted of a Series 200 quaternary pump and autosampler (Perkin-Elmer, Foster City, CA) and an API 2000 MS system (Applied Biosystems, Foster City, CA) equipped with a Turbo-Ion-Spray source operated in ESI-positive mode. The turbo probe vaporizer temperature of the interface was fixed at 400°C. Surfactin-like peptides were elucidated in the *m/z* range from 1,008 through 1,076.

Pigmentation. All OTU representative isolates showing visible colony pigmentation were selected for further pigment characterization by LC-MS. Isolates were cultured on TSA plates and incubated at 30°C for 48 h. Using a standard laboratory scale, ~200 mg of biomass was scraped using a loop into aluminum foil-wrapped polypropylene tubes (1.5 ml; Axygen). The biomass was washed (1 ml) and resuspended (500 μl) in phosphate-buffered saline before the addition of Ready-Lyse lysozyme (EpiCentre, Madison, WI) to a final concentration of 10 U μl^{-1}, followed by incubation at room temperature for 10 min. The biomass was pelleted (10,000 × *g*, 5 min) and lyophilized to facilitate extraction in organic solvents. Organic extraction of pigments was performed with methanol (200 μl) on a shaker plate at room temperature for 1 h. After centrifugation (10,000 × *g*, 5 min), the supernatant was transferred into amber glass vials and stored at −80°C. LC-MS analysis was performed on an Agilent TOF mass spectrometer coupled to an Agilent 1100 series LC system equipped with a diode array detector recording the UV/VIS spectra between 200 and 650 nm. Mobile phases were methanol-water (50:50) in channel A and dichloromethane-methanol-water (45:50:5) in channel B. A Zorbax RP C_8 4.6-by-150-mm column was used, the flow was kept at 800 μl min^{-1}, and 20 μl of the extract was injected for each run. It was assumed that most of the pigments belong to the chemical group of carotenoids and, hence, the chromatographic conditions were optimized using astaxanthin (Sigma-Aldrich) and β-carotene (Sigma-Aldrich) as representative external standards. The hydrophilic initial elution conditions should also retain more hydrophilic pigments, if present in the extract. The following gradient was used: 0% B for 0 to 2 min, linear gradient to 100% B after 10 min, and then 100% B for additional 5 min. The data acquisition was also performed during the 6 min re-equilibration period.

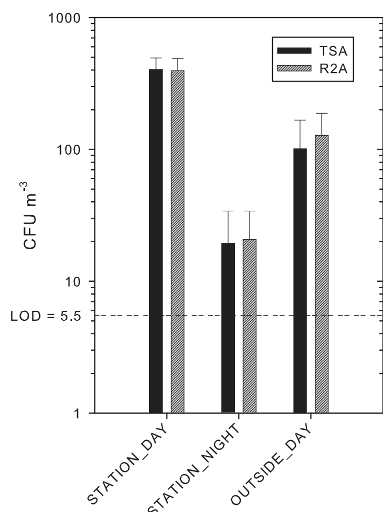

FIG 1 Average total cultivable airborne bacterial concentration levels on TSA and R2A from the three environments studied: daytime station ($n = 8$), nighttime station ($n = 6$), and daytime outdoor reference ($n = 5$). Standard deviations are indicated by error bars.

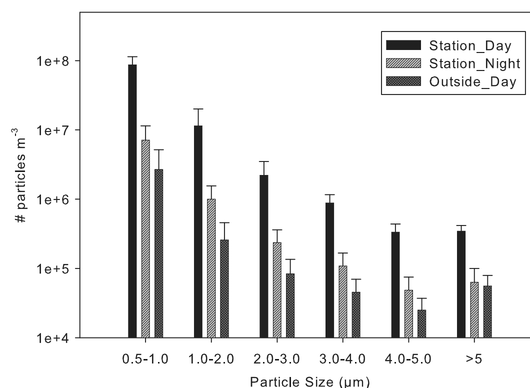

FIG 2 Average airborne particle concentrations and size distributions from the three environments studied: daytime station ($n = 8$), nighttime station ($n = 6$), and daytime outdoor reference ($n = 5$). Standard deviations are indicated by error bars.

Analytes were ionized using atmospheric pressure chemical ionization with following settings: 325°C dry temperature, 350°C vaporizer temperature, 50-lb/in^2 nebulizer pressure, and 5.0 liters min^{-1} dry gas. Reference solution for correction of mass axes on the mass spectrometer was continuously added after column chromatography to the mobile phase using a T-fitting. Tentative identification of pigments was performed by inspection of the diode array chromatograms and corresponding total ion chromatograms. The UV/VIS peaks were assigned peak maximum values, and the corresponding mass spectra were evaluated for correlating m/z peaks (i.e., extracted ion chromatogram with the same profile as the extracted wavelength chromatogram). Determined accurate mass and absorption peak maximum values were used to search the Dictionary of Natural Products (version 19.2) database (http://dnp.chemnetbase.com).

Statistical analysis. Airborne cultivable bacterial concentrations and particle data were found to be far from well modeled by a normal distribution based on a normality plot and the Lillefors test (43). The nonparametric Mann-Whitney U test (47) was therefore used to test for differences in the concentration of airborne bacteria between the environments and the cultivation media and also to test for differences in the total particle concentrations between environments. Particle size distribution data were analyzed using the Pearson product-moment correlation coefficient (52) to determine the correlation among the various particle size bins within and between the environments. Pearson product-moment correlation was also used to investigate the correlation between the total particle concentrations and total airborne bacterial concentrations independent of sampling time and location. Differences in the observed bacterial diversity between environments and cultivation media were tested using the Pearson chi-square test (53). The significance level was set at $P < 0.05$ for all statistical tests.

RESULTS

Airborne bacterial concentration. Total and spore-specific airborne bacterial concentrations were determined on TSA and R2A growth media for each individual sample (Table 1). The average concentrations on TSA were 403 ± 91, 19 ± 15, and 101 ± 66 CFU m^{-3} for daytime and nighttime sampling at the station and daytime outdoor reference sampling, respectively (Fig. 1). The average concentrations on R2A were 396 ± 93, 21 ± 13, and 128 ± 60 CFU m^{-3} for daytime and nighttime sampling at the station and daytime outdoor reference sampling, respectively (Fig. 1). The average aerobic spore concentrations on TSA were 17 ± 21 and 14 ± 7 CFU m^{-3}, for daytime sampling at the station and daytime outdoor reference sampling, respectively. All average anaerobic spore concentrations and aerobic spore concentrations for the nighttime sampling at the station were below the LOD (5.5 CFU m^{-3}) on TSA and are therefore not reported. The average aerobic spore concentrations on R2A were 7 ± 5 and 23 ± 30 CFU m^{-3} for daytime sampling at the station and daytime outdoor reference sampling, respectively. Except for the average daytime outdoor concentration of 6 ± 13 CFU m^{-3}, all average anaerobic spore concentrations and the aerobic spore concentration for the nighttime station samples were below the LOD (5.5 CFU m^{-3}) on R2A and are therefore not reported. The obtained results showed that daytime concentrations at the subway station were 20- and 3.5-fold higher ($P < 0.05$) than the nighttime concentrations at the station and the daytime outdoor reference concentrations, respectively. The aerobic spore fraction (spore specific to total cultivation ratio) was 4.7-fold higher ($P < 0.05$) at the outdoor reference compared to the subway station, accounting for ca. 16% of the total concentration of cultivable airborne bacteria at the outdoor location and ca. 3.4% at the station. The two growth media (TSA and R2A) did not show significant differences ($P > 0.586$) in total cultivable bacterial concentrations. No bacterial growth was observed in any of the field blanks during this study.

Particle concentration and size distribution. Particle concentrations and size distributions were stable within each environment throughout the study, and the relative differences between the sampled environments were consistently observed (Fig. 2). The average cumulative particle concentrations over the entire measured size range showed that daytime station samples had 11.9-fold higher ($P < 0.05$) particle level than the nighttime station samples and that the nighttime station samples had 2.8-fold higher ($P < 0.05$) particle level than the daytime outdoor reference samples. The particle size distribution profiles showed a de-

crease in particle concentrations related to increased particle size, and this observation was conserved between all samples and environments ($r = 0.995$, $P < 0.05$), with the smallest size bin (0.5 to 1.0 μm) accounting for >80% of the total particle counts. The average count median diameters were similar between the environments, 0.86 ± 0.01, 0.88 ± 0.03, and 0.88 ± 0.02 μm, while the average mass median diameters varied, 3.79 ± 0.37, 4.71 ± 0.50, and 5.80 ± 0.21 μm, for the daytime station, nighttime station, and daytime outdoor samples, respectively. The total particle concentrations and total cultivable airborne bacterial concentrations showed a significant correlation when calculated independent of time and location ($r = 0.896$, $P < 0.05$), indicating that the level of airborne bacteria was related to the total particle concentration.

Meteorological data. Temperature and relative humidity (RH) are reported as an average of a 2-h sampling period for each individual sample (Table 1). Average temperatures of 19.3 ± 0.4, 19.3 ± 0.4, and 18.2 ± 1.2°C were observed for the daytime station, nighttime station, and the daytime outdoor samples, respectively. The average RH values were 69.9% ± 4.9%, 67.6% ± 1.9%, and 58.7% ± 4.9% for the daytime station, nighttime station, and daytime outdoor samples, respectively.

Airborne bacterial diversity. A total of 429 bacterial colonies were isolated from TSA and R2A primary cultivation plates. High-quality partial 16S rRNA gene sequences were obtained from 94% (291/308) and 92% (111/121) of the isolates from total and spore-specific cultivations, respectively. Thus, 402 bacterial isolates were successfully characterized, while the remaining 27 were discarded due to no growth after freeze storage or inability to yield 16S rRNA gene sequence data with >400 acceptable base calls and <1% ambiguities. Isolates were distributed between three major phyla, *Actinobacteria* (48%), *Firmicutes* (43%), and *Proteobacteria* (9%) from total cultivation and *Actinobacteria* (3%), *Firmicutes* (96%), and *Proteobacteria* (1%) from spore-specific cultivation, respectively. A total of 37 bacterial genera were observed (Table 2), with the majority belonging to the genera *Micrococcus* (32%), *Staphylococcus* (20%), *Bacillus* (18%), *Pseudomonas* (4%), *Microbacterium* (3%), and *Streptomyces* (3%) from total cultivation and *Bacillus* (68%), *Staphylococcus* (15%), and *Paenibacillus* (9%) from spore-specific cultivation. More than 80% of the isolates originating from spore-specific cultivations were classified into the bacterial genus *Bacillus* or the closely related genera *Paenibacillus*, *Viridibacillus*, *Tumebacillus*, *Brevibacillus*, and *Lysinibacillus*, consistent with their spore-forming capabilities, but also into the genus *Staphylococcus* (15%), which is not consistent with spore-forming capabilities. Although there was no significant difference in the distribution of bacterial genera at the subway station and the outside reference environment ($P = 0.19$), *Bacillus* spp. were more frequently observed at the outside reference location than at the station, accounting for 28 and 17% of the total cultivation isolates, respectively. The opposite was observed for *Micrococcus* spp. and *Streptomyces* spp., which accounted for 36 and 3% of the total cultivation isolates at the station and 23 and 0% at the outside reference location, respectively. There was a significant difference between the daytime and nighttime distributions of bacterial genera at the station ($P < 0.05$). *Bacillus* and *Micrococcus* spp. were observed more frequently during the daytime, where they accounted for 17 and 36% of the isolates from daytime samples and 5 and 27% from nighttime samples, respectively, whereas *Staphylococcus* spp. were found to account for 25% of the isolates at night and 19% during the day. Several bacterial genera were uniquely present, although in low abundance, at nighttime compared to daytime, such as the genera *Corynebacterium*, *Erwinia*, *Gordonia*, *Rothia*, and *Serratia*. A significant difference in the genus distribution was observed between the two culture media that we used ($P < 0.05$). *Micrococcus*, *Pseudomonas*, and *Staphylococcus* spp. were recovered more frequently from TSA than R2A, accounting for 40, 6, and 23% of the isolates on TSA and 24, 2, and 16% on R2A, respectively. *Dermacoccus* and *Rhodococcus* spp. were recovered only from R2A, where each genus accounted for 4% of the isolates.

Biotyper 2.0 taxonomical classification. Using the MALDI-TOF MS-based Biotyper 2.0 microbial identification system to classify the OTU representative isolates ($n = 84$) resulted in 40% species-consistent and 60% genus-consistent results compared to the 16S rRNA gene-based classifications (see Table S1 in the supplemental material). Only one isolate failed to generate an approvable mass spectrum, whereas 36% of the isolates yielded only low-scoring classification results, i.e., score values below 1.7. When considering the 64% ($n = 54$) of the isolates with score values above 1.7, 61% of the isolates were species-consistently classified and a total of 94% were genus-consistently classified, respectively. Of the 6% of isolates ($n = 3$) with score values above 1.7 that did not show genus-consistent classification results, the bacterial genera ($n = 2$) or species ($n = 1$) given by the 16S rRNA gene-based classification were missing from the Bruker Taxonomy database. Of the total number of isolates that were analyzed ($n = 84$), seven genera and 24 species given by the 16S rRNA gene-based classification were missing from the Bruker Taxonomy database.

Antibiotic resistance. About 27% of the total cultivation isolates ($n = 291$) showed antibiotic resistance against at least two of the antibiotics tested, whereas 10% did not show resistance against any of the antibiotics tested. Resistance against three, four, and all of the antibiotics used was seen in 12, 1, and 1 of the isolates, respectively. The distribution of resistance in the total cultivation isolates were as follows: 27% AMP, 9% STR, 75% NAL, 4% TET, and 5% CHL. About 32% of the spore-specific cultivation isolates ($n = 111$) showed antibiotic resistance against at least two of the antibiotics tested, whereas 22% did not show resistance against any of the antibiotics tested. Resistance against 3, 4, and all of the antibiotics used was seen in 3, 1, and none of the isolates, respectively. The distribution of resistance in the spore-specific cultivation isolates was as follows: 44% AMP, 19% STR, 37% NAL, 2% TET, and 14% CHL, showing that these isolates had less frequent resistance to NAL and TET and more frequent resistance against AMP, STR, and CHL than total cultivation isolates. The majority of isolates from the dominant genera had frequent resistance against NAL (Table 3), except isolates of *Bacillus* (14%) and *Paenibacillus* (0%) spp. *Arthrobacter* and *Kocuria* spp. showed no resistance, while *Micrococcus* spp. had a very low frequency of resistance (1 to 6%), against all of the antibiotics that were tested except NAL. *Paenibacillus* spp. isolates were the only isolates to show frequent (57%) resistance against STR. *Paenibacillus* spp. isolates also had a very low frequency of resistance against all of the other antibiotics that were tested. Very few isolates displayed resistance against TET and CHL, except isolates of *Pseudomonas*, *Microbacterium*, and *Rhodococcus* spp., which still had a relatively low frequency of resistance (10 to 20%). No differences in the distribution of antibiotic resistance were observed between the environments, except that isolates derived from spore-specific

TABLE 2 Genus distribution of the obtained bacterial isolates

	Total cultivation								Spore-specific cultivation[a]							
	Station				Outside (day)		Total		Station				Outside (day)		Total	
	Day		Night						Day		Night					
Genus	n^b	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Arthrobacter	1	<1	2	5	2	4	5	2								
Bacillus	33	17	2	5	16	28	51	18	44	73	1	50	30	61	75	68
Brevibacillus													1	2	1	<1
Brevundimonas	2	1					2	<1								
Cellulosimicrobium					1	2	1	<1								
Comamonas	1	<1					1	<1								
Corynebacterium			2	5			2	<1								
Curtobacterium					1	2	1	<1								
Dermacoccus	5	3			1	2	6	2								
Dietzia	1	<1					1	<1								
Enhydrobacter	1	<1					1	<1								
Erwinia			1	2			1	<1								
Exiguobacterium	1	<1					1	<1								
Gordonia			1	2			1	<1								
Janibacter	1	<1					1	<1								
Kocuria	3	2	1	2			4	1	1	2					1	<1
Lysinibacillus	1	<1			1	2	2	<1					1	2	1	<1
Microbacterium	6	3	2	5	1	2	9	3								
Micrococcus	69	36	12	27	13	23	94	32								
Paenibacillus	3	2	1	2	3	5	7	2	4	7			6	12	10	9
Paenisporosarcina	1	<1					1	<1	1	2					1	<1
Pantoea	1	<1	1	2			2	<1								
Paracoccus	3	2					3	1								
Planococcus	2	1					2	<1								
Plantibacter					1	2	1	<1								
Pseudomonas	9	5	1	2	3	5	13	4								
Rhodococcus	1	<1	3	7	1	2	5	2								
Roseomonas	1	<1			1	2	2	<1								
Rothia			1	2			1	<1	1	2			1	2	2	2
Serratia			1	2			1	<1								
Sphingomonas									1	2					1	<1
Sporosarcina					1	2	1	<1								
Staphylococcus	37	19	11	25	10	18	58	20	6	10	1	50	10	20	17	15
Streptomyces	6	3	2	5			8	3								
Tumebacillus									1	2					1	<1
Viridibacillus					1	2	1	<1	1	2					1	<1
Weissella	1	<1					1	<1								
Sum	190		44		57		291		60		2		49		111	

[a] Some isolates obtained from the spore-specific cultivations were from bacterial genera not known to harbor spore-forming members, such as *Staphylococcus*.
[b] n, number of isolates.

cultivation had a higher frequency of CHL resistance at the station (17%) compared to the outdoor reference location (8%).

Hemolytic activity. About 22% (87/402) of the bacterial isolates showed hemolysis in the initial screening on sheep blood agar plates. These were distributed between the bacterial genera *Bacillus* (87%), *Staphylococcus* (10%), *Erwinia* (1%), and *Pseudomonas* (1%). Of the total number of *Bacillus* spp. isolates obtained (n = 125), 61% were shown to be hemolytic in the initial screening on sheep blood agar plates. All isolates showed beta-hemolysis, except two that showed alpha-hemolysis. All of the hemolytic *Bacillus* isolates (n = 76) were further investigated to elucidate hemolytic and virulence-associated properties (see Table S2 in the supplemental material). In the subsequent analysis performed on bovine blood agar plates, 87% of the isolates from the initial screen on sheep blood were hemolytic. The two isolates that induced alpha-hemolysis on sheep blood were nonhemolytic on bovine blood. Phylogenetic clustering of the hemolytic *Bacillus* spp. isolates and type strains of closely related *Bacillus* spp. showed that the obtained isolates clustered into at least three distinct groups (see Fig. S1 in the supplemental material). Each group harbored known producers of SCPs, including *B. cereus* (cereulide), *B. pumilus* (pumilacidin), and *B. subtilis* (surfactin). A total of 30% of the isolates showing hemolysis on bovine blood displayed putative SCLPT-induced hemolysis, characterized by an opaque and slow-growing clearing zone. These isolates were limited to the *B. pumilus* and *B. subtilis* groups, except for one isolate that was closely related to *B. mycoides* and *B. weihenstephanensis* in the *B. cereus* group. A total of 35% of all of the *Bacillus* spp. isolates

TABLE 3 Distribution of antibiotic resistance in the dominant bacterial genera

	Total cultivation										Spore-specific cultivation[a]									
	NAL		AMP		STR		CHL		TET		NAL		AMP		STR		CHL		TET	
Genus	%	*n*[b]	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*
Arthrobacter	100	5/5	0	0/5	0	0/5	0	0/5	0	0/5										
Bacillus	14	7/51	61	31/51	16	8/51	8	4/51	2	1/51	20	15/75	40	30/75	13	10/75	17	13/75	3	2/75
Dermacoccus	100	6/6	0	0/6	17	1/6	0	0/6	0	0/6										
Kocuria	100	4/4	0	0/4	0	0/4	0	0/4	0	0/4										
Microbacterium	100	9/9	0	0/9	0	0/9	11	1/9	11	1/9										
Micrococcus	98	92/94	4	4/94	6	6/94	2	2/94	1	1/94										
Paenibacillus	0	0/7	0	0/7	57	4/7	0	0/7	0	0/7	20	2/10	20	2/10	90	9/10	20	2/10	0	0/10
Pseudomonas	54	7/13	31	4/13	8	1/13	23	3/13	15	2/13										
Rhodococcus	100	5/5	40	2/5	0	0/5	0	0/5	20	1/5										
Staphylococcus	93	54/58	31	18/58	10	6/58	5	3/58	2	1/58	100	17/17	88	15/17	6	1/17	0	0/17	0	0/17
Streptomyces	100	8/8	38	3/8	0	0/8	0	0/8	0	0/8										

[a] Some isolates obtained from the spore-specific cultivations were from bacterial genera not known to harbor spore-forming members, such as *Staphylococcus*.
[b] *n*, number of resistant isolates/total number of isolates.

obtained and 60% of the *Bacillus* spp. isolates that were hemolytic on bovine blood,were positive for NRPS. NRPS-positive isolates were distributed between all three identified *Bacillus* groups but, interestingly, all hemolytic isolates showing high similarity to *B. pumilus* ($n = 5$) were NRPS positive. The presence of cereulide synthase (*ces*) or cereulide were not detected in any of the hemolytic *Bacillus* spp. isolates analyzed here. The LC-MS analysis performed on all NRPS- positive isolates, except for one isolate that was discarded due to slow growth, revealed that 77% of the isolates produced SCLPTs. This corresponded to 50% of the *Bacillus* isolates that were hemolytic on bovine blood and 26% of the total number of *Bacillus* isolates obtained in the present study. Isolates positive for SCLPTs were limited to the *B. pumilus* and *B. subtilis* groups, except for three isolates that were closely related to *B. thuringiensis* ($n = 2$) and *B. cereus* ($n = 1$) in the *B. cereus* group. More than 60% of the SCLPT-positive isolates were classified as *B. altitudinis* and *B. stratosphericus* in the *B. pumilus* group.

Pigmentation. About 48% (141/291) of the total cultivation isolates displayed visible colony pigmentation, while 8% (9/111) of the spore-specific cultivation isolates displayed visible colony pigmentation. The observed pigmentation included many shades of yellow, orange, pink, and red, with the majority being yellow. No major differences in colony pigmentation were observed between the environments. Bacterial isolates from the station showed a 50% frequency of pigmentation, while the outdoor isolates showed ca. 40%, which could probably be attributed to the presence of more *Micrococcus* spp. and fewer *Bacillus* spp., typically yellow pigmented and nonpigmented, respectively, at the station compared to the outdoor reference. A total of 28% (24/84) of the OTU representative isolates displayed visible colony pigmentation and were further characterized by a LC-MS method optimized for detection of carotenoids (see Table S3 in the supplemental material). From these, 54% of the isolates yielded data that led to tentative pigment identification. About 29% of the isolates led to appropriate UV/VIS spectra but no corresponding ion masses that enabled determination of accurate mass, while 17% of the isolates yielded no appropriate UV/VIS spectra. No further attempts were made to identify colored compounds in these 11 extracts. Several carotenoids were observed, and although only tentatively identified, triophaxanthin, alloxanthin, amarouciaxanthin, sarcinaxanthin, cycloviolaxanthin, a diglycosylated variant of zeaxanthin, a glycosylated variant of OH-chlorobactene, and a derivative of staphyloxanthin (8-apo-caroten-8-oic acid) were observed. Sarcinaxanthin was observed in six different isolates from the bacterial phylum *Actinobacteria*, distributed between the bacterial families *Microbacteriaceae* ($n = 5$) and *Micrococcaceae* ($n = 1$).

DISCUSSION

We evaluated here the airborne bacterial community at a subway station in Norway. The concentration level, composition, and variability of airborne bacteria were investigated, along with virulence- and survival-associated bacterial properties, such as hemolytic activity, antibiotic resistance, pigmentation, and spore fraction. A methodology scheme for investigating airborne bacteria based on the SASS 3100 high-volume electret filter-based air sampler and for rapid classification of bacterial isolates using the Biotyper 2.0 MALDI-TOF MS-based microbial identification system was also demonstrated. The observed bacterial content, particle size distribution, and concentration level were stable, except for some sporadic deviations, within each environment (daytime station, nighttime station, and daytime outdoor reference) despite the seasonal variation (spring-summer-fall) throughout the study (May to September 2010). In contrast to this, several differences were seen between the sampled airborne environments, such as a higher daytime concentration level of airborne bacteria at the subway station compared to the outdoor location and a higher concentration level of airborne bacteria at the subway station during the day than at night. These results, combined with observed differences in the bacterial diversity between the environments, suggested that anthropogenic sources, such as passengers and train traffic, were major contributors to airborne bacteria at the Norwegian subway station.

This study used a SASS 3100 high-volume electret filter-based air sampler, and comparable airborne bacterial concentration levels to previous studies in subway environments were obtained (34, 38, 62), suggesting that this instrument may be suitable for sampling of airborne bacteria for cultivation analysis. Preliminary results from another sampling campaign performed by the Norwegian Defense Research Establishment at the same subway station (unpublished data) show that the SASS 3100 provides airborne bacterial concentration level estimates comparable to those obtained with the MAS-100 (high-volume impactor) and Andersen

sampler (six-stage cascade impactor). Future harmonization or standardization of the sampling, processing, and analysis methodologies (e.g., air sampling equipment and cultivation conditions) used in bioaerosol characterization studies of indoor/outdoor environments is needed to facilitate improved interpretation and comparison of the obtained results.

The results reported here may aid in improving the development of biological detection/surveillance equipment and enable T&E schemes that can be used to evaluate biological detectors' operational performance by simulating complex real-life environments in controlled aerosol test chambers during challenge tests with biological threat agents. Such T&E schemes could improve the performance criteria of biological detection/surveillance equipment to meet the users' requirements to such equipment with respect to reliable sensitive and specific real-time monitoring of biological threat agents in different operational environments. Furthermore, the present report may provide public health authorities with baseline data for airborne bacteria, which can be used when assessing human exposure limits or evaluating other aspects of air quality, in subways or other enclosed environments where people may be confined.

Airborne bacterial concentration. Bioaerosol surveys investigating airborne bacteria have been performed at subway stations in various countries, including the United States (7), Japan (35, 62), Korea (34, 38, 41), China (18), Russia (8), Egypt (4), the United Kingdom (27), and Hungary (66, 67), but have not previously been carried out in Norway. The airborne bacterial concentrations reported in these studies ranged from not detected to 10^4 CFU m^{-3}, and, in general, the content of airborne bacteria was higher in subway stations compared to adjacent outdoor air and also higher at stations located deeper underground than in more shallow stations (34). Due to the lack of standardized equipment, analytical methods, and variable goals of the different studies, several different air sampling instruments and cultivation conditions have been used in the published studies. Thus, the comparison of the results is challenging since the various methodologies have been shown to provide different results even when subjected to the same bioaerosol challenge (3, 42, 64, 76). The daytime concentration of airborne cultivable bacteria ($\sim$400 CFU m^{-3}) found at the subway station in Oslo was within the range of previous reports from Korea and Japan (34, 38, 62) but 10- to 100-fold lower than that found in China and Egypt (4, 18). This discrepancy could be due to the use of different air sampling and cultivation methods, geographical differences in the atmospheric concentration of airborne bacteria, and physical differences between the subway stations, such as their size, layout, type of ventilation system, and not least the number of passengers. The daytime concentration of airborne bacteria at the station was higher (3.5-fold) than in adjacent outdoor air, a finding consistent with previous reports (34). The increased daytime concentration observed at the station compared to outdoor air could suggest that anthropogenic sources are major contributors to airborne bacteria in the subway station since these sources were more abundant at the station compared to the outdoor location. The observation that the nighttime airborne bacterial concentration at the station was lower than the station (20-fold) and outdoor (5.7-fold) daytime concentrations further strengthens this hypothesis since anthropogenic sources were nearly absent from the station during the night. Bacterial spores are considered the most resistant form of bacteria, and spores of *B. anthracis* (the causative agent of anthrax) are on the Centers for Disease Control and Prevention's Category A list of biological threat agents (12). However, little information is available about the amount of airborne bacterial spores in the subway environment. To our knowledge, the study by Awad (4) in Egypt is the only previous report addressing the airborne bacterial spore content in the subway environment. Our results showed that the bacterial spore fraction was higher (4.7-fold) in outdoor air than at the subway station, which is consistent with the previous report from Egypt that also found a higher spore fraction in outdoor air (4). These observations are probably due to exposure to solar/UV-radiation in the outdoor environment that primarily inactivates vegetative bacteria or to differences in the composition of bacterial sources between the subway station and the outside environment. The daytime concentration of airborne bacterial spores that we observed at the subway station, about 12 CFU m^{-3}, is 100-fold lower than that noted in the study from Egypt (4), thus indicating that only low airborne concentrations of bacterial spores were present at the Norwegian subway station. The LOD for the cultivation assay used here was 5.5 CFU m^{-3}, and many of the spore-specific cultivations fell below this threshold.

Airborne bacterial diversity. The presence and activities of anthropogenic sources might be linked to increased concentrations of airborne bacteria in subway stations, which is consistent with the observation that these environments frequently are dominated by members of the bacterial genera *Micrococcus*, *Bacillus* and *Staphylococcus*, all of which contain several species representatives associated with an anthropogenic origin (4, 62). The dominant bacterial genera observed in all sampled environments in the present study were *Micrococcus*, *Bacillus*, and *Staphylococcus*, which is consistent with previous cultivation studies in the subway environment (38). *Micrococcus* and *Bacillus* spp., which are regularly found on human skin (39), were observed at higher frequencies at the subway station during the day than at night, and *Micrococcus* spp. were observed at higher frequencies at the subway station than in outdoor air, thus further strengthening the hypothesis that anthropogenic sources are a major contributor to airborne bacteria in subway environments. *Bacillus* spp. were observed more frequently in outdoor air than at the station, suggesting local source differences or specific inactivation of non-spore-forming bacteria in the outdoor atmosphere. Several bacterial genera, such as *Corynebacterium*, *Erwinia*, *Gordonia*, *Rothia*, and *Serratia*, were exclusively recovered from the station at nighttime but might still be present at the station during daytime even if they were not recovered. A plausible explanation for this might be that the higher cultivable airborne bacterial concentration observed at the station during daytime and also the higher frequency of *Bacillus* spp. and *Micrococcus* spp. could bias the isolation process into missing genera present only in very low abundances. Our results also showed differences in the airborne bacterial diversity obtained with the two cultivation media (TSA and R2A), thus highlighting the importance of using multiple growth media to reveal a more complete diversity estimate and the challenges associated with comparing results obtained with different growth media. Our spore-specific cultivation assay used a heat-shock procedure to inactivate vegetative bacterial cells and activate bacterial spores, which have been commonly applied to determine the content of bacterial spores in food products and soil (28). As expected, most isolates originating from spore-specific cultivations were classified into the bacterial genus *Bacillus* or the closely related genera such as *Paenibacillus*, *Viridibacillus*, *Tumebacillus*, *Brevibacillus*, and

Lysinibacillus, consistent with their spore-forming capabilities, but also into the genus *Staphylococcus* (15%), which is not consistent with spore-forming capabilities. This discrepancy might indicate that the obtained *Staphylococcus* spp. isolates were heat resistant. Even though an extensive literature survey was performed by the authors, no reports were found that have previously described the finding of *Staphylococcus* spp. that were heat resistant to the heat-shocking procedure used here (75°C, 20 min). However, ongoing work at the Norwegian Defense Research Establishment has provided similar observations of heat resistance in *Staphylococcus* spp. found in wastewater treatment plants (unpublished data). Alternatively, the bacterial cells might have been protected from the treatment if they were imbedded in larger airborne particles, such as cell aggregates, biofilms, skin flakes, or other composite matrices, as have been shown for other injuring stresses such as solar/UV radiation and desiccation (46, 71). It is generally acknowledged that spore-forming bacteria, such as *Bacillus* spp., are present mainly as spores in the environment (65). This is supported by the high amount of *Bacillus* spp. that were derived from spore-specific cultivations in the present study, although we cannot exclude the possibility of *Bacillus* spp. being present as both spores and vegetative cells in the airborne environment. Direct comparison of the number of *Bacillus* spp. isolates derived from the total and spore-specific cultivations, which could have been used to elucidate whether *Bacillus* spp. were exclusively present as spores, was not appropriate on the basis of some important cultivation and isolation differences between the total and spore-specific cultivations. All colonies from spore-specific cultivation plates were isolated, whereas only a representative selection was isolated from total cultivation plates. In addition, the germination efficiencies could be different for bacterial spores that were activated by heat shocking prior to cultivation and those that were not (16, 37).

Particle concentration and size distribution. The particle concentration and size distribution obtained at the subway station were comparable to those reported by Birenzvige et al. from the Washington, DC, subway (7). Over the entire measured size range, increased particle concentrations were seen at the station during daytime compared to nighttime, and this is probably related to anthropogenic daytime sources that were nearly absent at night, such as passengers and train traffic. The total particle concentrations and total cultivable airborne bacterial concentrations showed significant correlation ($r = 0.896$). This indicated that the level of airborne bacteria was related to the total particle concentration, but the ratio (total particles to cultivable bacteria) was as high as 10^6 airborne particles per CFU detected when considering the entire particle size range measured. This is in agreement with the study from Washington, DC, which showed that fluorescent airborne particles accounted for <1% of the total particle counts (7), which would translate into a total particle to total cultivable bacteria ratio much higher than 10^2 airborne particles per CFU detected.

Biotyper 2.0 MALDI-TOF MS-based classification. We focused here on rapid screening of bacterial isolates obtained from airborne bacteria at a subway station using the Biotyper 2.0 microbial identification system, and thus the standard direct transfer method recommended by the manufacturer for screening purposes was exclusively used. The obtained results showed correlation, but also some discrepancies, between the time-consuming and costly 16S rRNA gene-based classification, and the rapid MALDI-TOF MS-based Biotyper 2.0 system. About 64% of the isolates analyzed with the Biotyper 2.0 yielded a score value above 1.7, and hence 36% were assigned with no reliable identification since 1.7 is the lower cutoff score value used by the Biotyper 2.0 system. Still, one and six of these low-scoring isolates yielded species- and genus-consistent classification results compared to the 16S rRNA gene-based classification, respectively, although this could not have been appreciated without the *a priori* knowledge from the 16S rRNA gene-based classification. When only considering the isolates that yielded a score value above 1.7, 61% were species-consistently classified and 94% were genus-consistently classified compared to the 16S rRNA gene-based classification. Of the 84 isolates that were analyzed (Biotyper 2.0 system), seven genera and 24 species given by the 16S rRNA gene-based classification were missing from the Bruker Taxonomy database. Adding these into the database could probably increase the consistency between the two classification methods. The coverage of environmental bacteria in the Bruker Taxonomy database and other taxonomic reference databases in general are gradually increasing, but there is still a bias toward clinically relevant and highly studied bacteria, and the coverage is still low for the myriad of newly identified environmental bacteria (11). The Biotyper 2.0 system allows users to construct their own libraries, thus enabling them to increase the database coverage for organisms of special interest in their research. Taken together, the Biotyper 2.0 microbial identification platform is a rapid and powerful tool for low-cost screening of environmental bacterial isolates, but an increased coverage of such bacteria is urgently needed in the reference database before the full potential of this screening method can be appreciated. Care must also be taken to obtain high-quality spectra, balancing between the rapidness of the direct transfer method and the more laborious protein extraction method that is recommended by the manufacturer to increase spectrum quality.

Antibiotic resistance. To our knowledge, antibiotic resistance profiling of the airborne bacterial community in a subway station has not previously been addressed, but several studies have addressed antibiotic resistance of airborne bacteria in other specific environments (14, 17, 25, 30, 58), such as cattle, swine, or poultry farms (1, 26, 57). The results obtained in the present study show that most dominant bacterial genera have a very high frequency of resistance to NAL, except for *Bacillus* and *Paenibacillus* spp., which had 14 and 0% frequencies of resistance, respectively. *Bacillus* was the only genus to show a high (>50%) frequency of resistance against AMP, while *Paenibacillus* was the only genus to show a high (>50%) frequency of resistance against STR. The airborne environment harbored bacteria with antibiotic resistance that covered all of the antibiotics tested, and more than 27% of the obtained isolates were resistant against at least two of them. This shows that airborne bacteria in the subway environment contain a pool of antibiotic resistance determinants against a broad range of antibiotics. If these determinants are mobile, or mobilized, they could be transferred to pathogenic or opportunistic pathogenic bacteria, either in the environment or after human inhalation. This could possibly have an impact on public health, and further elucidation of this pool of antibiotic resistance determinants and their presence on mobile genetic elements (e.g., plasmids, integrons, and transposons) is warranted.

Hemolytic activity and small cyclic peptides. The hemolytic properties of airborne microorganisms in the subway environment have only been briefly studied previously (8, 62). About 22%

of the obtained bacterial isolates in the present study were hemolytic. This is less than what was found in a study from Korea (62), which reported that 34% of the isolates were hemolytic. These researchers reported 27% beta-hemolysis and 7% alpha-hemolysis, while we found ca. 21% beta-hemolysis and less than 1% alpha-hemolysis in our initial screen on sheep blood and ca. 19% beta-hemolysis and no alpha-hemolysis in the subsequent screen on bovine blood. The majority of our hemolytic isolates (87%) were members of the bacterial genus *Bacillus*. More than 50% of the total number of *Bacillus* isolates obtained here were hemolytic on both sheep and bovine blood. The hemolytic *Bacillus* isolates clustered into at least three distinct phylogenetic groups, corresponding to known producers of NRPS-produced SCPs, including *B. cereus* (cereulide), *B. pumilus* (pumilacidin), and *B. subtilis* (surfactin). Cereulide (*B. cereus* emetic toxin) causes food poisoning (22, 65). We investigated here the ecology of cereulide-producing strains in airborne bacteria and in a subway environment. In our study, the presence of cereulide or the NRPS gene (*ces*) was not detected in any of the hemolytic *Bacillus* isolates, while in foods usually ca. 5% of strains are *ces* positive (22). Surfactin-like hemolytic peptides, such as surfactins, pumilacidins, and lichenycins, are a family of structurally similar SCLPTs that are produced by several *B. subtilis* group species, such as *B. subtilis*, *B. pumilus*, and *B. licheniformis*. In the present study, we investigated the distribution of SCLPTs in airborne *Bacillus* spp. from a subway environment, showing that 50% of the hemolytic *Bacillus* spp. isolates and 26% of the total number of *Bacillus* spp. isolates produced SCLPTs. Surprisingly, SCLPT-positive isolates were limited to the *B. pumilus* and *B. subtilis* group, except for three isolates that were closely related to *B. thuringiensis* ($n = 2$) and *B. cereus* ($n = 1$) in the *B. cereus* group. The percentage of SCLPT-producing isolates is very high compared to what has been reported by From et al., who found that only 8 of 333 isolates from food and water produced SCLPTs (24). We currently have no explanation for the finding of such a high number of SCLPT-positive airborne isolates compared to that found in foods and water. To our knowledge, there are no available surveys on the percentage of SCLPT-containing *Bacillus* spp. in the airborne environment.

Pigmentation. The atmosphere is generally considered a hostile environment for microorganisms due to stress, such as desiccation, radiation, oxygen toxicity, and pollutants (13). Solar/UV radiation has been shown to influence the airborne survival of bacteria, and pigmentation is a mechanism adopted by several types of bacteria to protect them against photo-oxidative damage (72). Our study presented an in-depth characterization of pigmentation in airborne bacteria at a subway station. About 48% of the total cultivation isolates were pigmented, while only 8% of the spore-specific cultivation isolates were pigmented. One of the main protective effects of pigmentation is protection against oxidative damage due to solar/UV radiation, and since bacterial spores are highly resistant to UV/solar radiation (63), the finding of a substantially lower percentage of pigmented spore-forming bacteria is not surprising but still noteworthy. A high percentage of pigmented bacteria has been found in ambient outdoor atmosphere, and positive selection toward pigmented bacteria has been demonstrated (73). Carotenoids, with more than 700 different representatives isolated from natural sources, represent an abundant group of natural pigments in both the prokaryote and eukaryote kingdoms, exhibiting colors from dark red to bright yellow (15). Yellow, orange, and red pigments are abundantly found in members of the bacterial genera *Micrococcus*, *Corynebacterium*, *Mycobacterium*, and *Nocardia*, all of which contain carotenoids (61). More than 50% of the pigmented isolates analyzed by LC-MS in the present study led to tentative pigment identification, spanning a broad range of different carotenoids. Although the pigments were only tentatively identified, the finding of sarcinaxanthin in an isolate classified as *M. luteus* further strengthens our identifications since this pigment was originally isolated from *S. lutea* (the former name of *M. luteus*) (45). Carotenoids have more recently also been implicated as potential bacterial virulence factors, since they may quench destructive effects of oxygen radicals, a killing mechanism used by immune cells during the innate immune response to bacterial infections (29, 44, 54). A derivative of staphyloxanthin, 8-apo-caroten-8-oic acid, was found in an isolate that was classified as *S. warneri*, and this is of interest since staphyloxanthin is the predominant pigment in another bacterial species within the same genus, namely, *S. aureus* (48). Staphyloxanthin has recently been implicated as a potential virulence factor involved in protecting the bacteria against oxygen radical killing used by neutrophils during the innate immune response to bacterial infections (44).

Future directions. We have addressed the bacterial fraction of the bioaerosol community and its characteristics at a subway station. Our aim was to investigate the presence of naturally occurring airborne bacteria in an environment regarded as a potential target for incidents involving the deliberate use of biological threat agents and where biological detectors might be used. Information about the bioaerosol background of the detectors operational location is important for its performance criteria. However, other types of airborne microorganisms (e.g., fungi and viruses) and their products (e.g., toxins) could also be important for biological detection/surveillance, as well as for public health. Further investigations of the subway environment, or other operational environments, should therefore seek to also address airborne fungi, viruses, and toxins.

The temporal variability of the bioaerosol background might influence the operational performance of biological detectors, and while the present study revealed differences between the daytime and nighttime bioaerosol environment at a subway station, an apparent limitation is the temporal resolution of the cultivation data since only two samples were collected each day. In an attempt to more specifically address the diurnal variation of the bioaerosol background at the subway station, we are currently conducting follow-up studies involving a multiday continuous sampling campaign using the high-volume impactor MAS-100. This approach will hopefully yield cultivation data with a much higher temporal resolution than what was obtained here and thereby allow a more in-depth investigation of the diurnal variability of the bioaerosol background.

Cultivation has been the traditional method used to determine airborne bacterial concentrations, but since generally $<$1% of environmental bacteria are cultivable by standard laboratory methods (2), cultivation-independent analyses have become widespread. In the present study, the obtained bacterial isolates were distributed among only three phyla, *Actinobacteria*, *Proteobacteria*, and *Firmicutes*, and the proportion of Gram-positive bacteria was $>$90%. The predominance of these phyla and the high content of Gram-positive bacteria are common for cultivation-dependent studies of airborne bacteria (36) but likely represent

only a fraction of the complete diversity (32, 33, 51). To further elucidate the airborne bacterial diversity at the Norwegian subway station, we are currently conducting a culture-independent diversity study using 16S rRNA gene-based PhyloChip microarrays (9, 10).

The regional deposition of bioaerosols inside the human respiratory tract is related to particle size, which is an important property of airborne bacteria-containing particles when assessing their potential health hazard (70). To our knowledge, only one study (available in Korean) has previously addressed the size distribution of cultivable bacterium-containing particles in a subway environment (41). We are currently conducting further studies at the Nationaltheatret subway station using the Andersen sampler (six-stage cascade impactor) to investigate the size distribution of airborne bacterium-containing particles.

ACKNOWLEDGMENTS

This study was funded by the Norwegian Defense Research Establishment.

We thank Abdelghani Ahmed and Victor Hormazabal at the Norwegian School of Veterinary Science for technical assistance during the characterization of bacterial hemolytic activity and LC-MS studies on cereulide and surfactin-like peptides. We thank Anja Valen and Vilde Sørvik Eggen, Norwegian Defense Research Establishment, who contributed to the project with technical and statistical assistance, respectively.

REFERENCES

1. **Alvarado CS, et al.** 2009. Seasonal changes in airborne fungi and bacteria at a dairy cattle concentrated animal feeding operation in the southwest United States. J. Environ. Health **71**:40–44.
2. **Amann R, Ludwig W, Schleifer K.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59**:143–169.
3. **An HR, Mainelis G, Yao M.** 2004. Evaluation of a high-volume portable bioaerosol sampler in laboratory and field environments. Indoor Air **14**: 385–393.
4. **Awad AHA.** 2002. Environmental study in subway metro stations in Cairo, Egypt. J. Occup. Health **44**:112–118.
5. **Barbeau B, et al.** 1997. A modified method for the enumeration of aerobic spore-forming bacteria. Can. J. Microbiol. **43**:976–980.
6. **Baron P, Willeke K, Kulkarni P (ed).** 2011. Aerosol measurement: principles, techniques, and applications, 3rd ed. John Wiley & Sons, Inc, Hoboken, NJ.
7. **Birenzvige A, et al.** 2003. Aerosol characteristics in a subway environment. Aerosol Sci. Technol. **37**:210–220.
8. **Bogomolova E, Kirtsideli I.** 2009. Airborne fungi in four stations of the St. Petersburg underground railway system. Int. Biodeterior. Biodegrad. **63**:156–160.
9. **Brodie EL, et al.** 2006. Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Appl. Environ. Microbiol. **72**:6288–6298.
10. **Brodie EL, et al.** 2007. Urban aerosols harbor diverse and dynamic bacterial populations. Proc. Natl. Acad. Sci. U. S. A. **104**:299–304.
11. **Cayrou C, Raoult D, Drancourt M.** 2010. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the identification of environmental organisms: the *Planctomycetes* paradigm. Environ. Microbiol. Rep. **2**:752–760.
12. **Centers for Disease Control and Prevention.** 2011. Category A list. Centers for Disease Control and Prevention, Atlanta, GA. [Online.]
13. **Cox CS, Wathes CM.** 1995. Bioaerosols handbook. Lewis Publishers, Boca Raton, FL.
14. **Decraene V, Ready D, Pratten J, Wilson M.** 2008. Air-borne microbial contamination of surfaces in a UK dental clinic. J. Gen. Appl. Microbiol. **54**:195–203.
15. **Dembitsky V.** 2005. Astonishing diversity of natural surfactants. III. Carotenoid glycosides and isoprenoid glycolipids. Lipids **40**:535–557.
16. **Desrosier NW, Heiligman F.** 1956. Heat activation of bacterial spores. J. Food Sci. **21**:54–62.
17. **Donderski W.** 2005. Microbiological contamination of air within the city of Torun. Pol. J. Environ. Studies **14**:223–230.
18. **Dong S, Yao M.** 2010. Exposure assessment in Beijing, China: biological agents, ultrafine particles, and lead. Environ. Monitoring Assess. **170**:331–343.
19. **Douwes J, Thorne P, Pearce N, Heederik D.** 2003. Bioaerosol health effects and exposure assessment: progress and prospects. Ann. Occup. Hyg. **47**:187–200.
20. **Emanuel P, Caples M.** 2011. Chemical, biological, radiological technology survey, 2011. U.S. Army Edgewood Chemical Biological Center, APG, MD.
21. **Fricker M, Messelhausser U, Busch U, Scherer S, Ehling-Schulz M.** 2007. Diagnostic real-time PCR assays for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. Appl. Environ. Microbiol. **73**:1892–1898.
22. **From C, Hormazabal V, Granum PE.** 2007. Food poisoning associated with pumilacidin-producing *Bacillus* pumilus in rice. Int. J. Food Microbiol. **115**:319–324.
23. **From C, Hormazabal V, Hardy SP, Granum PE.** 2007. Cytotoxicity in *Bacillus mojavensis* is abolished following loss of surfactin synthesis: implications for assessment of toxicity and food poisoning potential. International J. Food Microbiol. **117**:43–49.
24. **From C, Pukall R, Schumann P, Hormazabal V, Granum PE.** 2005. Toxin-producing ability among *Bacillus* spp. outside the *Bacillus cereus* group. Appl. Environ. Microbiol. **71**:1178–1183.
25. **Gandara A, et al.** 2006. Isolation of *Staphylococcus aureus* and antibiotic-resistant *Staphylococcus aureus* from residential indoor bioaerosols. Environ. Health Perspect. **114**:1859–1864.
26. **Gibbs SG, et al.** 2006. Isolation of antibiotic-resistant bacteria from the air plume downwind of a swine confined or concentrated animal feeding operation. Environ. Health Perspect. **114**:1032–1037.
27. **Gilleberg SB, Faull JL, Graeme-Cook KA.** 1998. A preliminary survey of aerial biocontaminants at six London underground stations. Int. Biodeterior. Biodegrad. **41**:149–152.
28. **Gould GW (ed).** 1971. Methods for studying bacterial spores, vol 6A. Academic Press, London, England.
29. **Hassett D, Cohen M.** 1989. Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. FASEB J. **3**:2574–2582.
30. **Heo Y, et al.** 2010. Size-resolved culturable airborne bacteria sampled in rice field, sanitary landfill, and waste incineration sites. J. Environ. Monit. **12**:1619–1624.
31. **Hormazábal V, Østensvik Ø, O'Sullivan K, Granum PE.** 2004. Quantification of *Bacillus cereus* emetic toxin (cereulide) in figs using LC/MS. J. Liquid Chromatogr. Relat. Technol. **27**:2531–2538.
32. **Hugenholtz P, Goebel BM, Pace NR.** 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. **180**:4765–4774.
33. **Hugenholtz P, Pace NR.** 1996. Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. Trends Biotechnol. **14**:190–197.
34. **Hwang SH, Yoon CS, Ryu KN, Paik SY, Cho JH.** 2010. Assessment of airborne environmental bacteria and related factors in 25 underground railway stations in Seoul, Korea. Atmos. Environ. **44**:1658–1662.
35. **Kawasaki T, et al.** 2010. Distribution and identification of airborne fungi in railway stations in Tokyo, Japan. J. Occup. Health **52**:186–193.
36. **Kellogg CA, Griffin DW.** 2006. Aerobiology and the global transport of desert dust. Trends Ecol. Evol. **21**:638–644.
37. **Keynan A, Evenchik Z, Halvorson HO, Hastings JW.** 1964. Activation of bacterial endospores. J. Bacteriol. **88**:313–318.
38. **Kim KY, Kim YS, Kim D, Kim HT.** 2011. Exposure level and distribution characteristics of airborne bacteria and fungi in Seoul metropolitan subway stations. Ind. Health **49**:242–248.
39. **Kloos WE, Musselwhite MS.** 1975. Distribution and persistence of *Staphylococcus* and *Micrococcus* species and other aerobic bacteria on human skin. Appl. Environ. Microbiol. **30**:381–395.
40. **Lane DJ.** 1991. 16S/23S rRNA sequencing, p 115–175. *In* Stackebrandt, EGM (ed), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Inc, New York, NY.
41. **Lee CM.** 2004. Characterization of airborne bioaerosol concentration in public facilities (Korean). J. Environ. Sci. **13**:215–222.
42. **Li C-S.** 1999. Evaluation of microbial samplers for bacterial microorganisms. Aerosol Sci. Technol. **30**:100–108.
43. **Liaaen-Jensen S, Weeks OB, Strang RH, Thirkell D.** 1967. Identity of the

C-50-carotenoid dehydrogenans-P439 and sarcinaxanthin. Nature **214**: 379–380.
44. **Lighthart B, Shaffer BT.** 1997. Increased airborne bacterial survival as a function of particle content and size. Aerosol Sci. Technol. **27**:439–446.
45. **Lilliefors H.** 1967. On the Kolmogorov-Smirnov test for normality with mean and variance unknown. J. Am. Stat. Assoc. **62**:399–402.
46. **Liu GY, et al.** 2005. *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. J. Exp. Med. **202**:209–215.
47. **Mann HB, Whitney DR.** 1947. On a test of whether one of two random variables is stochastically larger than the other. Ann. Mathematical Stat. **18**:50–60.
48. **Marshall JH, Wilmoth GJ.** 1981. Pigments of *Staphylococcus aureus*, a series of triterpenoid carotenoids. J. Bacteriol. **147**:900–913.
49. **National Research Council.** 2005. 2005. Sensor systems for biological agent attacks: protecting buildings and military bases. NRC Committee on Materials and Manufacturing Processes for Advanced Sensors. National Academies Press, Washington, DC.
50. **Okumura T, et al.** 1996. Report on 640 victims of the Tokyo subway sarin attack. Ann. Emerg. Med. **28**:129–135.
51. **Pace NR.** 1997. A molecular view of microbial diversity and the biosphere. Science **276**:734–740.
52. **Pearson K.** 1895. Note on regression and inheritance in the case of two parents. Proc. R. Soc. **58**:241.
53. **Pearson K.** 1900. On the criterion that a given system of deviations from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling. Philos. Magazine **50**:157–175.
54. **Provvedi R, et al.** 2008. SigF controls carotenoid pigment production and affects transformation efficiency and hydrogen peroxide sensitivity in *Mycobacterium smegmatis.* J. Bacteriol. **190**:7859–7863.
55. **Ratnesar-Shumate S, et al.** 2011. Improved method for the evaluation of real-time biological aerosol detection technologies. Aerosol Sci. Technol. **45**:635–644.
56. **Research International.** 2011, posting date. Evaporation rates for microorganisms captured by Research International's electret filters. Research International, Seattle, WA. [Online.]
57. **Rule AM, Evans SL, Silbergeld EK.** 2008. Food animal transport: a potential source of community exposures to health hazards from industrial farming (CAFOs). J. Infect. Public Health **1**:33–39.
58. **Safatov AS, et al.** 2008. To what extent can viable bacteria in atmospheric aerosols be dangerous for humans? Clean Soil Air Water **36**:564–571.
59. **Samuels AC, et al. (ed).** 2006. Test methodology development for biological agent detection systems. Proc. SPIE **6378**:637802–1.
60. **Sánchez-Monedero MA, Aguilar MI, Fenoll R, Roig A.** 2008. Effect of the aeration system on the levels of airborne microorganisms generated at wastewater treatment plants. Water Res. **42**:3739–3744.
61. **Schlegel H, Jannasch H.** 2006. Prokaryotes and their habitats, p 137–184. *In* Dworkin M, et al. (ed), The prokaryotes. Springer, New York, NY.
62. **Seino K, Takano T, Nakamura K, Watanabe M.** 2005. An evidential example of airborne bacteria in a crowded, underground public concourse in Tokyo. Atmos. Environ. **39**:337–341.
63. **Setlow P.** 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. **101**:514–525.
64. **Shahamat M, et al.** 1997. Evaluation of media for recovery of aerosolized bacteria. Aerobiologia **13**:219–226.
65. **Stenfors Arnesen LP, Fagerlund A, Granum PE.** 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol. Rev. **32**: 579–606.
66. **Szam L, Nikodemusz I, Csatai L, Vedres I, Dakay M.** 1980. Airborne microflora found in some stations of the metro in the Hungarian capital of Budapest (author's transl). Zentralbl. Bakteriol. B **170**:199–208.
67. **Szam L, Vedres I, Csatai L, Nikodemusz I.** 1983. Further microbiological studies of the air in a newly built (under the pavement) section of the underground railway in Budapest (author's transl.). Zentralbl. Bakteriol. Mikrobiol. Hyg. B **177**:312–318.
68. **Takahashi H, et al.** 2004. *Bacillus anthracis* incident, Kameido, Tokyo, 1993. Emerg. Infect. Dis. **10**:117–120.
69. **Tapi A, Chollet-Imbert M, Scherens B, Jacques P.** 2010. New approach for the detection of non-ribosomal peptide synthetase genes in *Bacillus* strains by polymerase chain reaction. Appl. Microbiol. Biotechnol. **85**: 1521–1531.
70. **Thomas RJ, et al.** 2008. Characterization and deposition of respirable large- and small-particle bioaerosols. Appl. Environ. Microbiol. **74**:6437–6443.
71. **Tong Y, Lighthart B.** 1998. Effect of simulated solar radiation on mixed outdoor atmospheric bacterial populations. FEMS Microbiol. Ecol. **26**: 311–316.
72. **Tong Y, Lighthart B.** 1997. Solar radiation has a lethal effect on natural populations of culturable outdoor atmospheric bacteria. Atmos. Environ. **31**:897–900.
73. **Tong Y, Lighthart B.** 1997. Solar radiation is shown to select for pigmented bacteria in the ambient outdoor atmosphere. Photochem. Photobiol. **65**:103–106.
74. **Valdes JJ, et al.** 2011. Background discrimination of bioaerosols. ITEA J. **32**:181–190.
75. **Wang Q, Garrity GM, Tiedje JM, Cole JR.** 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. **73**:5261–5267.
76. **Yao M, Mainelis G.** 2007. Analysis of portable impactor performance for enumeration of viable bioaerosols. J. Occup. Environ. Hyg. **4**:514–524.

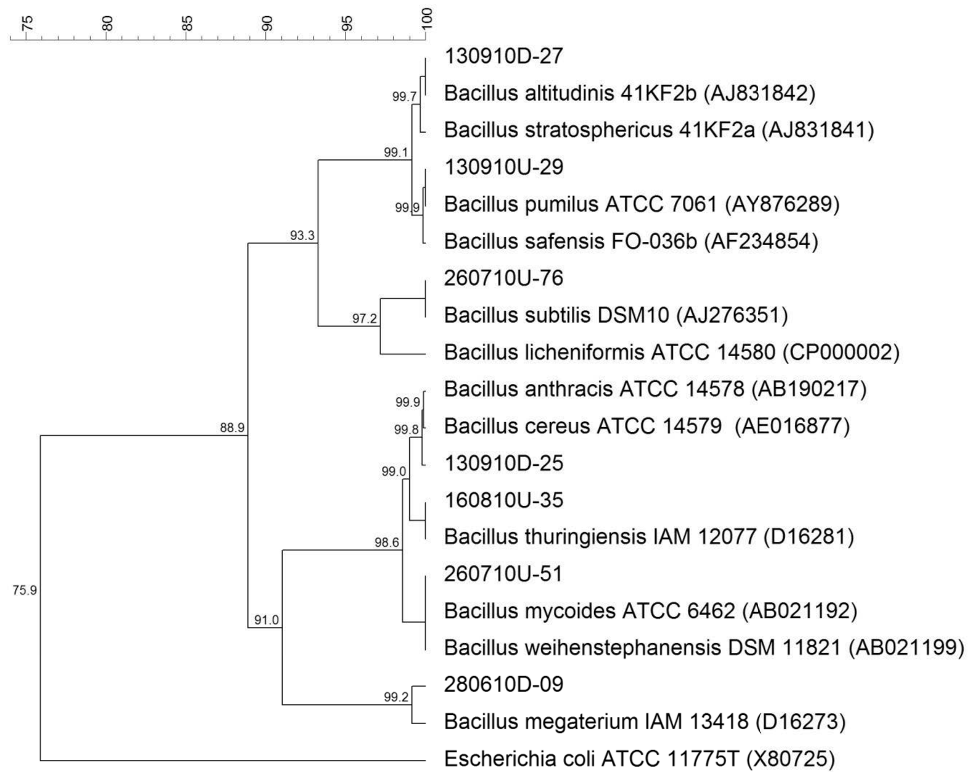

FIG S1. The phylogenetic relationship between the hemolytic *Bacillus* spp. isolates that were sequenced in this study (partial 16SrDNA) and type strains of several closely-related *Bacillus* spp. (RDP-derived 16SrDNA sequences). The obtained hemolytic *Bacillus* spp. isolates clustered into three distinct groups, the "*B. pumilus* group", the "*B. subtilis* group" and the "*B. cereus* group". The type strain of *B. megaterium* (RDP-derived 16SrDNA sequence) and a closely related non-hemolytic isolate obtained in this study (partial 16SrDNA) were included in the analysis. The type strain of *E. coli* was included as an out-group (RDP-derived 16SrDNA sequence).

TABLE S1. Comparison between MALDI-ToF MS-based Biotyper 2.0 and 16SrDNA-based taxonomical classification

Isolate[1]	16SrDNA classification	Score	Biotyper 2.0 classification	Score	Quality[2]	Genus[3]	Species[3]	Consistency
180510D-18	*Kocuria rosea*	0.978	*Kocuria rosea*	2.510	(+++)			Species
180510D-16	*Staphylococcus aureus*	0.990	*Staphylococcus aureus*	2.428	(+++)			Species
280610D-28	*Paenibacillus glucanolyticus*	0.942	*Paenibacillus glucanolyticus*	2.392	(+++)			Species
130910D-15	*Roseomonas mucosa*	1.000	*Roseomonas mucosa*	2.359	(+++)			Species
300810N-68	*Rhodococcus erythropolis*	0.987	*Rhodococcus erythropolis*	2.319	(+++)			Species
280610N-02A	*Corynebacterium callunae*	0.987	*Corynebacterium callunae*	2.300	(+++)			Species
260710R-76	*Bacillus subtilis*	1.000	*Bacillus subtilis*	2.247	(++)			Species
140610D-14	*Pseudomonas stutzeri*	1.000	*Pseudomonas stutzeri*	2.228	(++)			Species
270910D-33	*Kocuria kristinae*	0.980	*Kocuria kristinae*	2.199	(++)			Species
130910D-20	*Bacillus asahii*	1.000	*Bacillus asahii*	2.193	(++)			Species
260710N-44	*Gordonia alkanivorans*	1.000	*Gordonia alkanivorans*	2.191	(++)			Species
140610D-35	*Staphylococcus saprophyticus*	1.000	*Staphylococcus saprophyticus*	2.149	(++)			Species
270910D-24	*Pseudomonas poae*	1.000	*Pseudomonas poae*	2.142	(++)			Species
280610N-03	*Micrococcus luteus*	1.000	*Micrococcus luteus*	2.137	(++)			Species
160810R-26	*Bacillus thuringiensis*	1.000	*Bacillus thuringiensis*	2.060	(++)			Species
160810D-41	*Staphylococcus epidermidis*	1.000	*Staphylococcus epidermidis*	2.055	(++)			Species
260710N-39	*Staphylococcus lentus*	0.990	*Staphylococcus lentus*	2.005	(++)			Species
280610N-06	*Staphylococcus equorum*	0.990	*Staphylococcus equorum*	2.002	(++)			Species
180510D-48	*Staphylococcus haemolyticus*	0.971	*Staphylococcus haemolyticus*	2.000	(++)			Species
270910D-18	*Bacillus psychrodurans*	0.976	*Bacillus psychrodurans*	1.997	(+)			Species
300810R-17	*Rothia amarae*	1.000	*Rothia amarae*	1.960	(+)			Species
300810D-37	*Kocuria palustris*	0.998	*Kocuria palustris*	1.946	(+)			Species
160810R-16	*Dermacoccus nishinomiyaensis*	0.959	*Dermacoccus nishinomiyaensis*	1.938	(+)			Species
130910D-14	*Bacillus megaterium*	0.967	*Bacillus megaterium*	1.933	(+)			Species
130910D-11	*Bacillus flexus*	1.000	*Bacillus flexus*	1.927	(+)			Species
260710R-63	*Staphylococcus warneri*	0.990	*Staphylococcus warneri*	1.924	(+)			Species
160810R-11	*Bacillus simplex*	1.000	*Bacillus simplex*	1.901	(+)			Species
140610D-20	*Staphylococcus succinus*	0.984	*Staphylococcus succinus*	1.849	(+)			Species

130910R-44	*Paenibacillus odorifer*	0.973	*Paenibacillus odorifer*	1.829	(+)			Species
180510D-10	*Staphylococcus cohnii*	0.992	*Staphylococcus cohnii*	1.815	(+)			Species
280610D-21	*Lysinibacillus sphaericus*	0.906	*Lysinibacillus sphaericus*	1.809	(+)			Species
180510D-06	*Brevundimonas vesicularis*	1.000	*Brevundimonas vesicularis*	1.790	(+)			Species
260710R-69	*Brevibacillus choshinensis*	0.928	*Brevibacillus choshinensis*	1.736	(+)			Species
130910N-51	*Rothia nasimurium*	0.911	*Rothia nasimurium*	1.481	(-)			Species
300810R-04	*Arthrobacter koreensis*	1.000	*Arthrobacter gandavensis*	2.545	(+++)			Genus
260710D-29	*Sphingomonas sanguinis*	0.944	*Sphingomonas paucimobilis*	2.412	(+++)			Genus
300810N-64	*Microbacterium phyllosphaerae*	0.838	*Microbacterium paludicola*	2.396	(+++)			Genus
130910N-50	*Rhodococcus pyridinivorans*	1.000	*Rhodococcus rhodochrous*	2.366	(+++)			Genus
160810R-19	*Cellulosimicrobium funkei*	0.961	*Cellulosimicrobium cellulans*	2.156	(++)		x	Genus
140610D-32	*Streptomyces luridiscabiei*	1.000	*Streptomyces badius*	2.083	(++)		x	Genus
260710R-60	*Paenibacillus favisporus*	0.984	*Paenibacillus rhizosphaerae*	2.055	(++)			Genus
130910D-18	*Paenibacillus peoriae*	1.000	*Paenibacillus polymyxa*	1.972	(+)			Genus
140610D-03	*Viridibacillus arvi*	1.000	*Viridibacillus neidei*	1.961	(+)			Genus
280610D-30	*Bacillus altitudinis*	1.000	*Bacillus pseudofirmus*	1.938	(+)		x	Genus
140610D-25B1	*Pseudomonas psychrotolerans*	0.964	*Pseudomonas oryzihabitans*	1.866	(+)		x	Genus
160810N-82	*Serratia grimesii*	0.921	*Serratia liquefaciens*	1.835	(+)			Genus
260710R-56	*Arthrobacter humicola*	1.000	*Arthrobacter polychromogenes*	1.832	(+)		x	Genus
280610D-15	*Pseudomonas fulva*	0.945	*Pseudomonas fuscovaginae*	1.823	(+)			Genus
160810R-06	*Pseudomonas asplenii*	0.956	*Pseudomonas brenneri*	1.822	(+)			Genus
160810N-80	*Paenibacillus pabuli*	0.983	*Paenibacillus amylolyticus*	1.758	(+)			Genus
160810R-36	*Bacillus drentensis*	0.891	*Bacillus novalis*	1.756	(+)			Genus
130910D-04	*Staphylococcus kloosii*	1.000	*Staphylococcus equorum*	1.718	(+)			Genus
300810R-16	*Paenibacillus konsidensis*	0.853	*Paenibacillus glucanolyticus*	1.695	(-)		x	Genus
260710N-40	*Arthrobacter tumbae*	0.913	*Arthrobacter ilicis*	1.689	(-)			Genus
260710D-15	*Paenibacillus kobensis*	0.928	*Paenibacillus agaridevorans*	1.536	(-)			Genus
280610D-36	*Bacillus licheniformis*	0.952	*Bacillus endophyticus*	1.447	(-)			Genus
300810R-12	*Curtobacterium pusillum*	0.946	*Curtobacterium flaccumfaciens*	1.443	(-)		x	Genus
260710D-06	*Microbacterium oxydans*	0.940	*Microbacterium saperdae*	1.346	(-)			Genus
270910D-29	*Janibacter limosus*	0.955	*Staphylococcus saprophyticus*	2.064	(++)	x	x	

260710D-10B	*Enhydrobacter aerosaccus*	0.950	*Moraxella osloensis*	1.846	(+)	x	x	
300810D-42	*Microbacterium lacus*	0.943	*Arthrobacter castelli*	1.838	(+)		x	
160810N-87	*Pectobacterium cypripedii*	0.918	*Escherichia coli*	1.661	(-)			
140610D-05A	*Paenibacillus hodogayensis*	0.645	*Staphylococcus cohnii*	1.597	(-)		x	
270910R-07	*Bacillus litoralis*	0.953	*Aeromonas schubertii*	1.483	(-)			
160810N-84	*Erwinia tasmaniensis*	0.934	*Burkholderia sacchari*	1.474	(-)			
180510D-01	*Bacillus bataviensis*	0.893	*Burkholderia xenovorans*	1.466	(-)			
180510D-02	*Paenibacillus turicensis*	0.746	*Lactobacillus curvatus*	1.459	(-)		x	
300810D-44	*Microbacterium hatanonis*	0.907	*Pseudomonas pictorum*	1.452	(-)		x	
300810R-18	*Bacillus massiliensis*	0.889	*Lysinibacillus sphaericus*	1.438	(-)		x	
270910D-38	*Bacillus luciferensis*	0.889	*Rhizobium rhizogenes*	1.427	(-)			
160810R-04	*Sporosarcina ureae*	0.789	*Lactobacillus parabuchneri*	1.418	(-)		x	
130910R-40	*Paenibacillus woosongensis*	1.000	*Pandoraea pnomenusa*	1.414	(-)		x	
160810R-22	*Microbacterium oleivorans*	0.978	*Lactobacillus kimchii*	1.399	(-)			
300810R-09	*Plantibacter flavus*	0.982	*Kytococcus sedentarius*	1.399	(-)	x	x	
160810D-50	*Paenisporosarcina quisquiliarum*	1.000	*Bacillus bataviensis*	1.388	(-)	x	x	
140610D-04	*Tumebacillus permanentifrigoris*	0.648	*Staphylococcus auricularis*	1.383	(-)	x	x	
130910D-06	*Weissella confusa*	1.000	*Bacillus asahii*	1.378	(-)			
260710D-19	*Exiguobacterium indicum*	0.966	*Clostridium novyi*	1.377	(-)	x	x	
130910D-16	*Comamonas koreensis*	0.913	*Alcaligenes faecalis*	1.373	(-)		x	
300810D-34	*Microbacterium esteraromaticum*	0.984	*Curtobacterium albidum*	1.372	(-)		x	
130910D-09	*Planococcus rifietoensis*	1.000	*Achromobacter xylosoxidans*	1.349	(-)	x	x	
140610D-33A	*Dietzia cinnamea*	0.964	*Sphingobium cloacae*	1.329	(-)		x	
180510D-20	*Pseudomonas asplenii*	0.964	*Lactobacillus vitulinus*	1.239	(-)			
140610D-24A	*Paracoccus yeei*	1.000	no peaks found	0.000	(-)			Failed

[1] D = daytime station, N = nighttime station, R = daytime outdoor reference

[2] (+++) secure species identification, (++) probable species, secure genus identification; (+) probable genus identification; (-) no reliable identification

[3] Not represented in the Bruker Taxonomy database (V3.1.1.0, containing 3740 library entries)

TABLE S2. Hemolytic activity and virulence-associated properties of the obtained *Bacillus* spp. isolates.

Isolate[1]	**Cultivation**		**PCR**		**HPLC/LC-MS**		**16SrDNA-based classification**	**RDP score**
	Sheep	**Bovine**	**NRPS**[3]	***ces*** [4]	**Cereulide**	**SCLPT**[5]		
130910D-13	β	β	+	-	-	-	*Bacillus cereus*	1.000
260710D-07	β	β	+	-	-	-	*Bacillus cereus*	1.000
260710R-78	β	β	+	-	-	-	*Bacillus cereus*	0.978
300810D-53	β	β	+	-	-	-	*Bacillus mycoides/weihenstephanensis*	0.979
140610D-29	β	β	+	-	-	-	*Bacillus thuringiensis*	1.000
160810D-60	β	β	+	-	-	-	*Bacillus thuringiensis*	0.995
180510D-41	β	β	+	-	-	-	*Bacillus thuringiensis*	1.000
270910D-21	β	β	+	-	-	-	*Bacillus thuringiensis*	1.000
270910D-36	β	β	+	-	-	-	*Bacillus thuringiensis*	1.000
300810R-13	β	β	+	-	-	-	*Bacillus thuringiensis*	1.000
260710R-64	β	β	+	-	-	+	*Bacillus cereus*	1.000
160810R-29	β	-	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
260710R-74	β	-	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
280610D-10A	β	-	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
140610D-12	β	-	+	-	-	+	*Bacillus stratosphericus/aerophilus*	0.948
130910R-39	β	β	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
260710N-45	β	β	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
280610D-10B	β	β	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
280610D-29	β	β	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
280610D-30	β	β	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
260710R-57	β	β	+	-	-	+	*Bacillus safensis/pumilus*	1.000
130910D-17	β	β	+	-	-	+	*Bacillus thuringiensis*	1.000
130910D-27	β	β/p[2]	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
160810R-30	β	β/p	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
260710D-30	β	β/p	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
260710R-71	β	β/p	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
280610D-26	β	β/p	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000

280610D-32	β	β/p	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
300810R-15	β	β/p	+	-	-	+	*Bacillus altitudinis/stratosphericus*	0.995
300810R-22	β	β/p	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
130910R-29	β	β/p	+	-	-	+	*Bacillus safensis/pumilus*	0.948
270910R-10	β	β/p	+	-	-	+	*Bacillus safensis/pumilus*	0.949
260710R-52	β	β	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
280610D-33	β	β	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
260710R-72	β	β	+	-	-	+	*Bacillus safensis/pumilus*	1.000
260710R-73	β	β	+	-	-	+	*Bacillus safensis/pumilus*	1.000
130910D-19	β	β	+	-	-	+	*Bacillus subtilis*	1.000
140610D-06	β	β	+	-	-	+	*Bacillus subtilis*	1.000
180510D-29	β	β	+	-	-	+	*Bacillus thuringiensis*	1.000
160810R-21	β	β/p	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
280610D-27A	β	β/p	+	-	-	+	*Bacillus altitudinis/stratosphericus*	1.000
260710R-76	β	β/p	+	-	-	+	*Bacillus subtilis*	1.000
270910R-09	β	β/p	+	-	-	+	*Bacillus subtilis*	1.000
160810R-27	β	β/p	+	-	-	n.d.[6]	*Bacillus subtilis*	1.000
280610D-35	β	-	-	-	-	n.d.	*Bacillus altitudinis/stratosphericus*	1.000
280610D-37	β	-	-	-	-	n.d.	*Bacillus altitudinis/stratosphericus*	1.000
280610D-31	α	-	-	-	-	n.d.	*Bacillus cereus*	1.000
300810D-32	β	-	-	-	-	n.d.	*Bacillus thuringiensis*	1.000
300810D-36	β	-	-	-	-	n.d.	*Bacillus thuringiensis*	1.000
270910D-34	α	-	-	-	-	n.d.	*Bacillus weihenstephanensis*	0.990
130910D-12	β	β	-	-	-	n.d.	*Bacillus altitudinis/stratosphericus*	1.000
160810R-09	β	β	-	-	-	n.d.	*Bacillus altitudinis/stratosphericus*	1.000
280610D-34	β	β	-	-	-	n.d.	*Bacillus altitudinis/stratosphericus*	1.000
130910D-25	β	β	-	-	-	n.d.	*Bacillus cereus*	0.977
140610D-08	β	β	-	-	-	n.d.	*Bacillus cereus*	1.000
180510D-39	β	β	-	-	-	n.d.	*Bacillus cereus*	1.000
260710D-12	β	β	-	-	-	n.d.	*Bacillus cereus*	1.000
260710D-27	β	β	-	-	-	n.d.	*Bacillus cereus*	1.000

300810D-28	β	β	-	-	-	n.d.	*Bacillus cereus*	1.000
300810D-45	β	β	-	-	-	n.d.	*Bacillus cereus*	1.000
300810D-47	β	β	-	-	-	n.d.	*Bacillus cereus*	1.000
160810R-08	β	β	-	-	-	n.d.	*Bacillus mycoides/weihenstephanensis*	0.980
260710R-65	β	β	-	-	-	n.d.	*Bacillus mycoides/weihenstephanensis*	1.000
300810N-61	β	β	-	-	-	n.d.	*Bacillus mycoides/weihenstephanensis*	1.000
140610D-07	β	β	-	-	-	n.d.	*Bacillus thuringiensis*	0.984
140610D-11	β	β	-	-	-	n.d.	*Bacillus thuringiensis*	1.000
160810D-49	β	β	-	-	-	n.d.	*Bacillus thuringiensis*	1.000
160810R-33	β	β	-	-	-	n.d.	*Bacillus thuringiensis*	1.000
160810R-35	β	β	-	-	-	n.d.	*Bacillus thuringiensis*	1.000
300810D-52	β	β	-	-	-	n.d.	*Bacillus thuringiensis*	0.956
260710R-51	β	β	-	-	-	n.d.	*Bacillus weihenstephanensis*	1.000
280610D-27B	β	β/p	-	-	-	n.d.	*Bacillus altitudinis/stratosphericus*	1.000
300810D-46	β	β/p	-	-	-	n.d.	*Bacillus altitudinis/stratosphericus*	1.000
300810D-48	β	β/p	-	-	-	n.d.	*Bacillus altitudinis/stratosphericus*	1.000
300810R-01	β	β/p	-	-	-	n.d.	*Bacillus mycoides/weihenstephanensis*	1.000
300810D-51	β	β/p	-	-	-	n.d.	*Bacillus subtilis*	0.985
160810R-34	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus altitudinis*	1.000
260710R-49	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus altitudinis*	0.990
130910D-20	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus asahii*	1.000
160810R-31	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus asahii*	1.000
140610D-10	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus atrophaeus*	1.000
160810R-28	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus cereus*	1.000
270910R-14	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus cereus*	1.000
130910R-38	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus drentensis*	0.949
160810R-36	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus drentensis*	0.891
130910D-11	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus flexus*	1.000
140610D-09	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus flexus*	1.000
160810D-55	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus flexus*	0.986
160810D-59	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus flexus*	1.000

270910D-25	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus flexus*	0.985
300810D-49	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus flexus*	0.990
140610D-05B	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus licheniformis*	1.000
280610D-36	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus licheniformis*	0.952
270910R-07	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus litoralis*	0.953
270910D-38	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus luciferensis*	0.889
130910D-14	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.967
130910R-42	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.935
140610D-28	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.946
160810D-37	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.947
160810D-56	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.964
180510D-40	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.967
260710R-59	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.942
270910D-27	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.965
270910D-28	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.963
270910R-11	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.960
280610D-09	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.944
300810D-39	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus megaterium*	0.955
180510D-01	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus niacini*	0.934
270910D-18	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus psychrodurans/psychrotolerans*	0.976
140610D-23	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus safensis*	1.000
130910R-37	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus simplex*	1.000
140610D-01	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus simplex*	1.000
160810R-11	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus simplex*	1.000
160810R-14	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus simplex*	1.000
180510D-03	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus simplex*	0.970
270910D-39	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus simplex*	1.000
270910R-05	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus simplex*	1.000
270910R-13	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus simplex*	0.983
260710R-67	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus soli*	0.962
140610D-13A2	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus subtilis*	0.992

140610D-13B	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus subtilis*	1.000
160810R-10	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus thuringiensis*	0.992
160810R-26	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus thuringiensis*	1.000
300810D-50	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus thuringiensis*	0.954
300810N-65	-	n.d.	n.d.	n.d.	n.d.	n.d.	*Bacillus thuringiensis*	0.981

[1] D = daytime station, N = nighttime station, R = daytime outdoor reference

[2] β/p, putative SCLPT-induced β-hemolysis

[3] Non-ribosomal peptide synthetases (NRPS)

[4] Cereulide synthase gene (*ces*)

[5] Small cyclic lipopeptide toxins (SCLPT)

[6] n.d. = not determined. Isolates that were non-hemolytic on sheep blood in the initial screen were not further analyzed, hemolytic isolates that were negative for NRPS were not assayed for SCLPT, and one single hemolytic isolate that were positive for NRPS were not assayed for SCLPT because it had to be discarded due to low growth.

TABLE S3: Pigmented bacterial isolates and tentatively identified pigments

Isolate	Color[1]	16SrDNA-based classification	LC-MS results				Dictionary of Natural Products search results					
			Result[2]	Retention	λ-max	Mass	Best match	Formula	Source	λ-max	Mass	ppm[6]
130910D-09	Orange	*Planococcus rifietoensis*	(++)	11.80-12.00	475, 505, 445	444.30265	Triophaxanthin	c31h40o2	Mollusks	447	444.30283	-0.41
140610D-25B1	Yellow	*Pseudomonas psychrotolerans*	(++)	9.25-9.35	450, 478, 428	892.53235	Zeaxanthin[3]	c52h76o12	*Archaea*	449, 477	892.53368	-1.49
140610D-33A	Orange	*Dietzia cinnamea*	(++)	12.50-12.60	480	564.39615	Alloxanthin	c40h52o	Insects	452, 478	564.39673	-1.03
180510D-06	Orange	*Brevundimonas vesicularis*	(++)	9.88-9.96	470	614.39845	Amarouciaxanthin A	c40h54o5	Protozoa	408, 434, 460	614.39713	2.16
260710D-06	Yellow	*Microbacterium oxydans*	(++)	12.16-12.29	444, 474, 420	704.55285	Sarcinaxanthin	c50h72o2	*S. lutea*	416, 440, 470	704.55323	-0.54
260710D-29	Yellow	*Sphingomonas sanguinis*	(++)	9.91-10.00	455, 480, 420	600.41835	Cycloviolaxanthin	c40h56o4	Red Paprika	453, 483, 427	600.41786	0.82
260710N-44	Pink	*Gordonia alkanivorans*	(++)	10.18-10.28	478, 508, 455	712.47145	OH-chlorobactene[4]	c46h64o6	*Chlorobium*	465,493	712.47029	1.63
			(++)	13.60-13.75	478, 508, 455	550.41945	OH-chlorobactene	c40h54o	*Chlorobium*		550.41747	3.61
260710R-63	Yellow	*Staphylococcus warneri*	(++)	12.28-12.38	460, 480, 428	432.30245	8-apo-caroten-8-oic acid[5]	c30h40o2	*S. aureus*		432.30283	-0.88
280610N-03	Yellow	*Micrococcus luteus*	(++)	12.24-12.36	444, 473, 419	704.55245	Sarcinaxanthin	c50h72o2	*S. lutea*	416, 440, 470	704.55323	-1.11
300810D-37	Yellow	*Kocuria palustris*	(++)	12.24-12.42	444, 473, 419	704.55215	Sarcinaxanthin	c50h72o2	*S. lutea*	416, 440, 470	704.55323	-0.46
300810D-44	Yellow	*Microbacterium hatanonis*	(++)	12.33-12.46	444, 473, 419	704.55215	Sarcinaxanthin	c50h72o2	*S. lutea*	416, 440, 470	704.55323	-0.40
300810R-09	Yellow	*Plantibacter flavus*	(++)	12.35-12.47	455, 481, 432	704.55305	Sarcinaxanthin	c50h72o2	*S. lutea*	416, 440, 470	704.55323	-0.26
300810R-12	Yellow	*Curtobacterium pusillum*	(++)	12.37-12.47	455, 481, 432	704.55305	Sarcinaxanthin	c50h72o2	*S. lutea*	416, 440, 470	704.55323	0.03
130910D-15	Pink	*Roseomonas mucosa*	(+)	9.97-10.20	503, 533, 472							
160810N-84	Yellow	*Erwinia tasmaniensis*	(+)	9.26-9.34	456, 483, 425							
160810N-87	Yellow	*Pectobacterium cypripedii*	(+)	9.28-9.35	455, 485, 425							
160810R-19	Yellow	*Cellulosimicrobium funkei*	(+)	9.80-9.85	445, 475, 420							
300810R-04	Yellow	*Arthrobacter koreensis*	(+)	9.79-9.86	445, 474, 421							
280610N-15	Orange	*Staphylococcus aureus*	(+)	12.10-12.25	465, 493, 440							
140610D-10	Orange	*Bacillus subtilis*	(+)	8.95-9.05	367, 388, 349							
130910N-50	Pink	*Rhodococcus pyridinivorans*	(-)									
160810R-16	Orange	*Dermacoccus nishinomiyaensis*	(-)									
180510D-18	Pink	*Kocuria rosea*	(-)									
130910D-07	Orange	*Staphylococcus saprophyticus*	(-)									

[1] Colony pigmentation, determined visually after growth on TSA for 48 hours at 30°C.

[2] (++) Tentative pigment identification, (+) No appropriate pigment mass identified, (-) No appropriate UV-VIS spetrum identified

[3] Di-O-beta-D-glucopyanoside

[4] O-beta-D-glucopyanoside

[5] Derivative of staphyloxanthin from *S. aureus*

[6] Mass deviation in parts per million (ppm)

Paper II

Temporal Variability of the Bioaerosol Background at a Subway Station: Concentration Level, Size Distribution and Diversity of Airborne Bacteria

Dybwad, M, G Skogan, and JM Blatny

Applied and Environmental Microbiology, 2014, 80(1):257-270

AEM Journals.ASM.org

Temporal Variability of the Bioaerosol Background at a Subway Station: Concentration Level, Size Distribution, and Diversity of Airborne Bacteria

Marius Dybwad,[a,b] Gunnar Skogan,[a] Janet Martha Blatny[a]

Norwegian Defence Research Establishment, Kjeller, Norway[a]; Norwegian University of Science and Technology, Department of Biotechnology, Trondheim, Norway[b]

Naturally occurring bioaerosol environments may present a challenge to biological detection-identification-monitoring (BIODIM) systems aiming at rapid and reliable warning of bioterrorism incidents. One way to improve the operational performance of BIODIM systems is to increase our understanding of relevant bioaerosol backgrounds. Subway stations are enclosed public environments which may be regarded as potential bioterrorism targets. This study provides novel information concerning the temporal variability of the concentration level, size distribution, and diversity of airborne bacteria in a Norwegian subway station. Three different air samplers were used during a 72-h sampling campaign in February 2011. The results suggested that the airborne bacterial environment was stable between days and seasons, while the intraday variability was found to be substantial, although often following a consistent diurnal pattern. The bacterial levels ranged from not detected to 10^3 CFU m^{-3} and generally showed increased levels during the daytime compared to the nighttime levels, as well as during rush hours compared to non-rush hours. The airborne bacterial levels showed rapid temporal variation (up to 270-fold) on some occasions, both consistent and inconsistent with the diurnal profile. Airborne bacterium-containing particles were distributed between different sizes for particles of $>$1.1 μm, although ~50% were between 1.1 and 3.3 μm. Anthropogenic activities (mainly passengers) were demonstrated as major sources of airborne bacteria and predominantly contributed 1.1- to 3.3-μm bacterium-containing particles. Our findings contribute to the development of realistic testing and evaluation schemes for BIODIM equipment by providing information that may be used to simulate operational bioaerosol backgrounds during controlled aerosol chamber-based challenge tests with biological threat agents.

Subway transportation systems are found in at least 133 cities worldwide, transporting about 200 million passengers daily (www.uitp.org). Subway stations are typically confined and crowded underground public environments that may be regarded as potential bioterrorism targets.

A major challenge with bioterrorism incidents involving aerosolized biological threat agents is that exposure to even lethal doses will in most cases not induce any immediate symptoms. Thus, in the absence of on-site biological detection-identification-monitoring (BIODIM) capabilities, the public will most likely remain unaware of such potential health hazards until symptomatic individuals seek medical assistance up to several days later. Such a time frame can result in exposed individuals traveling worldwide before an alarm is raised, thus complicating postincident response efforts and potentially leading to the unnecessary loss of human life.

An urgent need for early-warning BIODIM systems capable of operating in complex environmental backgrounds has been expressed by both military and civilian authorities, exemplified by the Joint Biological Point Detection System (JBPDS) program of the U.S. Department of Defense (www.defense.gov) and the BioWatch Generation-3 program of the U.S. Department of Homeland Security (www.dhs.gov). Early-warning BIODIM systems may allow for timelier implementation of effective countermeasures (e.g., containment, evacuation, and prophylactic treatment with medical countermeasures) which could contribute to reduce the consequences of bioterrorism incidents.

In operational environments, the natural aerosol background will challenge BIODIM systems with a dynamic and complex mixture of both biological and nonbiological airborne particulate matter that could interfere with the system's performance. The natural occurrence of similar but nonpathogenic environmental relatives of biological threat agents may lead to false-positive or -negative detection responses by the BIODIM system. Currently, few if any available systems have been able to meet all of the user requirements regarding rapid, robust, reliable, cost-effective, sensitive, and specific surveillance of biological threat agents in different operational environments (2).

Obtaining more-detailed information about various bioaerosol backgrounds may contribute to provide an overview of bioaerosol background-related conditions that will be encountered by BIODIM systems. Such information may assist in the development and testing and evaluation (T&E) of BIODIM equipment by allowing more-realistic operational conditions to be taken into account.

Airborne bacteria have been surveyed at subway stations in several countries around the world, including the United States (3, 4), Japan (5), South Korea (6–8), China (9), Russia (10), Egypt (11), the United Kingdom (12), Hungary (13, 14), Iran (15), and Norway (16). However, certain characteristics of the airborne bac-

Received 23 August 2013 **Accepted** 17 October 2013
Published ahead of print 25 October 2013
Address correspondence to Janet Martha Blatny, janet-martha.blatny@ffi.no.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02849-13.
Copyright © 2014, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AEM.02849-13

terial environments encountered at subway stations have not previously been addressed in detail, including the size distribution of airborne bacterium-containing particles and the temporal variability of the concentration level, size distribution, and diversity of airborne bacteria.

The current work investigated airborne bacteria at the Nationaltheatret subway station in Oslo, Norway, aiming at providing detailed information concerning the temporal variability of airborne cultivable bacteria during a 72-h continuous-sampling campaign in the winter season of 2011. Three air samplers were used to obtain data sets with different properties, including (i) particle size-resolved bacterial concentration levels and diversity data using an Andersen six-stage cascade impactor, (ii) concentration levels with a high temporal resolution using a MAS-100 high-volume impactor, and (iii) concentration levels and diversity data using a SASS 3100 high-volume electret filter sampler, in accordance with a sampling scheme that was used during a previous study at the same station in the spring, summer, and fall seasons of 2010 (16). The airborne bacterial diversity was investigated by analyzing representative bacterial isolates using the Biotyper 3.0 MALDI-TOF MS microbial identification system. The sampling campaign also included meteorological and total particle measurements using a VXT520 weather station and an APS3321 aerodynamic particle sizer, respectively.

MATERIALS AND METHODS

Study location. The study was conducted at the Nationaltheatret subway station in Oslo (ca. 600,000 inhabitants), Norway. The subway transportation network in Oslo has six bidirectional lines which all pass through the Nationaltheatret station, transporting over 70 million people annually. The underground station consists of a single double-tracked tunnel hall housing both the eastbound and westbound routes. On average, one train departs every minute during the operating hours of the station. No heating, ventilation, and air conditioning (HVAC) or passenger screening door (PSD) systems are installed at the station. A previous survey of the airborne bacterial environment at the same station (May to September 2010) has been reported (16).

The sampling campaign was initiated on 14 February 2011 at 09:00 and ended on 17 February 2011 at 09:00 after 72 h of continuous sampling activities at the station. The air sampling and monitoring equipment were colocated in the middle of the westbound concourse about 4 m from the train tracks. Throughout the campaign, the subway lines were running, with (i) increasing train frequency from 05:10 (first train) to 06:00, (ii) constant train frequency from 06:00 to 22:00, and (iii) decreasing train frequency from 22:00 to 01:15 (last train). The station was nonoperative and closed to the public from 01:15 to 05:10. On some occasions during this period, maintenance personnel were working at the station and diesel-powered maintenance trains were operating in the adjacent tunnel network.

Bioaerosol collection. Air samples were collected during the sampling campaign using three different instruments, as follows: (i) an Andersen six-stage cascade impactor (28.3 liters of air per minute [lpm], TE-10-800; Tisch Environmental, Cleves, OH), (ii) a MAS-100 high-volume single-stage impactor (100 lpm; Merck, Billerica, MA), and (iii) an SASS 3100 high-volume electret filter sampler (300 lpm; Research International, Monroe, WA). The Andersen sampler was used to obtain particle size-resolved airborne cultivable-bacterial concentration levels by sampling for 20 min at the start of every h. The Andersen sampler separates aerosols based on their aerodynamic diameter by cascade impaction onto cultivation plates in six size-resolved stages, as follows: stage 1, $>$7.1 μm; stage 2, 4.7 to 7.1 μm; stage 3, 3.3 to 4.7 μm; stage 4, 2.1 to 3.3 μm; stage 5, 1.1 to 2.1 μm; and stage 6, 0.65 to 1.1 μm. The airflow through the Andersen sampler was monitored using a mass flow meter (TopTrak 826; Sierra Instruments, Monterey, CA). The MAS-100 was used to obtain airborne cultivable bacterial concentration levels with a higher temporal resolution than the Andersen sampler by sampling for 2.5 min at the start of every 10-min period. The MAS-100 was scheduled to continue sampling until 17 February at 09:00, but due to a battery problem, the sampling had to be discontinued on 16 February at 11:00. The SASS 3100 obtained airborne cultivable bacterial concentration levels by sampling for 2 h per sample, generating three different sample types, as follows: (i) nighttime samples (03:00 to 05:00) in the station, (ii) daytime samples (07:00 to 09:00) in the station, and (iii) daytime samples (07:00 to 09:00) at a square adjacent to the station's entrance (outdoor reference location). Two SASS 3100 samplers were used to allow simultaneous air sampling in the station and at the outdoor reference location.

The air samplers were mounted on tripods with inlet heights of about 1.5 m. The samplers were disinfected with ethanol (70%) between samples to avoid cross-contamination. Field blanks were generated by mounting cultivation plates (Andersen and MAS-100) or filters (SASS 3100) without sampling air and subjecting them to the same analyses as the air samples. The Andersen and MAS-100 impactors were operated with Reasoner's 2a (R2A) (Oxoid, Cambridge, United Kingdom) plates supplemented with 100 $\mu g\ ml^{-1}$ cycloheximide (Sigma-Aldrich, St. Louis, MO) (R2Ac) to avoid fungal growth. Air samples collected on SASS 3100 filters were extracted into liquid using an extraction buffer (phosphate-buffered saline with 0.05% Triton X-100 [pH 7.4]) and the SASS 3010 extractor instrument (Research International) according to the manufacturer's instructions. The filter extracts (100 μl) were diluted as needed with extraction buffer and plated as triplicates on R2Ac plates. A similar procedure, but including a heat shock (75°C for 20 min), was also performed on the SASS 3100 filter extracts to exclusively enumerate aerobic bacterial spores. The incubation of all cultivation plates was performed at 30°C for 48 h before the colonies were enumerated. The results obtained from SASS 3100 samples were expressed as an average of the triplicate cultivation plates, while the results from the Andersen and MAS-100 were corrected using the standard positive-hole correction method (17). The airborne bacterial levels were expressed as CFU per cubic meter of air (CFU m^{-3}). The limit of detection (LOD) was 6 CFU m^{-3} for the SASS 3100 cultivation assay, corresponding to the observation of at least one CFU on each of the triplicate cultivation plates. The LODs for the Andersen and MAS-100 were 2 and 4 CFU m^{-3}, respectively.

Total particle and meteorological data collection. The total particle concentration level and size distribution at the station were monitored with an APS 3321 aerodynamic particle sizer (TSI, Shoreview, MN). The APS 3321 was positioned on a table with an inlet height of about 1.3 m. The data were logged at 10-s intervals in 51 channels for particles with an aerodynamic diameter between 0.5 and 20 μm and reported as particles per cubic meter of air (particles m^{-3}). Meteorological parameters, including temperature, humidity, wind speed, and wind direction, were monitored using a VXT520 weather station (Vaisala, Helsinki, Finland) mounted on a tripod at a height of about 1.5 m. The number of people on the station's westbound concourse was regularly counted, averaged over a 30-min period, and reported as 0, 1 to 10, 10 to 50, 50 to 100, or $>$100 passengers. The outdoor meteorological conditions during the sampling campaign were retrieved from the Norwegian Meteorological Institute (www.yr.no).

Bacterial isolation. Representative selections of morphologically distinct bacterial colonies were isolated from 17 Andersen and 9 SASS 3100 samples. The triplicate primary cultivation plates from each SASS 3100 sample were considered together as one sample when colonies were selected. For each Andersen sample, the six size-resolved primary cultivation plates were considered separately during colony selection. Five daily samples were included for the Andersen sampler, two rush hour samples (08:00 and 16:00), two non-rush hour samples (12:00 and 21:00), and one nighttime sample (04:00). Two additional single samples were also included, (i) a morning sample on 14 February (09:00), since the sampling campaign was initiated after the peak morning rush on the first day, and

(ii) a nighttime sample on 16 February (03:00), when the airborne cultivable bacterial level was temporarily increased compared to the general nighttime level.

The selected colonies were transferred from the primary cultivation plates to fresh R2A plates (secondary cultivation plates) and incubated at 30°C for 48 h. This process was repeated twice or until pure isolates were observed by subculturing from the secondary cultivation plates. The final bacterial isolates were stored at −80°C in brain heart infusion broth (Oxoid) supplemented with 18% glycerol (Merck).

MALDI-TOF MS. The bacterial isolates were classified using the Biotyper 3.0 microbial identification system (Bruker Daltonics, Bremen, Germany) coupled with a MicroFlex LT matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) instrument (Bruker Daltonics), in accordance with the manufacturer's standard direct transfer method. The bacterial isolates were recovered from frozen stocks and cultivated on Trypticase soy agar (TSA) plates (Oxoid) at 30°C for 24 to 48 h before single colonies were transferred onto an MSP 96 ground-steel target (Bruker Daltonics) as triplicates. The α-cyano-4-hydroxycinnamic acid (HCCA) matrix (Bruker Daltonics) was prepared in accordance with the manufacturer's recommendations and overlaid on each target spot (1 μl). The Biotyper 3.0 was run in automatic classification mode, and the reference database used was the Bruker Taxonomy database (version 3.3.1.0, containing 4,613 library entries), coupled with the security-relevant add-on database (Bruker Daltonics). The Biotyper 3.0 reports classification score values (SV) of between 0 and 3, which are generally interpreted to suggest (i) probable species identification if the SV is ≥2.3, (ii) secure genus identification and probable species identification if the SV is ≥2.0, (iii) probable genus identification if the SV is ≥1.7, and (iv) no reliable identification if the SV is <1.7. Bacterial isolates that failed to generate an SV of ≥2.0 during the first analysis round were subsequently cultured and analyzed again.

Statistical analysis. The results were subjected to statistical analyses using SigmaPlot version 12.3 (Systat Software, Inc., San Jose, CA). Normality testing was done with the Shapiro-Wilk test, and depending on whether the normality and equal variance criteria were fulfilled or not, significance testing was performed with the Student *t* test or the Mann-Whitney rank-sum test, respectively. Consistencies regarding the temporal variation both within and between the bacterial and particle concentration level data sets were investigated using the Pearson product-moment correlation coefficient. The Pearson chi-square test was used for the bacterial diversity (categorical) data sets. The significance level was set at a P value of <0.05 for all statistical tests.

RESULTS

Airborne cultivable bacterial concentration level. During the sampling campaign (14 February at 09:00 to 17 February at 09:00), 72 and 300 air samples were collected at the subway station by the Andersen six-stage cascade impactor and the MAS-100 high-volume single-stage impactor, respectively. The average bacterial levels reported by the Andersen and MAS-100 were 377 ± 227 CFU m^{-3} (mean ± standard deviation) and 378 ± 288 CFU m^{-3}, respectively. Both samplers showed significant correlation (r = 0.86) concerning the temporal variation of the airborne bacterial level during the campaign and revealed several consistent diurnal trends, i.e., (i) the daytime level was higher than the nighttime level, (ii) during the daytime, the morning (~07:00 to 10:00) and afternoon (~15:00 to 18:00) rush hours showed higher levels than the noon (~10:00 to 15:00) and evening (~18:00 to 00:00) non-rush periods, and (iii) following the afternoon rush hours, the bacterial level typically decayed throughout the evening and night, with a diurnal minimum just before the train activity recommenced in the morning (Fig. 1).

During the nighttime period, when the bacterial levels were typically low and decaying, two atypical events occurred, and both samplers reported (i) a temporary strong increase in the bacterial level on 16 February between 02:00 and 04:00 and (ii) a similar but modest increase on 15 February at about 01:00 (Fig. 1). When these two atypical events were considered together, the Andersen sampler reported a significantly higher (4-fold) bacterial level (395 ± 213 CFU m^{-3}) during the events than during the same periods on nonevent days (98 ± 38 CFU m^{-3}). Similar results were observed with the MAS-100, which reported a significantly higher (7.2-fold) bacterial level during the events (819 ± 225 CFU m^{-3}) than during nonevent periods (113 ± 100 CFU m^{-3}).

Despite the nighttime atypical events and for both samplers, significant correlations were observed when the individual diurnal periods were compared to each other (Andersen, r = 0.61 to 0.85, and MAS-100, r = 0.46 to 0.71). Based on the day-to-day diurnal consistencies, the Andersen and MAS-100 results from individual sampling days were each merged into a single diurnal period averaged hourly (Table 1). The two nighttime atypical events were not included in the averaged data.

To compare the daytime and nighttime bacterial levels at the station, the following time period definitions were used: daytime, between 05:20 and 00:00, and nighttime, between 00:00 and 05:20. The nighttime-to-daytime boundary was based on the arrival of the first morning trains between 05:10 and 05:20, with the subsequent rapid increase in anthropogenic activities (i.e., trains and passengers). The daytime-to-nighttime boundary was chosen because the passenger counts (Fig. 1) and train frequency decreased rapidly after midnight. The Andersen results showed that the daytime bacterial level (452 ± 198 CFU m^{-3}) was significantly higher (4.2-fold) than the nighttime level (107 ± 68 CFU m^{-3}). Similarly, the daytime level was significantly higher (2.9-fold) than the unfiltered (i.e., including atypical events) nighttime level (154 ± 151 CFU m^{-3}). The MAS-100 results showed that the daytime level (443 ± 275 CFU m^{-3}) was significantly higher (4.3-fold) than the nighttime level (103 ± 102 CFU m^{-3}) and also significantly higher (3.3-fold) than the unfiltered nighttime level (137 ± 187 CFU m^{-3}).

A total of nine air samples were collected with the SASS 3100 high-volume electret filter sampler during the sampling campaign, corresponding to three daily samples, as follows: (i) nighttime in the station (03:00 to 05:00), (ii) daytime at the station (07:00 to 09:00), and (ii) daytime in the outdoor reference location (07:00 to 09:00). The average bacterial levels reported by the SASS 3100 were 493 ± 153, 25 ± 22, and 41 ± 17 CFU m^{-3}, for daytime and nighttime in the station and the daytime outdoor reference, respectively (Table 1). The daytime level in the station was significantly higher (19.4-fold) than the nighttime level and also significantly higher (11.9-fold) than the daytime outdoor level. The bacterial levels reported by the SASS 3100 in the station were not significantly different from those obtained with the Andersen and MAS-100 impactors when these were averaged using only the corresponding sampling periods. However, the nighttime bacterial level reported by the SASS 3100 was, although not significantly different, 1.3-fold and 3.8-fold lower than those reported by the Andersen and MAS-100, respectively.

The spore-specific cultivation analysis performed on the SASS 3100 samples consistently reported airborne bacterial spore levels of less than the LOD (5.5 CFU m^{-3}), except during daytime sampling in the station (7 ± 5 CFU m^{-3}). Still, the daytime level of cultivable bacterial spores corresponded to less than 2% of the total cultivable bacterial level at the station.

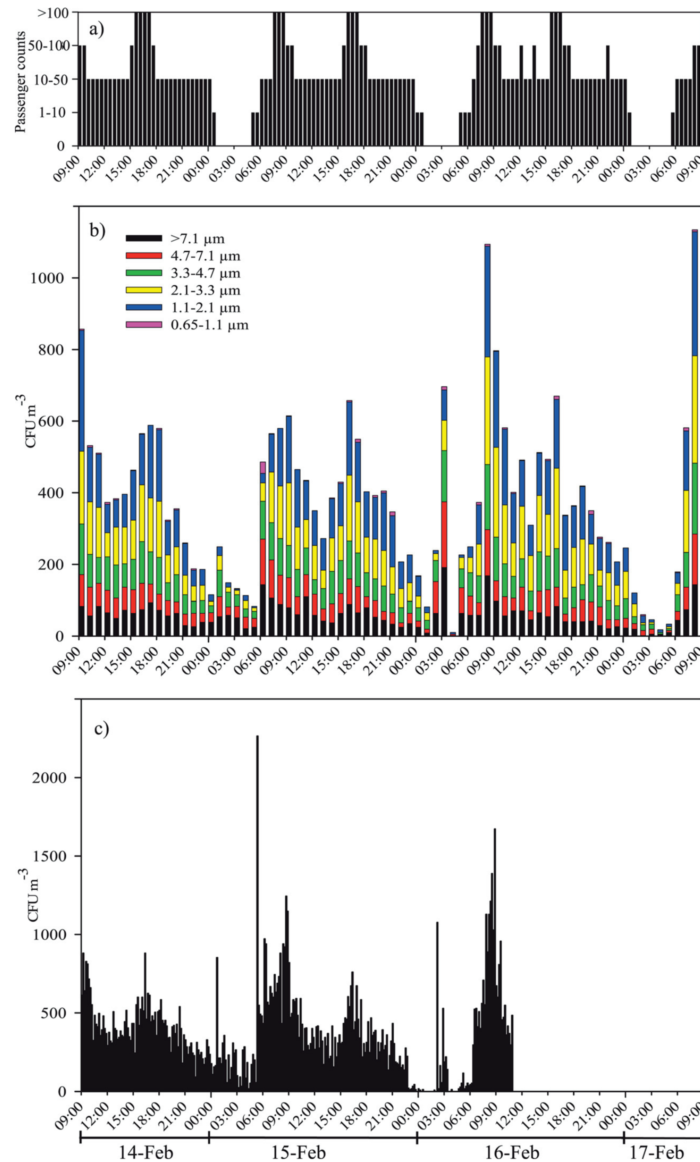

FIG 1 Airborne cultivable bacterial concentration levels and passenger counts during the 72-h sampling campaign at the subway station. (a) Numbers of passengers on the westbound concourse (30-min average). (b) Particle size-resolved airborne bacterial levels based on data from the Andersen six-stage cascade impactor. (c) High-temporal-resolution airborne bacterial levels based on data from the MAS-100 impactor. MAS-100 sampling was discontinued on 16 February at 11:00 due to a power supply failure.

Airborne cultivable-bacterium-containing particle size distribution. The average particle size-resolved bacterial levels reported by the Andersen six-stage cascade impactor were as follows: stage 1 (>7.1 μm), 58 ± 36 CFU m^{-3}; stage 2 (4.7 to 7.1 μm), 50 ± 33 CFU m^{-3}; stage 3 (3.3 to 4.7 μm), 69 ± 38 CFU m^{-3}; stage 4 (2.1 to 3.3 μm), 93 ± 67 CFU m^{-3}; stage 5 (1.1 to 2.1 μm), 105 ± 78 CFU m^{-3}; and stage 6 (0.65 to 1.1 μm), 3 ± 4 CFU m^{-3}. These results showed that the various particle sizes contributed to 17, 15, 19, 22, 26, and ≤1% of the total level, respectively (Fig. 1).

The bacterial levels reported within each particle size stage correlated significantly with the total level (r = 0.33 to 0.95). By

TABLE 1 Concentration levels and particle size distribution of airborne cultivable bacteria and total particles in the subway station[a]

Time period (hh:mm)	Bacterial level (mean CFU m^{-3} ± SD) for indicated sampler and location				Bacterial level (mean % contribution to total bacterial level ± SD) in airborne particle size (μm) stage:						Total particle level (mean particles m^{-3} ± SD)	Particle size distribution (0.5–20 μm) (mean ± SD)[c]	
	In station:		SASS 3100[b]										
	Andersen	MAS-100	In station	Outdoors	1 (>7.1)	2 (4.7–7.1)	3 (3.3–4.7)	4 (2.1–3.3)	5 (1.1–2.1)	6 (0.65–1.1)		CMAD (μm)	GSD
00:00–01:00	176 ± 54	79 ± 81			19 ± 11	15 ± 6	20 ± 2	20 ± 8	25 ± 7	<1	$3.2 \times 10^7 \pm 1.8 \times 10^7$	0.72 ± 0.04	1.56 ± 0.11
01:00–02:00	101 ± 19	111 ± 127			16 ± 5	16 ± 4	25 ± 7	24 ± 6	18 ± 6	<1	$4.0 \times 10^7 \pm 2.6 \times 10^7$	0.70 ± 0.05	1.48 ± 0.12
02:00–03:00	104 ± 44	152 ± 104			23 ± 15	25 ± 9	26 ± 1	9 ± 1	15 ± 13	2 ± 3	$4.0 \times 10^7 \pm 2.3 \times 10^7$	0.78 ± 0.10	1.62 ± 0.22
03:00–04:00	89 ± 43	114 ± 100	25 ± 22		26 ± 11	27 ± 3	25 ± 4	11 ± 1	10 ± 5	1 ± 1	$3.4 \times 10^7 \pm 2.7 \times 10^7$	0.72 ± 0.03	1.45 ± 0.10
04:00–05:00	47 ± 47	67 ± 80			25 ± 6	25 ± 10	23 ± 10	13 ± 4	13 ± 8	<1	$2.1 \times 10^7 \pm 5.8 \times 10^6$	0.70 ± 0.02	1.39 ± 0.07
05:00–06:00	114 ± 82	362 ± 605			30 ± 1	26 ± 7	21 ± 4	14 ± 1	9 ± 8	<1	$2.4 \times 10^7 \pm 8.3 \times 10^6$	0.76 ± 0.05	1.63 ± 0.17
06:00–07:00	304 ± 131	499 ± 276			26 ± 3	20 ± 5	25 ± 2	15 ± 3	11 ± 5	3 ± 3	$3.6 \times 10^7 \pm 1.0 \times 10^7$	0.86 ± 0.08	1.73 ± 0.04
07:00–08:00	506 ± 95	656 ± 171	493 ± 153	41 ± 17	16 ± 2	14 ± 4	18 ± 1	26 ± 3	26 ± 5	1 ± 1	$3.4 \times 10^7 \pm 8.7 \times 10^6$	0.81 ± 0.06	1.67 ± 0.04
08:00–09:00	936 ± 252	1073 ± 294			14 ± 1	13 ± 1	17 ± 1	26 ± 1	29 ± 1	<1	$3.2 \times 10^7 \pm 6.4 \times 10^6$	0.75 ± 0.03	1.61 ± 0.06
09:00–10:00	756 ± 103	666 ± 149			12 ± 1	10 ± 3	15 ± 1	28 ± 3	34 ± 4	<1	$3.7 \times 10^7 \pm 6.3 \times 10^6$	0.80 ± 0.06	1.64 ± 0.04
10:00–11:00	526 ± 48	435 ± 104			11 ± 1	12 ± 2	16 ± 1	27 ± 1	33 ± 3	<1	$3.7 \times 10^7 \pm 5.2 \times 10^6$	0.79 ± 0.05	1.66 ± 0.04
11:00–12:00	449 ± 46	375 ± 67			20 ± 4	12 ± 2	15 ± 1	23 ± 4	29 ± 4	<1	$3.8 \times 10^7 \pm 7.3 \times 10^6$	0.78 ± 0.04	1.64 ± 0.04
12:00–13:00	405 ± 62	357 ± 68			16 ± 1	16 ± 2	18 ± 6	25 ± 5	25 ± 3	<1	$4.3 \times 10^7 \pm 7.1 \times 10^6$	0.82 ± 0.05	1.66 ± 0.05
13:00–14:00	322 ± 46	313 ± 70			14 ± 1	12 ± 3	21 ± 3	26 ± 5	27 ± 5	<1	$4.3 \times 10^7 \pm 1.2 \times 10^7$	0.81 ± 0.06	1.65 ± 0.05
14:00–15:00	431 ± 58	356 ± 94			14 ± 4	14 ± 2	20 ± 3	27 ± 3	25 ± 2	<1	$4.1 \times 10^7 \pm 7.7 \times 10^6$	0.79 ± 0.05	1.62 ± 0.06
15:00–16:00	462 ± 26	446 ± 111			13 ± 2	14 ± 1	20 ± 1	23 ± 1	29 ± 1	<1	$4.1 \times 10^7 \pm 7.3 \times 10^6$	0.79 ± 0.06	1.63 ± 0.07
16:00–17:00	631 ± 46	597 ± 113			13 ± 1	10 ± 2	18 ± 2	30 ± 3	28 ± 3	<1	$4.3 \times 10^7 \pm 1.0 \times 10^7$	0.76 ± 0.05	1.59 ± 0.07
17:00–18:00	492 ± 110	408 ± 108			13 ± 2	9 ± 3	15 ± 2	25 ± 1	37 ± 6	<1	$4.5 \times 10^7 \pm 8.4 \times 10^6$	0.78 ± 0.06	1.63 ± 0.07
18:00–19:00	449 ± 94	421 ± 86			14 ± 4	9 ± 1	17 ± 1	27 ± 2	33 ± 1	<1	$4.4 \times 10^7 \pm 9.1 \times 10^6$	0.78 ± 0.07	1.62 ± 0.08
19:00–20:00	378 ± 40	332 ± 67			14 ± 3	13 ± 1	14 ± 2	28 ± 3	31 ± 3	1 ± 1	$4.5 \times 10^7 \pm 8.8 \times 10^6$	0.79 ± 0.09	1.62 ± 0.08
20:00–21:00	370 ± 25	316 ± 107			14 ± 3	10 ± 4	20 ± 2	24 ± 1	31 ± 7	2 ± 1	$4.4 \times 10^7 \pm 1.0 \times 10^7$	0.80 ± 0.11	1.62 ± 0.09
21:00–22:00	294 ± 38	265 ± 71			11 ± 11	13 ± 4	19 ± 3	20 ± 3	36 ± 4	2 ± 1	$4.3 \times 10^7 \pm 1.4 \times 10^7$	0.79 ± 0.11	1.61 ± 0.10
22:00–23:00	218 ± 31	196 ± 81			11 ± 2	12 ± 6	20 ± 1	26 ± 3	30 ± 5	1 ± 1	$3.9 \times 10^7 \pm 7.7 \times 10^6$	0.75 ± 0.06	1.58 ± 0.11
23:00–00:00	206 ± 17	130 ± 116			16 ± 3	12 ± 2	18 ± 2	24 ± 2	30 ± 4	<1	$3.3 \times 10^7 \pm 1.0 \times 10^7$	0.73 ± 0.04	1.58 ± 0.10

[a] Data from the 72-h sampling campaign are presented as 1-h averages over the diurnal period, unless otherwise indicated.
[b] Two-hour averages: the SASS 3100 sampled for 2 h during the nighttime (03:00 to 05:00) and daytime (07:00 to 09:00).
[c] CMAD, count median aerodynamic diameter; GSD, geometric standard deviation.

comparing the different stages to each other, significant correlations were also observed between stages ($r = 0.38$ to 0.94), except when stage 4 or 5 was compared to stage 6 ($r = 0.17$ and $r = 0.20$, respectively). The general trend was that the correlation strength was inversely related to the distance between the stages (i.e., neighboring stages correlated better than distant stages) (Fig. 1). The particle size-resolved bacterial levels also showed significant correlation ($r = 0.63$ to 0.89) between sampling days (Fig. 1), except for stage 2 on 14 February compared to 16 February and for stage 6 on all sampling days. The weak correlation observed between 14 February and 16 February for stage 2 could be explained by a higher variability in this stage on 16 February (Fig. 1). The weak correlations observed between all sampling days for stage 6 could be explained by frequent observations of bacterial levels that were ≤LOD, with sporadic increases on some occasions (Fig. 1). Based on the observed general temporal consistency concerning the various particle sizes' contributions to the total level, the results from individual sampling days were merged into a single diurnal period averaged hourly (Table 1).

Compared between sampling days, the size distribution of airborne bacterium-containing particles showed no significant differences, and similar results were obtained when different daytime periods (rush hours versus non-rush hours) were compared to each other (see Fig. S1 in the supplemental material). Taken together, these results suggested that the size distribution of airborne bacterium-containing particles at the station showed limited day-to-day variation and was also conserved between different daytime periods. The largest temporal variability concerning the size distribution of airborne bacterium-containing particles was consistently observed during two diurnal periods, (i) the daytime-to-nighttime transition period and (ii) the nighttime-to-daytime transition period (Table 1).

When daytime and nighttime periods were compared to each other, significant differences were revealed regarding the size distribution of airborne bacterium-containing particles in the station (Fig. 2). Bacterium-containing particles of between 1.1 and 3.3 μm corresponded to a significantly larger fraction during the daytime (56% ± 4% [mean ± standard deviation]) than at night (30% ± 8%), while a significantly greater fraction of bacterium-containing particles of >3.3 μm was observed at night (70% ± 7%) than during the day (44% ± 3%) (Fig. 2). By taking into account the general trend showing low and decaying bacterial levels at night (Fig. 1), the results suggested that the nighttime shift in size distribution was caused by a reduction in 1.1- to 3.3-μm bacterium-containing particles rather than an increase in >3.3-μm bacterium-containing particles. Taken together, these results suggested that the contributing sources for 1.1- to 3.3-μm bacterium-containing particles were predominantly present in the station only during daytime.

When the size distribution of bacterium-containing particles observed during nighttime atypical events (16 February from 02:00 to 04:00 and 15 February at 01:00) was compared to those observed during the temporally closest daytime periods showing corresponding total bacterial levels (15 February at 23:00, 16 February at 16:00, and 14 February at 21:00, respectively), bacterium-containing particles of >3.3 μm were found to be present as a significantly larger fraction during the nighttime atypical events (79% ± 4%) than during the corresponding daytime periods (44% ± 3%) (Fig. 2). The opposite result was observed for 1.1- to 3.3-μm bacterium-containing particles, which showed a significantly smaller fraction during the nighttime atypical events (21% ± 3%) than during the corresponding daytime periods (56% ± 4%) (Fig. 2). Taken together, these results suggested that the nighttime atypical events were caused by sources that generated a larger fraction of bacterium-containing particles of >3.3 μm and that these sources probably differed from the predomi-

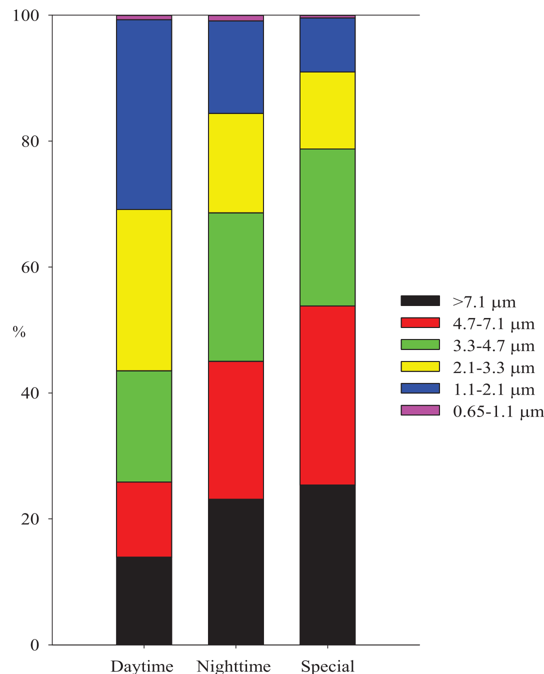

FIG 2 Size distribution of airborne cultivable-bacterium-containing particles at the subway station. Size-resolved airborne cultivable bacterial levels were obtained with the Andersen six-stage cascade impactor, expressed as percentages of the total level and categorized into three groups: daytime, nighttime, and atypical (special) nighttime events (16 February from 02:00 to 04:00 and 15 February at 01:00).

nant daytime sources, which generated a larger fraction of bacterium-containing particles between 1.1 and 3.3 μm.

Airborne cultivable bacterial diversity. The airborne bacterial diversity in the station was investigated by analyzing representative bacterial isolates using the Biotyper 3.0 MALDI-TOF MS system. A total of 1,832 bacterial isolates were recovered from the primary cultivation plates of 17 Andersen and 9 SASS 3100 samples and analyzed using the Biotyper 3.0 standard direct transfer method. The results showed that 1,293 isolates (71% of the total) were successfully classified at the species or genus level. Of the total number of isolates classified, 1,141 and 152 isolates were derived from the Andersen and SASS 3100 samples, respectively. The isolates recovered from the Andersen impactor consisted of 17% Gram-negative bacteria, while only 4% Gram negatives were recovered from the SASS 3100 electret filter sampler.

When considering the total bacterial diversity from both samplers, 39 different bacterial genera belonging to the following four bacterial phyla were observed at the station: *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteriodetes*/*Chlorobi* (Table 2). The predominant bacterial genera were *Micrococcus*, *Rhodococcus*, *Arthrobacter*, *Moraxella*, *Staphylococcus*, *Bacillus*, *Pseudomonas*, *Kocuria*, and *Dermacoccus*, corresponding to the following bacterial species: *Micrococcus luteus*, *Rhodococcus fascians*, *Arthrobacter* spp. (e.g., *Arthrobacter parietis*), *Moraxella osloensis*, *Staphylococcus* spp. (e.g., *Staphylococcus epidermis*), *Bacillus* spp. (e.g., *Bacillus megaterium*), *Pseudomonas* spp. (e.g., *Pseudomonas stutzeri*), *Kocuria* spp. (e.g., *Kocuria rosea*), and *Dermacoccus nishinomiyaensis* (Table 2).

Of the total number of SASS 3100-derived isolates classified, 60% were pigmented (92/152), showing yellow, orange, or pink colony pigmentation, and the predominant species were *M. luteus*, *R. fascians*, and *Rosemonas mucosa*/*K. rosea*, respectively. Of the total number of Andersen-derived isolates classified, 44% were pigmented (507/1,141), showing yellow, orange, or pink colony pigmentation, and the predominant species were *M. luteus*/*A. parietis*/*Kocuria marina*/*Kocuria rhizophila*, *R. fascians*/*Bacillus atrophaeus*, and *R. mucosa*/*K. rosea*, respectively.

The Andersen-derived bacterial diversity showed only limited day-to-day variation (see Fig. S2 in the supplemental material). Similarly, the Andersen-derived data for various daytime periods (morning rush hour, 08:00; noon non-rush period, 12:00; afternoon rush hour, 16:00; and evening non-rush period, 21:00) also corresponded to each other (see Fig. S2). These results suggested that the airborne bacterial diversity at the station was conserved between sampling days, as well as between different daytime periods. The Andersen-derived diversity results were therefore categorized into three groups, (i) daytime, (ii) nighttime, and (iii) nighttime atypical event (16 February at 03:00). Similarly, the SASS 3100-derived diversity results were also categorized into three groups, but the daytime outdoor reference location group replaced the nighttime station atypical event group. The results obtained with both samplers showed that the daytime bacterial diversity at the station differed significantly from the nighttime diversity (Fig. 3).

The Andersen-derived daytime diversity was dominated by *Micrococcus* (37%), followed by *Rhodococcus* (14%), *Arthrobacter* (11%), *Moraxella* (9%), *Staphylococcus* (6%), and *Dermacoccus* (5%), while the nighttime diversity was dominated by *Rhodococcus* (49%), followed by *Arthrobacter* (20%) and *Micrococcus* (8%) (Fig. 3). The diversity observed during the atypical event on 16 February at 03:00 was similar to the diversity of nonevent nighttime samples, being dominated by *Rhodococcus* (45%) and *Arthrobacter* (27%) (Fig. 3). However, no *Micrococcus* isolates were recovered during the nighttime atypical event, while *Pseudomonas* isolates (16%) were abundantly recovered. Interestingly, *Pseudomonas* was not observed during nonevent nighttime periods and was only at low abundance during daytime periods (3%).

The SASS 3100-derived daytime diversity at the station was dominated by *Micrococcus* (37%), followed by *Rhodococcus* (17%), *Staphylococcus* (14%), *Bacillus* (12%), and *Dermacoccus* (6%), while the nighttime diversity was dominated by *Rhodococcus* (46%), followed by *Staphylococcus* (17%), *Arthrobacter* (13%), and *Bacillus* (13%) (Fig. 3). The relative abundances of *Micrococcus*, *Rhodococcus*, and *Dermacoccus* observed with the SASS 3100 were consistent with the Andersen-derived results. However, *Bacillus* and *Staphylococcus* were observed more frequently with the SASS 3100 than with the Andersen impactor, while *Arthrobacter* (1%) and *Moraxella* (1%) were less frequently observed with the SASS 3100 (Fig. 3).

When the SASS 3100-derived daytime diversity in the station was compared to the daytime outdoor diversity, a higher abundance of *Micrococcus* (58%) and lower abundances of *Rhodococcus* (8%), *Staphylococcus* (8%), and *Bacillus* (8%) were observed at the outdoor location (Fig. 3). Additionally, several minor differences were observed for bacterial genera that were generally observed at

TABLE 2 Airborne cultivable bacterial diversity in the subway station

Genus[a]	Species	Occurrence using indicated sampler in:		
		Current study[b]		Previous study, SASS 3100[c]
		Andersen	SASS 3100	
Acinetobacter	*A. johnsonii*, *A. lwoffii*, *A. pittii*, *A. nosocomialis*, *A. schindleri*, *A. towneri*	X	X	
Aerococcus		X		
Agrococcus	*A. jenensis*	X		
Agromyces			X	
Arthrobacter	*A. parietis*, *A. scleromae*, *A. polychromogenes*, *A. oxydans*, *A. sulfonivorans*, *A. chlorophenolicus*, *A. castelli*, *A. crystallopoietes*	X	X	X
Bacillus	*B. megaterium*, *B. atrophaeus*, *B. simplex*, *B. flexus*, *B. pumilus*, *B. cereus sensu lato* group species	X	X	X
Brevibacillus	*B. choshinensis*	X		X
Brevibacterium		X		
Brevundimonas	*B. nasdae*, *B. vesicularis*, *B. diminuta*	X		X
Chryseobacterium		X		
Corynebacterium	*C. flavescens*	X		X
Curtobacterium	*C. flaccumfaciens*		X	X
Dermacoccus	*D. nishinomiyaensis*	X	X	X
Dietzia		X		X
Enterococcus	*E. gallinarum*	X		
Kocuria	*K. rosea*, *K. palustris*, *K. rhizophila*, *K. marina*, *K. polaris*	X	X	X
Lysinibacillus	*L. fusiformis*, *L. sphaericus*	X	X	X
Macrococcus		X		
Massilia	*M. timonae*	X	X	
Microbacterium	*M. phyllosphaerae*, *M. lacticum*	X		X
Micrococcus	*M. luteus*	X	X	X
Moraxella	*M. osloensis*	X	X	X
Ochrobactrum	*O. intermedium*	X		
Paenibacillus	*P. pasadenensis*, *P. amylolyticus*	X	X	X
Paracoccus	*P. yeei*	X		X
Pseudoclavibacter	*P. helvolus*	X		
Pseudomonas	*P. stutzeri*, *P. xanthomarina*, *P. gessardii*, *P. brenneri*, *P. libanensis*	X	X	X
Psychrobacillus		X		
Rhizobium	*R. rubi*	X		
Rhodococcus	*R. fascians*	X	X	X
Roseomonas	*R. mucosa*	X	X	X
Rothia	*R. amarae*	X		X
Sphingobacterium	*S. multivorum*	X		
Sphingobium		X		
Sphingomonas	*S. aerolata*	X		X
Staphylococcus	*S. aureus*, *S. hominis*, *S. capitis*, *S. haemolyticus*, *S. epidermis*, *S. saprophyticus*, *S. warneri*, *S. equorum*	X	X	X
Stenotrophomonas	*S. maltophilia*	X		
Streptococcus	*S. salivarius*	X		
Streptomyces	*S. badius*, *S. griseus*	X	X	X
Total		37	17	22

[a] The most-abundant bacterial genera in the current study are highlighted in boldface.
[b] Results were obtained using the Biotyper 3.0 MALDI-TOF MS system.
[c] Dybwad et al. (16); results were obtained using the Biotyper 2.0 MALDI-TOF MS system and partial 16S rRNA gene sequencing. Only genera corresponding to the current study are presented.

low frequencies. At the outdoor location, *Dermacoccus* (8%), *Roseomonas* (4%), and *Lysinibacillus* (4%) were more abundant than they were in the station, while *Acinetobacter* (1%), *Arthrobacter* (1%), *Massilia* (1%), *Moraxella* (1%), *Paenibacillus* (1%), *Pseudomonas* (2%), *Streptomyces* (2%), and *Agromyces* (1%) were only observed in the station (Fig. 3).

In an attempt to investigate particle size-dependent diversity differences, the 1,141 successfully classified Andersen-derived isolates were also categorized with respect to particle size based on the size-resolved stages of the Andersen sampler. The analysis was done without temporal considerations due to the limited number of isolates obtained during the nighttime periods. The size distribution observed for the isolates classified were as follows: stage 1 (>7.1 μm), 20% (228/1,141); stage 2 (4.7 to 7.1 μm), 18% (208/

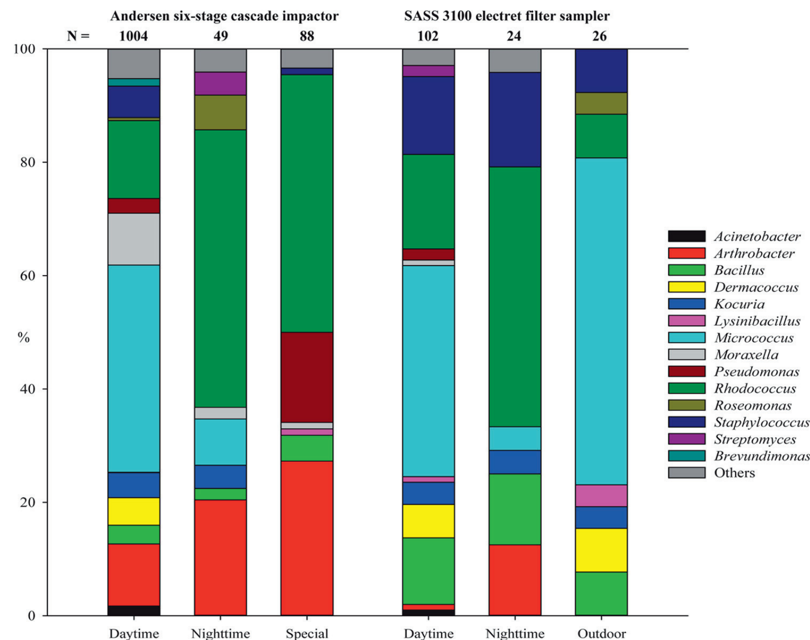

FIG 3 Airborne cultivable bacterial diversity in the subway station. The airborne bacterial diversity was based on Biotyper 3.0 MALDI-TOF MS analysis of representative bacterial isolates from 17 Andersen six-stage cascade impactor samples and 9 SASS 3100 electret filter samples. Andersen-derived results were categorized into three groups, daytime, nighttime, and nighttime atypical (special) events (16 February at 03:00) at the station. SASS 3100-derived results were categorized into three groups: daytime and nighttime in the station and daytime at the outdoor reference location. The number of bacterial isolates (N) classified within each category is also presented.

1,141); stage 3 (3.3 to 4.7 μm), 19% (218/1,141); stage 4 (2.1 to 3.3 μm), 19% (216/1,141); stage 5 (1.1 to 2.1 μm), 22% (253/1,141); and stage 6 (0.65 to 1.1 μm), 2% (18/1,141). The results showed that a very limited number of isolates were recovered from the 0.65- to 1.1-μm size range. However, by taking into account only major differences, the relative abundance of *Micrococcus* was low in stage 6 compared to its abundance in the other stages, while *Kocuria*, *Acinetobacter*, *Moraxella*, and *Pseudomonas* isolates were not observed in stage 6 (Fig. 4). Additionally, the relative abundance of *Arthrobacter*, *Bacillus*, *Rhodococcus*, *Dermacoccus*, *Brevundimonas*, and *Microbacterium* seemed to be higher in stage 6 than in the other stages, although for *Brevundimonas* (5%) and *Microbacterium* (5%), the abundance estimates were based on the observation of a single isolate (Fig. 4). When stage 6 was not taken into account, the observed bacterial diversity was similar for all other stages (Fig. 4). The only exception was an increased relative abundance of *Micrococcus* in the stages corresponding to particles of between 1.1 and 3.3 μm (stages 4 and 5) compared to particles of >3.3 μm (stages 1 to 3) (Fig. 4). These results suggested that the airborne cultivable bacterial diversity at the station was fairly conserved for bacterium-containing particles of different sizes greater than 1.1 μm (Fig. 4).

Total particle concentration level and size distribution. The average total particle level (mean ± standard deviation) at the station was $3.8 \times 10^7 \pm 1.4 \times 10^7$ particles m^{-3} (0.5 to 20 μm). The average count median aerodynamic diameter (CMAD) and geometric standard deviation (GSD) (mean ± standard deviation) were 0.77 ± 0.07 μm and 1.60 ± 0.11, respectively. The total particle levels were generally stable during the daytime periods, except for some sporadic short-duration events with strongly increased particle loads (Fig. 5). The daytime particle levels did not show a consistent temporal pattern that corresponded to the daytime bacterial levels (Fig. 5). The total particle levels generally decayed during the late evening period and continued to decay throughout the night, consistent with the general trend observed for the bacterial levels during the same period. The results suggested that the underlying baseline particle level was lower at night than in daytime but that the observed nighttime particle level variability was higher than the daytime variability, showing multiple short- and long-duration events with increased particle loads that even exceeded the daytime maximum levels (Fig. 5). The nighttime events showing increased particle loads commonly coincided with periods of increased maintenance activities at the station or in the adjacent tunnel network. Based on the observed diurnal consistencies, the total particle levels from the individual sampling days were merged into a single diurnal period averaged hourly (Table 1).

The average daytime total particle level was $3.9 \times 10^7 \pm 1.0 \times 10^7$ particles m^{-3}, while the average nighttime level was $3.3 \times 10^7 \pm 2.2 \times 10^7$ particles m^{-3}. The average daytime CMAD and GSD were 0.79 ± 0.07 μm and 1.63 ± 0.08, respectively, while the average nighttime CMAD and GSD were 0.72 ± 0.06 μm and 1.50 ± 0.16, respectively. No significant differences were observed with respect to total particle levels or particle size distributions

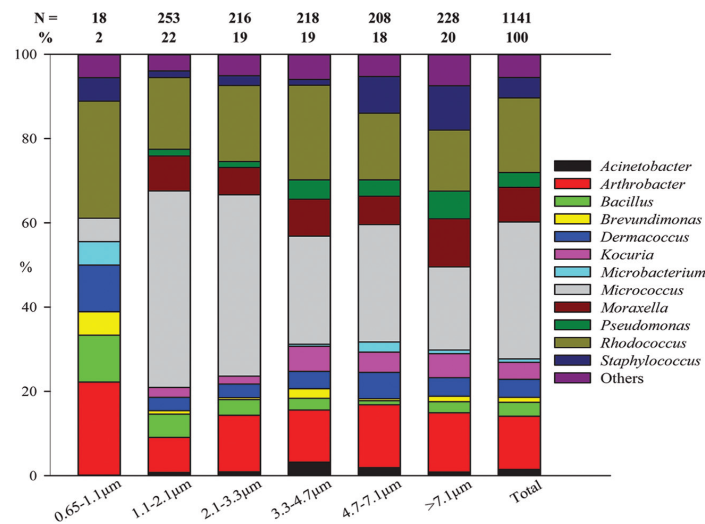

FIG 4 Particle size-resolved airborne cultivable bacterial diversity at the subway station. The particle size-resolved bacterial diversity data are based on Biotyper 3.0 MALDI-TOF MS analysis of representative bacterial isolates from 17 Andersen six-stage cascade impactor samples. The number of bacterial isolates (N) classified within each size bin and in total are presented.

when the daytime and nighttime periods were compared to each other (Table 1). Since the total particle levels did not show a temporal pattern that could be correlated with the bacterial levels, an attempt was made to investigate whether a high abundance of small particles could be masking an underlying correlation. Three different particle size ranges were extracted from the measured range (0.5 to 20 μm), as follows: (i) particles of ≥0.5 μm (i.e., total), (ii) particles of ≥1 μm, and (iii) particles of ≥3.3 μm. However, even after excluding particles of <1 μm and <3.3 μm, the correlation between the temporal variation profile of the total particle levels and the bacterial levels did not improve (Fig. 5).

Meteorological conditions. The average meteorological conditions (mean ± standard deviation) at the station were as follows: (i) temperature, 7.5 ± 0.4°C; (ii) relative humidity, 63 ± 8.4%; (iii) wind speed, 0.4 ± 0.2 m s^{-1}; and (iv) wind direction, 173 ± 69° with 0° west (i.e., wind from east toward west). The temperatures at the station did not show consistent diurnal or day-to-day trends, except that rapid fluctuations seemed to occur more frequently during the day than at night (Fig. 6). The humidity levels showed several consistent diurnal trends, including (i) lower levels at night than during the day, (ii) rapidly fluctuating but stable or increasing average daytime levels, and (iii) decaying levels during the late evening and nighttime periods, showing less fluctuation than in the daytime period (Fig. 6). Both the daytime and nighttime humidity levels showed day-to-day variation in terms of an overall increase throughout the sampling campaign, with the largest change observed for the nighttime levels (Fig. 6). The wind speed levels followed a trend similar to that of the humidity levels, i.e., (i) lower levels at night than during the day, (ii) rapid fluctuations during daytime, and (iii) rapid decay during the early nighttime period. The rapid drop in wind speed coincided with the daily shutdown of train operations, suggesting that train-induced piston effects were the main driving force (Fig. 6). During the nighttime, when no trains were running, the air movement was almost exclusively from east toward west (Fig. 6). During the daytime, the air movement fluctuated rapidly between all directions, although the dominant direction was the same as during night (Fig. 6). These results supported the hypothesis that train-induced piston effects were the main driving force behind the speed, as well as the direction, of air movements at the station but also suggested that a stable air movement from east toward west was present independent of the train activity. The average outdoor meteorological conditions were representative of typical winter conditions in the region and remained stable throughout the sampling campaign, i.e., (i) temperature of −7.4 ± 1.6°C, (ii) relative humidity of 79.0 ± 4.8%, (iii) wind speed of 4.1 ± 0.6 m s^{-1}, wind direction 36 ± 10° with 0° north (i.e., wind from the north-northeast), (iv) cloud cover of ≥90%, and (v) snow depth of ~0.5 m.

DISCUSSION

The current study characterized airborne bacteria during a 72-h continuous sampling campaign at a Norwegian subway station. The results obtained contribute novel information concerning the airborne bacterial community encountered in a subway station environment, including previously unaddressed properties like the size distribution of airborne bacterium-containing particles and the temporal variability of airborne bacteria with respect to their concentration level, size distribution, and diversity on several different timescales. Additionally, the results contributed to confirm and extrapolate results from previous efforts to characterize airborne bacteria at subway stations (5–9, 11, 12, 15, 16). The diurnal and day-to-day variation was specifically addressed in the current work (winter 2011), while seasonal variability assessments were made possible by including results obtained during our previous study (16) at the same station (spring, summer, and fall of 2010).

The results reported here may assist in the development of BIODIM equipment and enable improved T&E schemes involv-

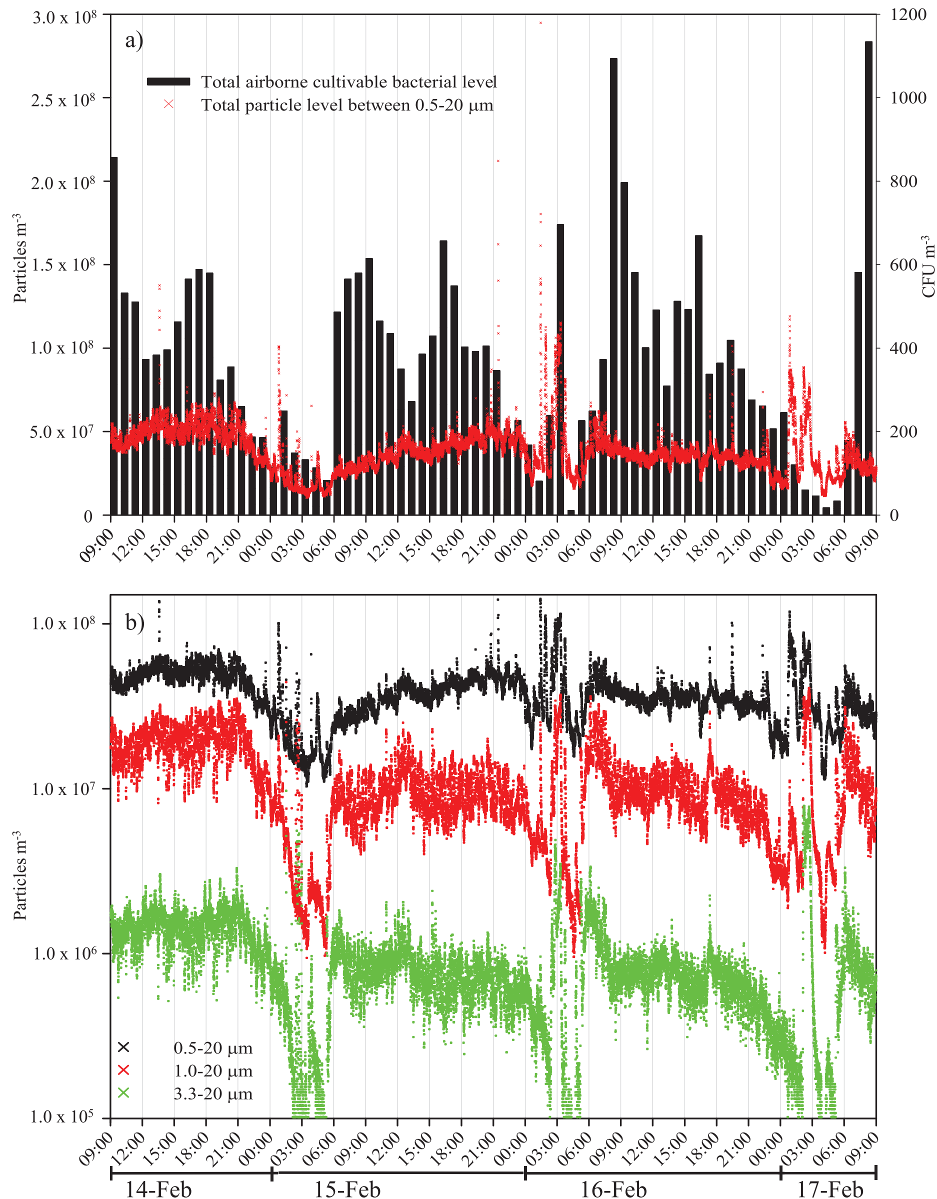

FIG 5 Total particle concentration levels during the 72-h sampling campaign at the subway station. (a) Levels of total particles between 0.5 and 20 μm obtained using the APS 3321 aerodynamic particle sizer (left *y* axis) and airborne cultivable bacterial levels obtained using the Andersen six-stage cascade impactor (right *y* axis). (b) Total levels of particles in various size ranges (indicated by the key), obtained using the APS 3321.

ing simulated operational conditions, including realistic bioaerosol backgrounds, during controlled aerosol chamber-based challenge tests with biological threat agents. Furthermore, the knowledge generated may also be of great interest to the public health, occupational health, and microbial ecology communities.

Airborne bacterial levels. Previous characterization efforts at subway stations have reported airborne cultivable bacterial levels ranging from not detected to 10^4 CFU m^{-3} (5–9, 11, 12, 15, 16). In the current work, the airborne cultivable bacterial levels, based on sampling with three air samplers possessing different properties

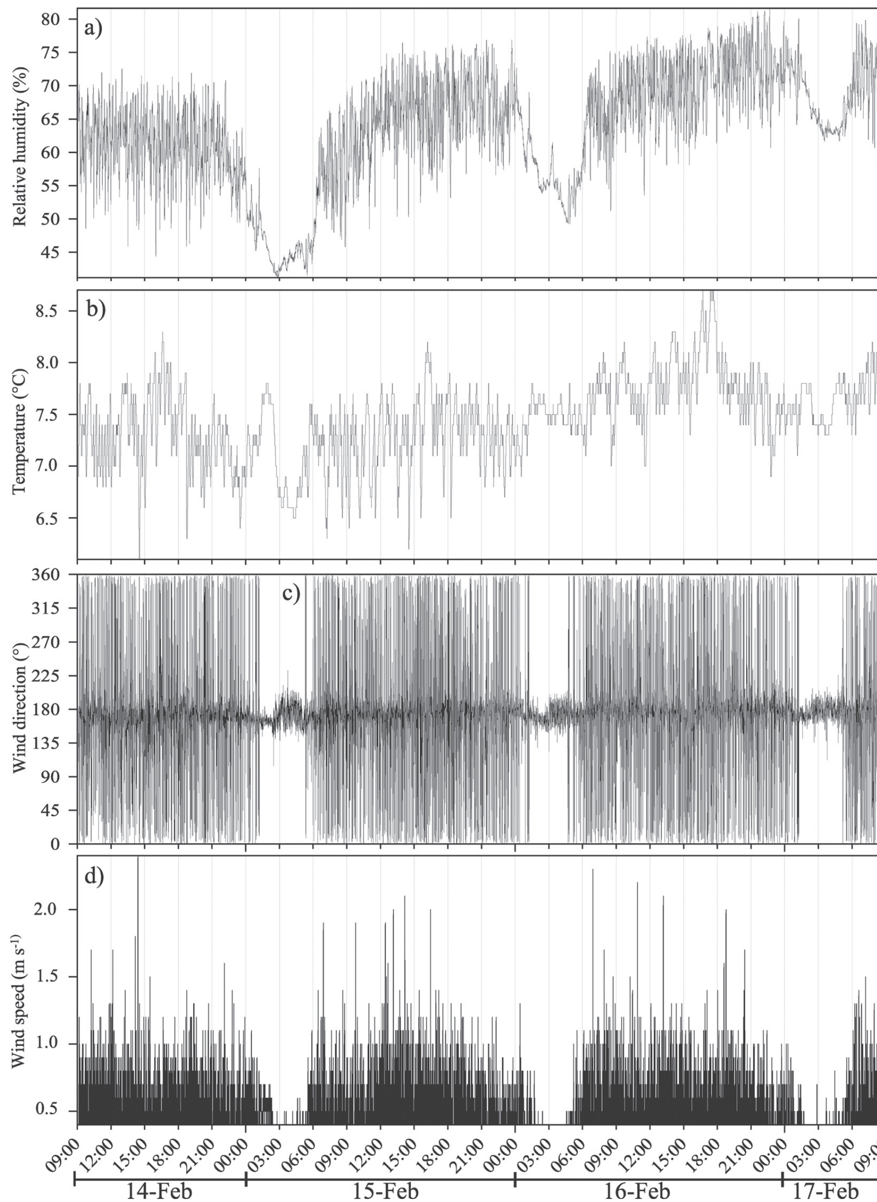

FIG 6 Meteorological conditions during the 72-h sampling campaign at the subway station. The condition measured is indicated on the *y* axis of each panel. In panel c, 180° corresponds to wind directly from east to west.

(Andersen, MAS-100, and SASS 3100), ranged from not detected to 10^3 CFU m^{-3}.

A consistent diurnal pattern regarding the levels of airborne cultivable bacteria was observed at the station, as follows: (i) the daytime levels were higher than the nighttime levels, (ii) the diurnal maximum levels were generally observed during the morning and afternoon rush hours, which both showed increased levels compared to the noon and evening non-rush periods, and (iii) following the afternoon rush hours, the bacterial levels generally decayed throughout the evening and nighttime periods, with diurnal minimum levels just before the train activity recommenced in the morning.

To the authors' knowledge, the diurnal variability of airborne bacteria in a subway station environment has not previously been addressed in detail. However, Birenzvige et al. (3) characterized the diurnal variation of the aerosol background at the Pentagon subway station (Washington, DC) and also attempted to address the diurnal variation of airborne bacteria. Unfortunately, airborne bacterial concentration levels could not be established due to an erratic air sampler, although some diurnal trends were proposed (3). A comparison between the diurnal concentration profile of airborne bacteria observed in the current work and the trends proposed by Birenzvige et al. (3) suggested that the profiles at both stations were highly similar. The observed diurnal concentration profiles of airborne bacteria were also found to correspond to the diurnal PM10 (airborne particulate matter of <10 μm) mass concentration profile reported by Salma et al. (18) from a subway station in Budapest, Hungary.

On two separate occasions during the current sampling campaign, atypical nighttime events deviating from the general nighttime trend occurred. The atypical events showed temporarily increased bacterial levels, which commonly exceeded daytime levels except for the rush hour maxima. The atypical events seemed to coincide with periods of increased maintenance activities at the station or in the adjacent tunnel network.

The largest temporal variability of the airborne bacterial levels was consistently observed during two distinct diurnal periods at the subway station, i.e., (i) the nighttime-to-daytime transition period, when the diurnal minimum levels at the end of the night were followed by a rapid increase in anthropogenic activities (i.e., passengers and trains) and the diurnal maximum levels during the peak morning rush hours, and (ii) the two nighttime atypical events, which showed increased bacterial levels that deviated from the general nighttime trend of low and decaying levels. During the nighttime-to-daytime transition period, the largest observed differences in the bacterial levels of adjacent samples were 20- and 40-fold, based on hourly (Andersen) and 10-min (MAS-100) sampling frequencies, respectively. Similarly, during the nighttime atypical events, the largest differences between adjacent samples were 66- and 270-fold, based on the Andersen and MAS-100 results, respectively. These results demonstrated that the airborne bacterial levels in subway stations may be associated with significant temporal variation over a short period of time and that such changes occur as a consequence of both predictable (e.g., nighttime-to-daytime transition periods) and unpredictable (e.g., atypical nighttime events) events.

The average daytime levels of cultivable airborne bacteria found at the station were 452 ± 198 (Andersen), 443 ± 275 (MAS-100), and 493 ± 153 (SASS 3100) CFU m^{-3}. In addition to being highly consistent when compared, these results were also in close agreement with the daytime level (396 ± 93 CFU m^{-3}) reported by the SASS 3100 during the previous study (16) at the same station. The average nighttime bacterial levels found in the current study were 107 ± 68 (Andersen), 103 ± 102 (MAS-100), and 25 ± 22 (SASS 3100) CFU m^{-3}. The average nighttime bacterial level (21 ± 13 CFU m^{-3}) reported by the SASS 3100 during the previous study (16) corresponded better to the SASS 3100 results in the current work than to the Andersen and MAS-100 results. This could be explained by the fact that the SASS 3100 collected a single 2-h nighttime sample during a period commonly corresponding to the diurnal minimum bacterial levels, while the Andersen and MAS-100 sampled throughout the night at an hourly and a 10-min sampling frequency, respectively. In summary, these results suggested that the airborne bacterial levels at the station appeared to be similar during both studies, with a consistent daytime-to-nighttime difference.

Considering the fact that the current study was performed during the winter season (February 2011), while the previous study (16) was performed during the spring, summer, and fall seasons (May to September 2010), the combined results suggested that the airborne bacterial levels at the station were conserved on a seasonal time scale. Additionally, the current results, which show a consistent diurnal pattern on three consecutive days, in combination with the previous results (16), which show consistent daytime and nighttime levels over a period of several months, suggested that the airborne bacterial levels at the station were also conserved on a day-to-day time scale.

Based on the fact that the station was nonoperational and closed to the public during the night, it could be suggested that anthropogenic activities, such as passengers and trains, were the major sources of airborne bacteria at the station. The increased bacterial levels observed during the morning and afternoon rush hours compared to the levels in the noon and evening periods corresponded to increased passenger numbers, while the train frequency remained constant. This result demonstrated more specifically that passengers were a major source of airborne bacteria at the station.

Airborne bacterium-containing particle size distribution. The size distribution of airborne particles containing cultivable bacteria has, to the authors' knowledge, not previously been studied in a subway environment. Lee et al. (8) described the use of an Andersen six-stage cascade impactor in various public environments, including a subway station, but did not report size-resolved results from the station. The size distribution of bacterium-containing particles can have an impact on the regional deposition of airborne bacteria in the human respiratory tract upon inhalation and, therefore, may be of importance for health hazard assessments (19). Furthermore, information concerning the size distribution of bacterium-containing particles in various operational environments is also of interest to the BIODIM community (2).

The current work revealed that airborne bacterium-containing particles of between 0.65 and 1.1 μm contributed on average less than 1% of such particles at the station, while particles of between 1.1 and 2.1, 2.1 and 3.3, 3.3 and 4.7, 4.7 and 7.1, and >7 μm contributed 26, 22, 19, 15, and 17% of the total level, respectively. The averaged results suggested that bacterium-containing particles of >1.1 μm were relatively evenly distributed between different particle sizes, although the maximum level was observed for particles of between 1.1 and 2.1 μm and ~50% of the particles were between 1.1 and 3.3 μm.

The size distribution of airborne bacterium-containing particles was found to be conserved between sampling days, as well as between different daytime periods (rush hours versus non-rush hours). The largest temporal variation in the size distribution of airborne bacterium-containing particles was consistently observed during two distinct diurnal periods at the station, i.e., (i) the daytime-to-nighttime transition period and (ii) the nighttime-to-daytime transition period.

Both the relative and absolute amounts of 1.1- to 3.3-μm bacterium-containing particles were found to be higher during the daytime than at night, suggesting that the contributing sources for

such particles were primarily present in the station during the daytime. Since the major difference between daytime and nighttime periods was an almost complete absence of passengers and trains during the latter, it is reasonable to suggest that anthropogenic activities were the predominant sources of 1.1- to 3.3-μm bacterium-containing particles at the station.

The relative amount of airborne bacterium-containing particles of >3.3 μm observed during the nighttime atypical events was found to be much higher than the amounts observed during the temporally closest daytime periods that showed comparable bacterial levels, while the reverse was observed for 1.1- to 3.3-μm particles. Interestingly, these observations suggested that the nighttime atypical events were caused by different sources than the daytime sources, which generated a larger relative amount of 1.1- to 3.3-μm airborne bacterium-containing particles.

Airborne bacterial diversity. To our knowledge, no previous study has investigated the temporal variability of the airborne bacterial diversity at a subway station. The airborne cultivable bacterial diversity at subway stations has been shown to consist largely of the bacterial genera *Micrococcus*, *Staphylococcus*, and *Bacillus*, but several other genera, such as *Pseudomonas*, *Corynebacterium*, *Paracoccus*, *Kocuria*, *Aerococcus*, *Moraxella*, and *Enterococcus*, have also been observed, although generally less consistently (3, 5, 7, 11, 15).

The previous study (16) identified 37 bacterial genera in the station air and revealed that the airborne bacterial diversity appeared to be comparable with the diversity found in other stations (7). In the current work, 39 bacterial genera were observed in the subway air, and the most frequently observed were *Micrococcus*, *Rhodococcus*, *Arthrobacter*, *Moraxella*, *Staphylococcus*, *Bacillus*, *Pseudomonas*, *Kocuria*, and *Dermacoccus*. Of the total number of observed genera, 56% (22/39) were also encountered during the previous study (16). If only the most frequently observed genera in the current work were taken into account, a complete qualitative consistency was observed between the two studies concerning the diversity of airborne cultivable bacteria.

The results obtained for the temporal variability of the airborne bacterial diversity in the current study, in combination with our previous results (16), suggested that the diversity was conserved (i) between seasons, at least qualitatively, (ii) between days, and (iii) between different daytime periods, such as rush hours and non-rush hours.

The airborne bacterial diversities obtained with the Andersen impactor and the SASS 3100 electret filter sampler were generally similar, although some differences were observed, i.e., (i) the Andersen-derived diversity corresponded to 17% Gram-negative bacteria, while only 4% was observed with the SASS 3100, (ii) the Andersen-derived diversity showed a higher relative abundance of *Arthrobacter* and *Moraxella* than the SASS 3100, while the SASS 3100 showed a higher relative abundance of *Staphylococcus* and *Bacillus*, and (iii) the Andersen-derived diversity corresponded to 44% pigmented bacteria, while 60% was observed with the SASS 3100.

The results from both samplers suggested that the daytime diversity was strongly dominated by *Micrococcus* (*M. luteus*), while the nighttime diversity was strongly dominated by *Rhodococcus* (*R. fascians*) and had a very low relative abundance of *Micrococcus* compared to the daytime period. Several other bacterial genera appeared to have higher relative abundances in the station during the daytime than at night, including *Moraxella*, *Dermacoccus*, *Pseudomonas*, *Brevundimonas*, and *Acinetobacter*, while *Arthrobacter* and *Roseomonas* appeared to have higher relative abundances during the night. Compared to the previous study (16), the relative abundance of *Rhodococcus* (*R. fascians*) reported by both samplers was much higher in the current work.

An interesting observation was made regarding the airborne bacterial diversity during an atypical nighttime event (16 February at 03:00) that showed strongly increased bacterial levels at night. While the atypical event diversity generally resembled the nighttime diversity with respect to the dominance of *Rhodococcus* and *Arthrobacter*, a high relative abundance of *Pseudomonas* that deviated from both the typical daytime and nighttime abundances was observed. Additionally, no *Micrococcus* was observed during the atypical event. Taken together, these observations suggested that the nighttime atypical event sources differed from the predominant daytime sources and that the atypical event sources appeared to aerosolize a larger relative fraction of *Pseudomonas*.

When the daytime bacterial diversity in the station was compared to that of the equivalent outdoor reference location, a higher relative abundance of *Micrococcus* and lower relative abundances of *Rhodococcus*, *Staphylococcus*, and *Bacillus* were observed outdoors. The results concerning the difference between the station diversity and the outdoor bacterial diversity did not correspond to those obtained in our previous study (16), which showed a higher relative abundance of *Bacillus* and a lower relative abundance of *Micrococcus* at the outdoor location than in the station. The reasons behind these discrepancies are not known, although the composition of outdoor airborne bacterial communities has been shown to display seasonal variation (20).

The airborne cultivable bacterial diversity in the station generally showed a conserved diversity profile for bacterium-containing particles of different sizes >1.1 μm. Interestingly, the largest deviation from the general trend was observed for *Micrococcus*, which showed an increased relative abundance in airborne bacterium-containing particles of between 1.1 and 3.3 μm compared to its abundance in particles of >3.3 μm. Compared to the diversity observed for airborne bacterium-containing particles of >1.1 μm, particles of between 0.65 and 1.1 μm appeared to have very low relative abundances of *Micrococcus*, *Moraxella*, and *Pseudomonas*.

Total particle level and size distribution. The total particle levels and size distributions observed in this study were comparable to those reported previously at the same station (16), as well as to those reported by Birenzvige et al. (3). However, at the same time, several consistent differences were identified concerning the diurnal profiles and short-term temporal variability of the total particle levels. While the previous study (16) revealed a significant difference between the daytime and nighttime particle levels, the current work showed no significant difference between particle levels categorized into daytime and nighttime periods. The current results nevertheless revealed that the underlying baseline particle level was lower at night than during the day but that the particle level variability was higher at night, with multiple short- and long-duration events showing increased particle loads that exceeded the daytime maximum levels. The nighttime events commonly coincided with periods of increased maintenance activities at the station or in the adjacent tunnels, which suggested that a causal link existed. However, a direct correlation between specific maintenance activities and the nighttime events was not identified.

A low temporal correlation was observed between the total particle levels and the airborne bacterial levels at the station during the

current work, even when particles of <1 and <3.3 μm were excluded from the total particle size range (0.5 to 20 μm). However, some general consistencies were observed between the total particle levels and the airborne bacterial levels, i.e., (i) the baseline total particle levels and the airborne bacterial levels were both higher during the day than at night and (ii) the baseline total particle levels and airborne bacterial levels both showed a generally decaying trend during the late evening period that continued throughout the night.

Implication and future directions. This work represents an important step forward in obtaining a more-complete understanding of the bioaerosol backgrounds that will be encountered by BIODIM systems at subway stations. The knowledge generated helps to define a range of relevant bioaerosol background-related conditions that may be used to improve the development and T&E of BIODIM equipment by allowing realistic operational conditions to be taken into account. A set of representative and well-defined bioaerosol backgrounds may be developed and standardized for use in aerosol test chambers, thus simulating realistic operational conditions during controlled aerosol chamber-based challenge tests with biological threat agents.

This study specifically addressed airborne bacteria, although other types of airborne microorganisms (e.g., fungi and viruses), their products (e.g., toxins), and other types of nonmicrobial bioaerosols (e.g., pollen) may also correspond to relevant components of the bioaerosol background with respect to the development and T&E of BIODIM systems.

Since the current work provided novel information concerning the size distribution of airborne bacterium-containing particles and the temporal variability of airborne bacteria in a subway environment, very limited data were available for interstudy comparisons. To confirm and extend the results obtained in the current work, it is also of interest to address the spatiotemporal variability of airborne bacteria by performing similar studies in different subway stations.

Cultivation has traditionally been the method of choice when studying airborne bacteria, although cultivation-independent techniques are now becoming more widespread, also within aerobiological research (4). Cultivation-independent analyses (e.g., quantitative PCR, next-generation sequencing, and microarrays) are able to elucidate not only cultivable bacteria but also viable but not cultivable (VBNC) and dead bacterial fractions. The cultivable fractions of environmental bacteria have in some environments been shown to correspond to <1% of the total number (21), clearly suggesting that the use of cultivation-independent techniques may extend our understanding of the bioaerosol background. Currently, few reports exist on the use of cultivation-independent analyses to study airborne bacteria in subway stations (3, 4). We have recently performed a cultivation-independent diversity study of airborne bacteria using 16S rRNA gene-based PhyloChip microarrays at the same Norwegian subway station (unpublished data), which may contribute interesting information regarding differences between the cultivation-dependent and -independent airborne bacterial diversities.

ACKNOWLEDGMENTS

This work was funded by the Norwegian Defense Research Establishment.

We thank Anja Valen for technical assistance with the cultivation of SASS 3100 samples and SASS 3100 outdoor air sampling.

REFERENCES

1. Reference deleted.
2. **Committee on Materials and Manufacturing Processes for Advanced Sensors, National Research Council.** 2005. Sensor systems for biological agent attacks: protecting buildings and military bases. The National Academies Press, Washington, DC. http://books.nap.edu/catalog/11207.html.
3. **Birenzvige A, Eversole J, Seaver M, Francesconi S, Valdes E, Kulaga H.** 2003. Aerosol characteristics in a subway environment. Aerosol Sci. Technol. **37:**210–220. http://dx.doi.org/10.1080/02786820300941.
4. **Robertson CE, Baumgartner LK, Harris JK, Peterson KL, Stevens MJ, Frank DN, Pace NR.** 2013. Culture-independent analysis of aerosol microbiology in a metropolitan subway system. Appl. Environ. Microbiol. **79:**3485–3493. http://dx.doi.org/10.1128/AEM.00331-13.
5. **Seino K, Takano T, Nakamura K, Watanabe M.** 2005. An evidential example of airborne bacteria in a crowded, underground public concourse in Tokyo. Atmos. Environ. **39:**337–341. http://dx.doi.org/10.1016/j.atmosenv.2004.09.030.
6. **Hwang SH, Yoon CS, Ryu KN, Paik SY, Cho JH.** 2010. Assessment of airborne environmental bacteria and related factors in 25 underground railway stations in Seoul, Korea. Atmos. Environ. **44:**1658–1662. http://dx.doi.org/10.1016/j.atmosenv.2010.01.047.
7. **Kim KY, Kim YS, Kim D, Kim HT.** 2011. Exposure level and distribution characteristics of airborne bacteria and fungi in Seoul metropolitan subway stations. Ind. Health **49:**242–248. http://dx.doi.org/10.2486/indhealth.MS1199.
8. **Lee C, Kim Y, Lee T, Park W, Hong S.** 2004. Characterization of airborne bioaerosol concentration in public facilities. J. Environ. Sci. **13:**215–222. (In Korean.)
9. **Dong S, Yao M.** 2010. Exposure assessment in Beijing, China: biological agents, ultrafine particles, and lead. Environ. Monit. Assess. **170:**331–343. http://dx.doi.org/10.1007/s10661-009-1236-7.
10. **Bogomolova E, Kirtsideli I.** 2009. Airborne fungi in four stations of the St. Petersburg underground railway system. Int. Biodeterior. Biodegradation **63:**156–160. http://dx.doi.org/10.1016/j.ibiod.2008.05.008.
11. **Awad AHA.** 2002. Environmental study in subway metro stations in Cairo, Egypt. J. Occup. Health **44:**112–118. http://dx.doi.org/10.1539/joh.44.112.
12. **Gilleberg S, Faull J, Graeme-Cook K.** 1998. A preliminary survey of aerial biocontaminants at six London underground stations. Int. Biodeterior. Biodegradation **41:**149–152. http://dx.doi.org/10.1016/S0964-8305(98)00005-5.
13. **Szám L, Nikodemusz I, Csatai L, Vedres I, Dakay M.** 1980. Airborne microflora found in some stations of the metro in the Hungarian capital of Budapest (author's transl). Zentralbl. Bakteriol. B **170:**199–208. (In German.)
14. **Szám L, Vedres I, Csatai L, Nikodemusz I.** 1983. Further microbiological studies of the air in a newly built (under the pavement) section of the underground railway in Budapest. Zentralbl. Bakteriol. Mikrobiol. Hyg. B **177:**312–318. (In German.)
15. **Naddafi K, Jabbari H, Hoseini M, Nabizadeh R, Rahbar M, Younesian M.** 2011. Investigation of indoor and outdoor air bacterial density in Tehran subway system. Iranian J. Environ. Health Sci. Eng. **8:**381–386.
16. **Dybwad M, Granum PE, Bruheim P, Blatny JM.** 2012. Characterization of airborne bacteria at an underground subway station. Appl. Environ. Microbiol. **78:**1917–1929. http://dx.doi.org/10.1128/AEM.07212-11.
17. **Macher JM.** 1989. Positive-hole correction of multiple-jet impactors for collecting viable microorganisms. Am. Ind. Hyg. Assoc. J. **50:**561–568. http://dx.doi.org/10.1080/15298668991375164.
18. **Salma I, Weidinger T, Maenhaut W.** 2007. Time-resolved mass concentration, composition and sources of aerosol particles in a metropolitan underground railway station. Atmos. Environ. **41:**8391–8405. http://dx.doi.org/10.1016/j.atmosenv.2007.06.017.
19. **Thomas RJ, Webber D, Sellors W, Collinge A, Frost A, Stagg AJ, Bailey SC, Jayasekera PN, Taylor RR, Eley S.** 2008. Characterization and deposition of respirable large-and small-particle bioaerosols. Appl. Environ. Microbiol. **74:**6437–6443. http://dx.doi.org/10.1128/AEM.01194-08.
20. **Bowers RM, McCubbin IB, Hallar AG, Fierer N.** 2012. Seasonal variability in airborne bacterial communities at a high-elevation site. Atmos. Environ. **50:**41–49. http://dx.doi.org/10.1016/j.atmosenv.2012.01.005.
21. **Amann RI, Ludwig W, Schleifer K-H.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.

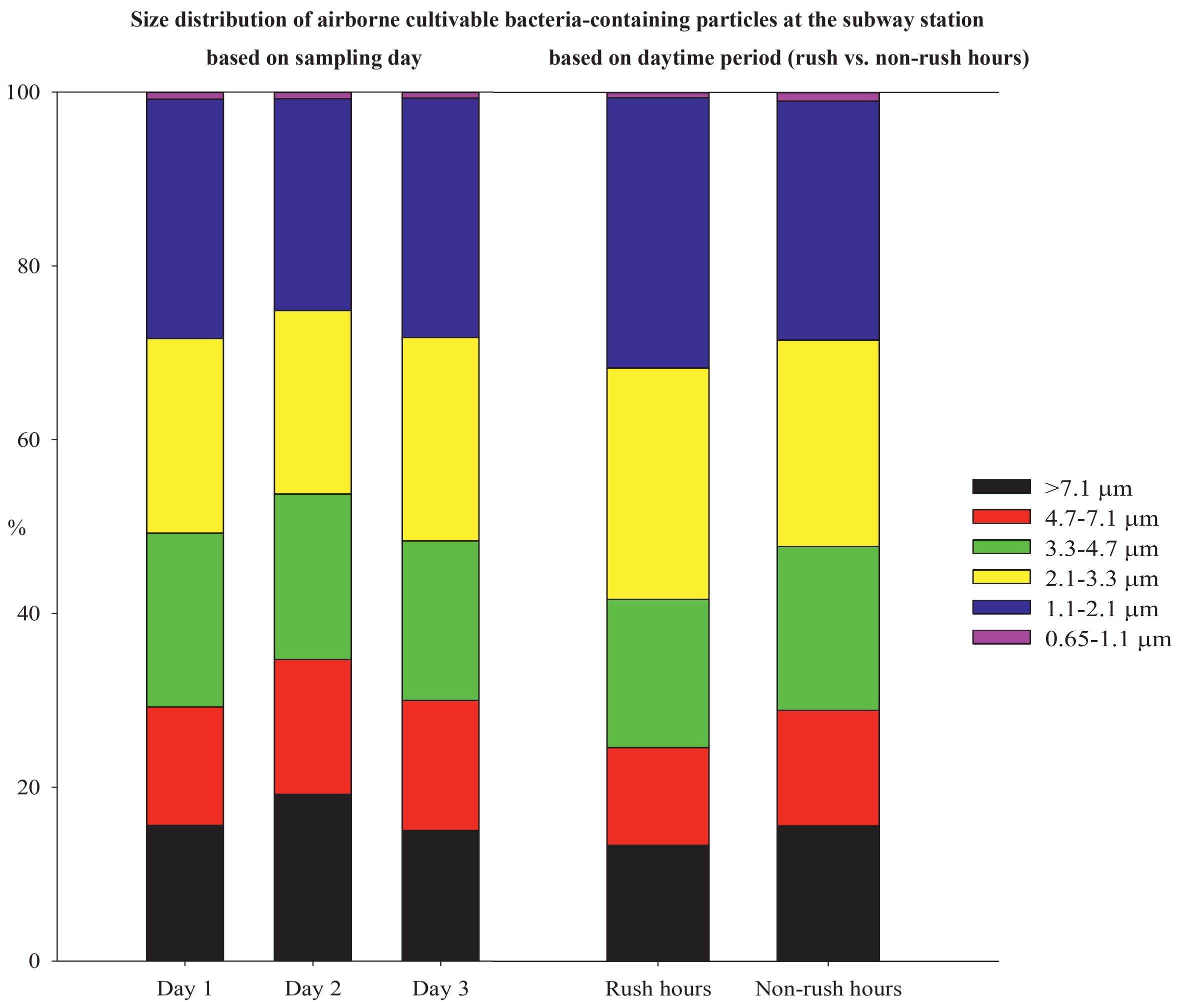

Figure S1. Size distribution of airborne cultivable bacteria-containing particles at the subway station. Size-resolved airborne cultivable bacterial levels were obtained with the Andersen six-stage cascade impactor. Left section: Size distribution on different days during the 72-h sampling campaign (**Day 1-3**). Right section; Size distribution during the daytime categorized into **rush hours** (morning and afternoon) and **non-rush hours** (noon and evening).

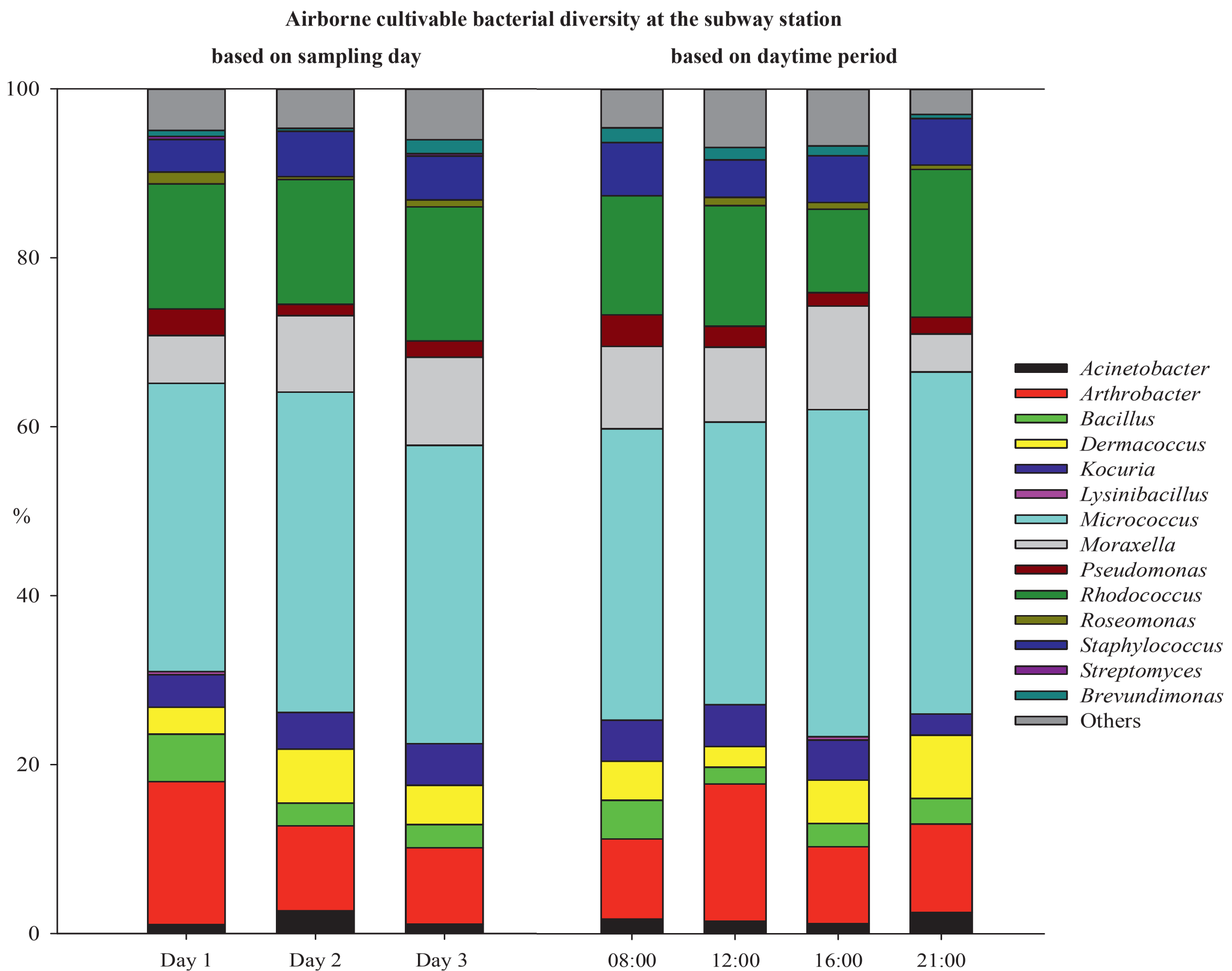

Figure S2. Airborne cultivable bacterial diversity at the subway station based on Biotyper 3.0 MALDI-TOF MS analysis of representative bacterial isolates from Andersen six-stage cascade impactor samples. Left section: Bacterial diversity on different days during the 72-h sampling campaign (**Day 1-3**). Right section; Bacterial diversity observed during different daytime periods (**08:00**: morning rush hour, **12:00**; noon non-rush period, **16:00**; afternoon rush hour, **21:00**; evening non-rush period).

Paper III

Comparative Testing and Evaluation of Nine Different Air Samplers: End-to-End Sampling Efficiencies as Specific Performance Measurements for Bioaerosol Applications

Dybwad, M, G Skogan, and JM Blatny

Aerosol Science and Technology, 2014, 48(3):281–294

Aerosol Science and Technology, 48:281–294, 2014
Copyright © American Association for Aerosol Research
ISSN: 0278-6826 print / 1521-7388 online
DOI: 10.1080/02786826.2013.871501

Comparative Testing and Evaluation of Nine Different Air Samplers: End-to-End Sampling Efficiencies as Specific Performance Measurements for Bioaerosol Applications

Marius Dybwad,[1,2] Gunnar Skogan,[1] and Janet Martha Blatny[1]
[1]*Norwegian Defence Research Establishment, Kjeller, Norway*
[2]*Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway*

Accurate exposure assessments are needed to evaluate health hazards caused by airborne microorganisms and require air samplers that efficiently capture representative samples. This highlights the need for samplers with well-defined performance characteristics. While generic aerosol performance measurements are fundamental to evaluate/compare samplers, the added complexity caused by the diversity of microorganisms, especially in combination with cultivation-based analysis methods, may render such measurements inadequate to assess suitability for bioaerosols. Specific performance measurements that take into account the end-to-end sampling process, targeted bioaerosol and analysis method could help guide selection of air samplers.

Nine different samplers (impactors/impingers/cyclones/electrostatic precipitators/filtration samplers) were subjected to comparative performance testing in this work. Their end-to-end cultivation-based biological sampling efficiencies (BSEs) and PCR-/microscopy-based physical sampling efficiencies (PSEs) relative to a reference sampler (BioSampler) were determined for gram-negative and gram-positive vegetative bacteria, bacterial spores, and viruses.

Significant differences were revealed among the samplers and shown to depend on the bioaerosol's stress–sensitivity and particle size. Samplers employing dry collection had lower BSEs for stress-sensitive bioaerosols than wet collection methods, while nonfilter-based samplers showed reduced PSEs for 1 μm compared to 4 μm bioaerosols. Several samplers were shown to underestimate bioaerosol concentration levels relative to the BioSampler due to having lower sampling efficiencies, although they generally obtained samples that were more concentrated due to having higher concentration factors.

Our work may help increase user awareness about important performance criteria for bioaerosol sampling, which could contribute to methodological harmonization/standardization and result in more reliable exposure assessments for airborne pathogens and other bioaerosols of interest.

Received 1 September 2013; accepted 28 November 2013.
Address correspondence to Janet Martha Blatny, Norwegian Defence Research Establishment, P.O. Box 25, NO-2027 Kjeller, Norway. E-mail: janet-martha.blatny@ffi.no

INTRODUCTION

Accurate and reliable quantification and identification of bioaerosols depends on several factors, including the use of air sampling equipment that efficiently capture representative samples and match the targeted bioaerosol (e.g., concentration level, particle size, and stress–sensitivity), the meteorological conditions (e.g., temperature, humidity, and wind speed) and the employed analysis methods (Alvarez et al. 1995). The latter commonly includes microscopy, microbiological, biochemical, immunological, and molecular techniques (Cox and Wathes 1995; Eduard and Heederik 1998; Buttner et al. 2002; Grinshpun and Clark 2005; Georgakopoulos et al. 2009; Reponen et al. 2011; Xu et al. 2011). Bioaerosol sampling is usually achieved through common aerosol collection principles such as impaction, impingement, cyclonic separation, filtration, and thermal or electrostatic precipitation (Burge and Solomon 1987; Macher and Willeke 1992; Nevalainen et al. 1992; Cox and Wathes 1995; Buttner et al. 2002; Grinshpun and Clark 2005; Reponen et al. 2011). A myriad of air samplers have been developed based on these principles and offer a wide range of different instrument properties with respect to sampling efficiency, collection size range, airflow rate, collection medium type and volume, physical properties (e.g., size, weight, ruggedness, and automation) and inflicted sampling-associated microbial stress (e.g., shear forces and desiccation) (Buttner and Stetzenbach 1991; Li 1999; Radosevich et al. 2002; An et al. 2004; Bergman et al. 2004; Yao and Mainelis 2007; Carvalho et al. 2008, Kesavan et al. 2008; 2010a; McFarland et al. 2010). Thus, no single air sampler is likely to be optimal, or even suitable, for all purposes (Macher and Willeke 1992; Nevalainen et al. 1992).

Different bioaerosol types and sampling applications may be associated with variable inherent challenges and study-specific requirements, which have made equipment and procedural standardization difficult within the bioaerosol community and consequently led to the use of several different air samplers and analysis methods. Interstudy data comparisons are therefore demanding since various methodologies provide different

results even when subjected to the same bioaerosol challenge (Shahamat et al. 1997; Eduard and Heederik 1998; Li 1999; Buttner et al. 2002; An et al. 2004; Yao and Mainelis 2007; Griffin et al. 2011). Bioaerosol investigations are therefore hampered by the lack of methods that can provide accurate, reliable, and comparable exposure estimates for airborne microorganisms (Macher 1999). Taken together, this highlights the need for comparative testing and evaluation (T&E) of air samplers and standardization of sampling procedures (Bartlett et al. 2002; An et al. 2004; Vitko Jr et al. 2005; Millner 2009; Xu et al. 2011). Such efforts will provide the users with important information upon selection of air samplers in order to meet their specific operational requirements.

Performance measurements for air samplers have been reported using several different, and sometimes redundant, efficiency terms (e.g., aspiration-, inlet-, transmission-, collection-, sampling-, recovery-, retention-, physical-, biological-, total-, and overall efficiency), taking into account different aspects or parts of the end-to-end sampling process (Nevalainen et al. 1992; Grinshpun et al. 1994; Grinshpun et al. 1996; Henningson et al. 1997; Li 1999; Brixey et al. 2002; Kesavan et al. 2003; An et al. 2004; Bergman et al. 2004; Yao and Mainelis 2007; Carvalho et al. 2008; Kesavan et al. 2008; 2010a; McFarland et al. 2010). The performance information supplied with commercially available samplers is often limited to collection efficiencies for different sizes of inert nonbiological particles (e.g., polystyrene latex spheres), which are used to define the sampler's particle cutoff diameter d_{50} at which 50% collection efficiency is observed. Such generic measurements do not, however, incorporate the sampler's propensity to induce sampling stress.

The well-characterized swirling liquid impinger (BioSampler) was used as a reference air sampler in this study (Willeke et al. 1998; Lin et al. 1999, 2000; Hermann et al. 2006; Rule et al. 2007; Fabian et al. 2009; Van Droogenbroeck et al. 2009; Chang et al. 2010; Kesavan et al. 2010b; Chang and Chou 2011; Kesavan et al. 2011). Compared to the BioSampler, the available performance data for the rest of the involved air samplers were more limited; gelatin filters (Li 1999; Lin and Li 1999; Tseng and Li 2005; Burton et al. 2007; Fabian et al. 2009; Van Droogenbroeck et al. 2009; Chang and Chou 2011; Estill et al. 2011; Zhao et al. 2011), Coriolis FR (or Coriolis μ and δ) (Carvalho et al. 2008; Gómez-Domenech et al. 2010; Ahmed et al. 2013), XMX-CV (or XMX/2L-MIL and XMX/2A) (Cooper 2010; Black 2011; Kesavan et al. 2011; Black and Cooper 2012; Enderby 2012), BioCapture 650 (Kesavan et al. 2011; Enderby 2012), OMNI-3000 (Kesavan et al. 2011; Zhao et al. 2011), SASS 2300 (or SASS 2000) (Kesavan and Stuebing 2009; Kesavan et al. 2011), and SASS 3100 (Kesavan et al. 2010b).

The aim of the present work was to perform aerosol chamber-based comparative T&E of nine air samplers representing different collection principles in order to establish their end-to-end cultivation-dependent biological sampling efficiencies (BSEs) and cultivation-independent physical sampling efficiencies (PSEs) for a selection of aerosol test agents relative to the BioSampler. The BSEs were based on plate count analyses, while the PSEs were based on quantitative real-time PCR (qPCR) or fluorescence microscopy direct count analyses. The aerosol test agents included Gram-positive and Gram-negative vegetative bacteria, bacterial spores and viruses, and also fluorescent polystyrene latex spheres. The T&E scheme was designed to provide the users of air sampling equipment with more specific end-to-end performance measurements for various bioaerosol sampling applications.

MATERIALS AND METHODS

Aerosol Test Agents and Spray Solutions

The biological test agents included Gram-negative (*Serratia marcescens*, SM) and Gram-positive (*Kocuria rhizophila*, KR) vegetative bacterial cells, bacterial spores (*Bacillus atrophaeus*, formerly *Bacillus globigii*, BG), and viruses (Bacteriophage MS2, non-enveloped ssRNA virus, MS2). A freeze-dried powder containing 2.0×10^8 cfu mg^{-1} of BG spores (DPG Lot 19076–03268) and a solution containing 3.5×10^{12} pfu ml^{-1} of MS2 phages (DPG Lot 2011JUN28AKS) in TNME buffer (10 mM Tris-HCl, 100 mM NaCl, 0.1 mM $MgSO_4$, 0.01 mM EDTA) were provided by Dugway Proving Ground (DPG, Dugway, UT, USA). A SM strain (ATCC 274) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and a KR strain (ATCC 9341, formerly classified as *Micrococcus luteus*) was provided by the Norwegian School of Veterinary Science (NVH, Oslo, Norway). Fluorescent polystyrene latex spheres (Fluospheres, FS) of two sizes, 1 μm with yellow-green fluorescence (505/515 nm) and 4 μm with red fluorescence (580/605 nm), respectively, were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA).

BG spores (5 mg ml^{-1}) were suspended in MilliQ water (Millipore, Billerica, MA) assisted by vortexing (5 min). SM and KR were seeded into nutrient broth (Oxoid, Cambridge, UK) or trypticase soy broth (Oxoid), respectively, followed by cultivation (18 h, 200 rpm) at 30°C (SM) or 37°C (KR). The BG, SM, and KR were washed by centrifugation (3,000 $\times$ g, 5 min) and re-dissolved in MilliQ water. The optical density (OD) was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and adjusted with MilliQ water to $OD_{600} = 1.0$ for BG ($\sim 1.0 \times 10^9$ cfu ml^{-1}), $OD_{600} = 0.5$ for SM ($\sim 5.0 \times 10^9$ cfu ml^{-1}), and $OD_{600} = 0.75$ for KR ($\sim 3.0 \times 10^9$ cfu ml^{-1}). The spray solutions' concentration levels (cfu ml^{-1}) were determined by plate count analyses before and after aerosol generation. Serial dilutions of the spray solutions were plated in triplicates on trypticase soy agar (TSA) for KR and nutrient agar (NA) for BG and SM, and incubated (18 h) at 37°C (KR) or 30°C (BG and SM). The MS2 stock solution was directly diluted with 12.5% TNME buffer to give

a spray solution concentration level of 1.0×10^9 pfu ml^{-1}. The MS2 spray solution's concentration level (pfu ml^{-1}) was determined using a pour-plate method before and after aerosol generation. Briefly, serial dilutions of the spray solution were mixed with 1.0×10^7 cfu of log-phase *Escherichia coli* (DSM 4230) cells in soft NA (0.7% agar), poured out on NA plates in triplicates, and incubated (18 h) at 37°C. The FS suspensions were directly diluted with MilliQ water to give spray solutions containing 1.0×10^9 spheres ml^{-1}. All spray solutions were prepared fresh each day.

Bioaerosol Test Chamber

The air sampler testing was performed in a 12 m^3 ($3 \times 2 \times 2$ m) stainless steel aerosol test chamber (ATC, Dycor Technologies, Edmonton, AB, Canada) fitted with external heating, ventilation and air conditioning (HVAC) and high-efficiency particulate air (HEPA)-filtration systems (Figure S1 in the online supplemental information [SI]). The ATC was equipped with two mixing fans (120 mm), meteorology sensors for temperature, humidity and pressure, optical particle counter (Grimm 1.108, Grimm Technologies, Douglasville, GA, USA), aerodynamic particle sizer (APS 3321, TSI, Shoreview, MN, USA), and two slit-to-agar samplers (STA-203, New Brunswick, Edison, NJ, USA). Real-time monitoring of the test aerosol concentration and size distribution was done with the Grimm 1.108 and APS 3321. The agent containing particles per liter of air (ACPLA) levels were monitored using sequentially operated STA-203 samplers (30 lpm, 0.5 rpm). The STA-203s were loaded with TSA (KR) or NA plates (BG and SM), and the plates were incubated (18 h) at 37°C (KR) or 30°C (BG and SM). The STA-203s were not used during MS2 and FS experiments.

Aerosol Generation

Aerosolization of the biological test agents (BG, SM, KR, and MS2) was achieved using a 48 kHz Sono-Tek ultrasonic atomizer nozzle (Sono-Tek, Milton, NY, USA), while FS was aerosolized using a Micro Mist nebulizer (Hudson RCI, Durham, NC, USA). The Sono-Tek nozzle was powered by a broadband ultrasonic generator (Model 06-5108, Sono-Tek) and the spray solution was fed from a syringe feeder (Model 997E, Sono-tek). The Micro Mist nebulizer was operated with N_2 gas (2.4 bar). Both dispersion devices were enclosed in an aerosol dilution system (ADS-A20, Dycor Technologies) which offered adjustable dilution of the aerosol with HEPA-filtered air before injection into the ATC. The targeted aerosol particle sizes were 1 and 4 μm mass median aerodynamic diameters (MMAD). The air sampler testing was performed with 4 μm aerosols for all test agents and additionally 1 μm aerosols for FS and BG. Appropriate instrument settings for the ATC and its subsystems were determined during pre-study experiments to generate reproducible concentration levels and size distributions for the test aerosols, and then kept static throughout the study.

Evaluated Air Samplers

Nine different air samplers based on various wet and dry aerosol collection principles, including filtration, impaction, impingement, cyclonic separation, and electrostatic precipitation, were subjected to aerosol chamber-based comparative T&E. The air samplers spanned a wide range of airflow rates (12.5–540 lpm) and ranged in technological sophistication from simple filter cassettes and glassware samplers that require external vacuum sources and manual handling to fully automated and ruggedized systems. The evaluated samplers (Table 1 and Figure S2) were SASS 2300, SASS 3100, gelatin filters, Coriolis FR, OMNI-3000, BioCapture 650, Electrostatic precipitator (ESP) prototype, XMX-CV, and BioSampler.

The BioSampler was used as a reference and the sampling efficiencies of the other samplers were reported relative to the reference sampler. The samplers were operated according to their respective manufacturers' instructions-for-use documents. The air flows through the BioSampler and the gelatin filter were monitored using mass flow meters (TopTrak 826, Sierra Instruments, Monterey, CA, USA). The SASS 3100's open-faced filter holder was covered with an aluminum cover cap to avoid deposition of test aerosols on the electret filter during aerosol generation and mixing.

The collection liquids used with Coriolis FR and BioCapture 650 were both phosphate buffered saline (PBS)-based surfactant-containing formulations supplied as single-use consumables. PBS supplemented with 0.05% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and 0.005% Antifoam A (Sigma-Aldrich, PBSTA) was used as collection liquid with the BioSampler and XMX-CV, and as extraction liquid with the SASS 3100, ESP, and gelatin filters. The OMNI-3000 was operated with collection cartridges containing PBS supplied as single-use consumables and MilliQ water as make-up liquid in the onboard reservoir. The SASS 2300 was operated with PBS as both collection liquid and make-up liquid. The OMNI-3000 and SASS 2300 samples were supplemented with Triton X-100 and Antifoam A to a final concentration of 0.05% and 0.005%, respectively, immediately after sampling.

Air Sampler Testing

The described aerosol chamber-based T&E scheme was consistently used unless otherwise stated. Each air sampler was tested with a minimum of five experimental repetitions for each aerosol test agent and particle size. The samplers were tested in groups with the number of simultaneously tested samplers ranging from 2 to 6. Fixed sampling positions were used in the ATC and the relative positions of the samplers were varied between experiments (Figure S1). Before each experiment, the ATC was sealed and purged using HVAC-conditioned HEPA-filtered air, until the Grimm 1.108 reported background concentration levels (<1 particles $liter^{-1}$ between 0.8 and 20 μm) and the targeted meteorological conditions were observed (55% relative humidity and 20°C). Test aerosol generation was initiated and continued ($\sim$2 min) until the Grimm 1.108 reached the

TABLE 1
Air sampling equipment subjected to comparative testing and evaluation (T&E) in this study

Air sampler	Manufacturer	Collection principle	Airflow rate ($l\ min^{-1}$)	Liquid sample volume (ml)[a]	Theoretical relative concentration factor[b]	Consumables (in this study)	Collection/ extraction liquid (in this study)	Additional equipment needs (in this study)
BioSampler	SKC (Eighty Four, PA, USA)	Liquid impingment (swirling liquid impingment)	12.5	19	Reference sampler		PBSTA	Vacuum pump (Picolino VTE 3, Gardner Denver Thomas, Sheboygan, WI) and liquid trap (SKC)
Gelatin filters	SKC	Filtration (gelatin filter)	15	10	2	Gelatin filters (3 μm pore size, 37 mm) with cellulose backing filters housed in conductive polypropylene filter cassettes	PBSTA	Vacuum pump (Picolino VTE 3) and flow restrictor (SKC)
SASS 2300	Research International (Monroe, WA, USA)	Cyclonic separation/liquid impingment (wetted-wall cyclone)	390	4	148		PBS[c]	

SASS 3100	Research International	Filtration (electret filter)	300	7.5	61	Electret filters	PBSTA	SASS 3010 filter extractor unit (Research International)
Coriolis FR	Bertin Technologies (Montigny-le-Bretonneux, France)	Cyclonic separation/liquid impingment (wet cyclone)	300	9	51	Cyclones	PBS-based surfactant-containing commercial formulation	
OMNI-3000	InnovaPrep (Drexel, MO, USA)	Cyclonic separation/liquid impingment (contactor/wetted-wall cyclone)	300	10	46	Cartridges for collection liquid injection and sample output	PBS[c]	
BioCapture 650	FLIR Systems (Arlington, VA, USA)	Impaction (spinning disk)	200	5	61	Collection cartridges with integrated spinning disk, tubing, extraction liquid reservoir, and sample output vial	PBS-based surfactant-containing commercial formulation	
ESP (prototype)	Dycor Technologies (Edmonton, AB, Canada)	Electrostatic precipitation	540	10	82	Collection pipes	PBSTA	
XMX-CV	Dycor Technologies	Liquid impingment[d]	530	4	201	Collection vials	PBSTA	

PBS, phosphate buffered saline; PBSTA, PBS supplemented with 0.05% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and 0.005% Antifoam A (Sigma–Aldrich).

[a]The reported value is the typical liquid volume recovered from the air sampler after 5 min of operation (20°C and 55% RH). For samplers that did not directly output a liquid sample (gelatin filters, SASS 3100, and ESP), the reported value is the typical liquid volume recovered after sample-to-liquid extraction.

[b]The air sampler's theoretical concentration factor (air to liquid) calculated relative to the reference sampler.

[c]The SASS 2300 and OMNI-3000 were not compatible with surfactant-based collection liquids. The samples were supplemented with 0.05% Triton X-100 (Sigma–Aldrich) and 0.005% Antifoam A (Sigma-Aldrich) after sampling.

[d]The XMX-CV may also be used with a filter cassette (filtration) instead of the collection vial (liquid impingment). Only the liquid impingment option was evaluated as part of this study.

targeted concentration level (∼160 particles liter^{-1}). The ventilation system, but not the mixing fans, was then switched off and the test aerosol mixed (1 min). The sampling period was 5 min, after which the ATC was purged down to background conditions. The collected samples were immediately transported to the laboratory, and the samplers decontaminated in accordance with their sample-to-sample decontamination procedures. The electret filters from SASS 3100 were extracted with PBSTA (8 ml) using a SASS 3010 extractor instrument (Research International). The collection pipes from the ESP were extracted with PBSTA (10 ml, hand-shaking, 30 s), while the gelatin filters were completely dissolved in PBSTA (10 ml, 37°C, hand-shaking).

Evaluation of Test Aerosol Homogeneity

The homogeneity of test aerosols inside the ATC was evaluated during pre-study experiments by simultaneous sampling with three STA-204 slit-to-agar samplers (30 lpm, 0.5 rpm, New Brunswick) and two STA-203 slit-to-agar samplers. These experiments were performed with 4 μm BG aerosols and repeated while changing the relative positions of the samplers between those used for air sampler testing (Figure S1). Two SASS 3100 electret filter samplers (300 lpm) and two SASS 2000 wetted-wall cyclones (325 lpm, older model of SASS 2300) were used to simulate ongoing air sampler testing during the homogeneity experiments. The ACPLA levels reported by the slit-to-agar samplers were used to assess the homogeneity of test aerosols inside the ATC during simulated air sampler testing. Additionally, wind speed measurements were recorded at multiple heights for each sampling position using a hotwire anemometer (VT200, Kimo Instruments, Montpon, France).

Cultivation Analysis

Plate count analyses (in the [SI]) were used to establish the air samplers' BSEs for BG, SM, KR, and MS2 aerosols. The BSEs were calculated relative to the reference sampler (BioSampler) using Equation (1) (Henningson et al. 1997; An et al. 2004; Yao and Mainelis 2007; Carvalho et al. 2008).

$$\text{BSE or PSE} = \frac{\dfrac{\text{sample concentration}\ (\#\,\text{ml}^{-1}) \times \text{collection medium volume (ml)}}{\text{airflow rate}\ (\text{l min}^{-1}) \times \text{duration (min)}}\ (\text{Sampler})}{\dfrac{\text{sample concentration}\ (\#\,\text{ml}^{-1}) \times \text{collection medium volume (ml)}}{\text{airflow rate}\ (\text{l min}^{-1}) \times \text{duration (min)}}\ (\text{Reference})} \quad [1]$$

Molecular Analysis

qPCR assays (in the SI) for BG (Buttner et al. 2004), SM (Saikaly et al. 2007) and KR (this study), and a reverse transcriptase qPCR (qRT-PCR) assay for MS2 (O'Connell et al. 2006), were used to determine the air samplers' PSEs. The PSEs were calculated relative to the reference sampler using Equation (1).

Direct Count Analysis

Fluorescence microscopy-based direct count analyses (in the SI) were used to determine the air samplers' PSEs for FS aerosols. The concentration levels of FS in the collected air samples were calculated using Equation (2). The PSEs were calculated relative to the reference sampler using Equation (1).

$$\text{Sample concentration (spheres ml}^{-1}) = \frac{\text{Counts per field (spheres field}^{-1}) \times \text{total filter area}\ (\mu\text{m}^2)}{\text{filtered volume (ml)} \times \text{area per field}\ (\mu\text{m}^2\text{field}^{-1})}. \quad [2]$$

Statistical Analysis

The results were subjected to statistical analyses using SigmaPlot (Systat Software, San Jose, CA, USA). Normality checking was done with the Shapiro–Wilk test and depending on whether the normality and equal variance criteria were fulfilled or not, significance testing was performed with the Student's *t*-test or the Mann–Whitney rank sum test, respectively. The significance level was set at $p < 0.05$ for all statistical tests.

RESULTS AND DISCUSSION

Spray Solutions and Test Aerosols

The spray solutions showed less than ±10% variability in cultivable counts (BG, KR, SM, and MS2) or direct microscopy counts (FS) throughout the period they were used for aerosol generation. Particle size calculations based on APS 3321 measurements from all experiments showed re-producible size distributions for all test aerosols with median sizes close to the targeted 1 and 4 μm MMAD (Table S2). The homogeneity of test aerosols in the ATC was evaluated to ensure that all samplers were exposed to the same challenge. Five simultaneously operated slit-to-agar samplers showed similar recoveries (±2%) of 4 μm BG aerosols at each sampling position, irrespectively of whether or not four high-volume air samplers were operated at the same time. The airflow velocities in the ATC were determined to evaluate the risk of observing over- or undersampling through the air samplers' inlets. Airflow velocities below 1 m s^{-1} were consistently observed at all sampling positions and inlet heights, suggesting that substantial over- or undersampling were unlikely for the involved particle sizes and inlet velocities (Grinshpun et al. 1994; Baron 1998; Li et al. 2000).

T&E Results (Test Agent Categorized)

The following sections present statistical interpretations of the T&E results assisted by two figures (Figures 1 and 2). A consolidated result summary showing BSEs and PSEs for each air sampler and test agent is provided as SI (Table S3).

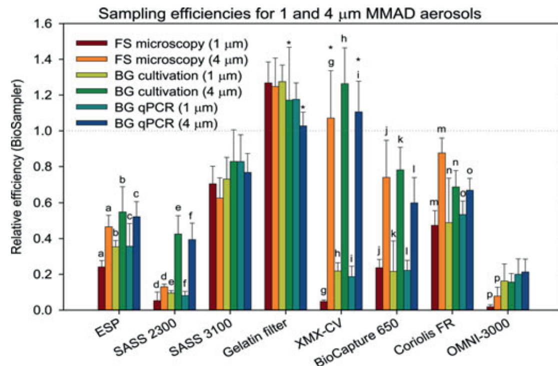

FIG. 1. The evaluated air samplers' end-to-end cultivation-based biological sampling efficiencies (BSEs) and qPCR- or fluorescence microscopy-based physical sampling efficiencies (PSEs) relative to a reference sampler (BioSampler) for 1 and 4 μm mass median aerodynamic diameter (MMAD) aerosols of FluoSpheres (FS) and *B. atrophaeus* spores (BG). The evaluated air samplers' sampling efficiencies were significantly different from the BioSampler's sampling efficiency (gray dotted line) except when specified with an asterisk (*). The evaluated air samplers' sampling efficiencies were significantly different for 1 μm aerosols compared to 4 μm aerosols when specified with the same letter.

FluoSpheres (FS)

The air samplers' PSEs relative to the reference sampler were determined for 1 and 4 μm FS aerosols (Figure 1). The filter-based samplers (SASS 3100 and gelatin filters) did not show significantly different PSEs for 1 μm compared to 4 μm. The other samplers showed significantly lower PSEs for 1 μm compared to 4 μm FS, although the reductions were smaller for ESP and Coriolis FR than for SASS 2300, XMX-CV, BioCapture 650, and OMNI-3000. The largest sampling efficiency reduction for 1 μm compared to 4 μm FS was observed for XMX-CV (∼26-fold).

In general, the evaluated samplers showed significantly lower PSEs for both sized FS compared to the reference sampler, except for: (i) gelatin filters that had significantly higher PSEs for both sized FS, and (ii) XMX-CV that did not have a significantly different PSE for 4 μm FS. Low PSEs ($\leq$0.13) were observed for both sized FS with SASS 2300 and OMNI-3000.

B. atrophaeus spores (BG)

The air samplers' BSEs and PSEs relative to the reference sampler were determined for 1 and 4 μm BG aerosols (Figure 1). The filter-based samplers (SASS 3100 and gelatin filters) and OMNI-3000 did not show significantly different BSEs or PSEs for 1 μm compared to 4 μm BG. The other samplers had significantly lower BSEs and PSEs for 1 μm compared to 4 μm BG, although the efficiency reductions were smaller for ESP and Coriolis FR compared to SASS 2300, XMX-CV, and BioCapture 650. The largest sampling efficiency reductions for 1 μm compared to 4 μm BG was observed for XMX-CV (∼6-fold) and SASS 2300 (∼5-fold).

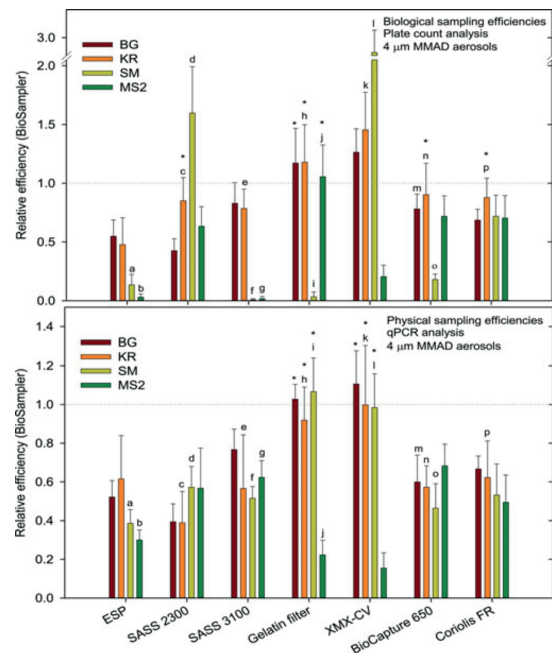

FIG. 2. The evaluated air samplers' end-to-end cultivation-based biological sampling efficiencies (BSEs, upper panel) and qPCR-based physical sampling efficiencies (PSEs, lower panel) relative to the reference sampler (BioSampler) for 4 μm mass median aerodynamic diameter (MMAD) aerosols of *B. atrophaeus* spores (BG), *Kocuria rhizophila* (KR), *Serratia marsescens* (SM), and bacteriophage MS2 (MS2). The evaluated air samplers' sampling efficiencies were significantly different from the BioSampler's sampling efficiency (gray dotted line) except when specified with an asterisk (*). The evaluated air samplers' BSEs and PSEs were significantly different from each other when specified with the same letter.

The evaluated samplers had significantly lower BSEs and PSEs for both sized BG compared to the reference sampler, except for: (i) gelatin filters that had a significantly higher BSE and PSE for 1 μm BG and not significantly different BSE and PSE for 4 μm BG, and (ii) XMX-CV that had a significantly higher BSE and not significantly different PSE for 4 μm BG only.

For both sized BG, no significant differences were observed between the BSEs and PSEs, except for 4 μm BG with BioCapture 650 that had a significantly lower PSE (∼1.5-fold).

Low BSEs and PSEs ($\leq$0.22) were observed for both sized BG with OMNI-3000 and for 1 μm BG with SASS 2300, XMX-CV, and BioCapture 650. Since OMNI-3000 showed low PSEs for both sized BG and FS, this sampler was excluded from further testing.

Kocuria rhizophila (KR)

The air samplers' BSEs and PSEs relative to the reference sampler were determined for 4 μm KR aerosols (Figure 2). The

evaluated samplers had significantly higher BSEs compared to PSEs, except for ESP that had a lower but not significantly different BSE.

The evaluated samplers also had significantly lower PSEs compared to the reference sampler, except for XMX-CV and gelatin filters that did not have significantly different PSEs. XMX-CV had a significantly higher BSE compared to the reference sampler, while ESP and SASS 3100 had significantly lower BSEs. The gelatin filters, SASS 2300, BioCapture 650, and Coriolis FR did not have significantly different BSEs compared to the reference sampler.

Serratia marcescens (SM)

The air samplers' BSEs and PSEs relative to the reference sampler were determined for 4 μm SM aerosols (Figure 2). Air samplers collecting directly into liquid had significantly higher (SASS2300 and XMX-CV) or not significantly different (Coriolis FR) BSEs compared to PSEs, while samplers employing dry collection principles (ESP, SASS 3100, gelatin filters, and BioCapture 650) had significantly lower BSEs compared to PSEs. The largest reductions in BSEs compared to PSEs were observed for the filter-based samplers, SASS 3100 ($\sim$52-fold) and gelatin filters ($\sim$36-fold), that both had very low BSEs ($\leq$0.03).

The evaluated samplers had significantly lower BSEs and PSEs compared to the reference sampler, except for: (i) XMX-CV that had significantly higher BSE and not significantly different PSE, (ii) SASS 2300 that had a significantly higher BSE, and (iii) gelatin filters that did not have a significantly different PSE.

Bacteriophage MS2

The air samplers' BSEs and PSEs relative to the reference sampler were determined for 4 μm MS2 aerosols (Figure 2). SASS 3100 and ESP had significantly lower BSEs than PSEs, while gelatin filters had a significantly higher BSE than PSE. The other samplers (SASS 2300, XMX-CV, BioCapture 650, and Coriolis FR) did not have significantly different BSEs compared to PSEs. Low BSEs and PSEs ($\leq$0.21) were observed with XMX-CV.

The evaluated air samplers had significantly lower BSEs and PSEs compared to the reference sampler, except for gelatin filters that did not have a significantly different BSE.

T&E Results (Test Agent- and Particle Size-Consolidated)

The following sections present and discuss the consolidated T&E results to identify and highlight performance differences and/or similarities between the evaluated air samplers.

Particle size

The particle size of the targeted bioaerosol is an important parameter to consider upon selection of air sampler. Our results showed that the BSEs and PSEs for BG and FS aerosols with the filter-based samplers (SASS 3100 and gelatin filters) were not affected by reducing the particle size from 4 μm to 1 μm, while the XMX-CV, SASS 2300, and BioCapture 650 showed strongly reduced BSEs and PSEs for 1 μm compared to 4 μm aerosols (Figure 1). The ESP and Coriolis FR also showed reduced BSEs and PSEs for 1 μm aerosols, but the reductions were only moderate compared to those observed for the XMX-CV, SASS 2300 and BioCapture 650 (Figure 1). These results are in agreement with previous studies showing that the d_{50} cutoff diameters are above 1 μm for XMX-CV, SASS 2300 and BioCapture 650, and below 1 μm for the BioSampler (Kesavan and Stuebing 2009; Kesavan et al. 2011).

Physical sampling efficiency (PSE)

The PSEs for 1 and 4 μm FS aerosols based on microscopy and 1 and 4 μm BG aerosols based on qPCR corresponded with each other (Figure 1). The observation that similar PSEs could be reproduced for two test agents even when based on different analysis methods, suggested that the PSEs were accurate and representative for the respective samplers. However, two minor exceptions were observed; SASS 2300 and OMNI-3000 had about two-fold and six-fold lower PSEs, respectively, for FS compared to similar sized BG. These air samplers had the largest internal surface areas and tubing coming in contact with the collection liquid during sampling. This could possibly lead to internal loss of sampled material due to adherence to the samplers' walls and tubing, thus having an impact on the end-to-end sampling efficiency. Observations also indicated that FS were more hydrophobic than BG, which could contribute to increase the internal loss of FS in the SASS 2300 and OMNI-3000. The OMNI-3000 was excluded from further testing due to its very low PSE for both sized FS and BG aerosols. It should however be noted that technical issues with this sampler's fluid monitoring system led to abnormal replenishment of collection liquid on some occasions.

When comparing the air samplers' PSEs for 4 μm BG, KR, SM, and MS2 aerosols consistency was observed, suggesting that the PSEs were similar for all test agents (Figure 2). However, for MS2, the gelatin filters and XMX-CV both had $\geq$5-fold lower PSEs for MS2 compared to BG, KR, and SM. A corresponding BSE result was seen for XMX-CV which had $\geq$5-fold lower BSE for MS2 compared to the other test agents, but this was not observed for the gelatin filters.

When the two discrepancies observed for MS2 were discarded, the PSEs for BG, KR, SM, and MS2 showed that gelatin filters and XMX-CV had an averaged PSE (1.01 $\pm$ 0.06 and 1.03 $\pm$ 0.07, respectively) close to the reference sampler, while ESP (0.46 $\pm$ 0.14), SASS 3100 (0.62 $\pm$ 0.11), SASS 2300 (0.48 $\pm$ 0.10), BioCapture 650 (0.58 $\pm$ 0.09), and Coriolis FR (0.58 $\pm$ 0.08) had an averaged PSE that was lower than the reference sampler.

Biological sampling efficiency (BSE)

The targeted bioaerosol's sensitivity to sampling stress and the sampler's propensity to induce such effects are important parameters to consider when cultivation-dependent analysis

methods are needed for quantification and identification of airborne microorganisms. By comparing the cultivation-dependent BSEs to the cultivation-independent PSEs, our results showed that the evaluated air samplers differed substantially regarding their propensity to induce sampling stress. The evaluated air samplers' BSEs for 4 μm BG, KR, SM, and MS2 aerosols differed more between the test agents than the PSEs (Figure 2), although this was not surprising since the test agents were selected to provide microorganisms with variable sensitivities to sampling stress.

The observation that the BSEs and PSEs for BG aerosols corresponded with each other provided additional support to the accuracy of the PSEs since *Bacillus* spores are known to be highly tolerant to microbial stress (Sinclair et al. 2008). These results also suggested that the BSEs and PSEs were comparable measurements that could be used to assess sampling-associated stress. The BSEs and PSEs for KR aerosols also corresponded with each other and with BG aerosols. The BSEs for SM and MS2 aerosols were however more variable, showing both lower and higher BSEs compared to PSEs. Our results (Figure 2) therefore suggested that BG (bacterial spores) and KR (gram-positive vegetative bacteria) were much more resistant to sampling-inflicted microbial stress than SM (gram-negative vegetative bacteria) and MS2 (non-enveloped ssRNA viruses). It should however be taken into account that these results were obtained with 4 μm aerosols consisting of cell/spore aggregates, which may respond differently to stresses than aerosols consisting of single cells/spores.

Coriolis FR was the only sampler that had similar BSEs for BG, KR, SM, and MS2, with an averaged BSE (0.75 $\pm$ 0.09) corresponding to the averaged PSE for the same test aerosols (0.58 $\pm$ 0.08), with a small offset (+30%).

BioCapture 650 had similar BSEs for BG, KR, and MS2, with an averaged BSE (0.80 $\pm$ 0.09) corresponding to the averaged PSE (0.58 $\pm$ 0.09) with a small offset (+38%). The BSE for SM was >4-fold reduced compared to that obtained for BG, KR and MS2, suggesting that that BioCapture 650 injured SM more than the reference sampler, but that the stress did not affect BG, KR, or MS2.

XMX-CV had similar BSEs for BG and KR, with an averaged BSE (1.36 $\pm$ 0.13) corresponding to the averaged PSE (1.03 $\pm$ 0.07) with a small offset (+32%). The BSE for SM was about two-fold higher compared to that obtained for BG and KR, suggesting that the reference sampler injured SM more than XMX-CV, but that the stress did not affect BG and KR. The BSE for MS2 was >6-fold lower compared to that obtained for BG and KR, but since the PSE for MS2 also showed a similarly reduced efficiency (>5-fold) compared to FS, BG, KR, and SM, these results could not be attributed to differences in sampling stress.

Gelatin filters had similar BSE for BG, KR, and MS2, with an averaged BSE (1.14 $\pm$ 0.07) corresponding to the averaged PSE (1.01 $\pm$ 0.06) with a small offset (+13%). The BSE for SM was about 38-fold lower compared to that obtained for BG, KR, and MS2, suggesting that the gelatin filters injured SM more than the reference sampler, but that the stress did not have an impact on BG, KR, and MS2.

SASS 3100 had similar BSEs for BG and KR, with an averaged BSE (0.80 $\pm$ 0.04) corresponding to the averaged PSE (0.62 $\pm$ 0.11) with a small offset (+30%). The BSEs for SM and MS2 were about 80-fold and 40-fold lower, respectively, compared to that obtained for BG and KR, suggesting that the SASS 3100 injured both SM and MS2 more than the reference sampler, but that no such effect was seen for BG and KR.

SASS 2300 had similar BSEs for BG, KR, and MS2, with an averaged BSE (0.64 $\pm$ 0.21) corresponding to the averaged PSE (0.48 $\pm$ 0.10) with a small offset (+33%). The BSE for SM was about 2.5-fold higher compared to that obtained for BG, KR, and MS2, suggesting that the reference sampler injured SM more than the SASS 2300, but that the stress did not have an impact on BG, KR, and MS2.

ESP had similar BSEs for BG and KR, with an averaged BSE (0.52 $\pm$ 0.05) corresponding to the averaged PSE (0.46 $\pm$ 0.14) with a small offset (+13%). The BSEs for SM and MS2 were about 4-fold and 17-fold lower, respectively, compared to the averaged BSE for BG and KR, suggesting that the ESP injured both SM and MS2 more than the reference sampler, but that the stress did not affect BG and KR.

The consistently observed positive offset (13%–38%) between BSEs and PSEs suggested that there was a small bias toward overestimating the BSEs, or underestimating the PSEs, of the evaluated samplers relative to the reference sampler.

Taken together our results suggested that samplers employing dry collection principles (ESP, SASS 3100, gelatin filters, and BioCapture 650) had a more dramatic impact on the cultivability/viability of SM than those employing wet collection (SASS 2300, XMX-CV, and Coriolis FR). The same pattern was not observed with MS2, for which two dry collection samplers (ESP and SASS 3100) seemed to injure MS2 more than the wet collection samplers (SASS 2300 and Coriolis FR), while the other two dry collection samplers (gelatin filters and BioCapture 650) did not seem to influence MS2 differently from the wet collection samplers. A possible explanation could be that MS2 was less sensitive to desiccation than SM, and remained infective with the gelatin filters and BioCapture 650 but not with the ESP and SASS 3100. The manufacturer's instructions-for-use document for the gelatin filters states that they contain 46%–49% residual dampness, while the BioCapture 650 automatically rinses the sampled material from its spinning disk impactor at the end of the sampling period. Thus, the sampled material had a short holding time in a desiccated state before transfer into liquid, in contrast to the ESP and SASS 3100 where the samples had to be manually extracted into liquid after sampling (i.e., the sampled material had a longer holding time in a dry state before transfer to liquid). For BG and KR, no

differences were observed between samplers that could be linked to specific collection principles, suggesting that both these test agents were resistant to the sampling stress inflicted by the evaluated air samplers, independent of whether they employed wet or dry collection principles.

Concentration Factor

In addition to the importance of the sampling efficiency, which is fundamental to ensure accurate quantification of the bioaerosols, the air sampler's concentration factor may also be important since it impacts the amount and concentration of sampled material than can be obtained and subjected to analysis. An air sampler's concentration factor will depend on its airflow rate, collection medium volume, and sampling efficiency. An air sampler with a relative concentration factor of 100 compared to the BioSampler will have a 100-fold lower detection limit for the same sampling operation, and which could be an important performance criterion when the targeted bioaerosol's concentration level is low. Similarly, for sampling applications requiring high temporal resolutions, or when other factors limit the sampling time, employing an air sampler with a high concentration factor may be crucial to ensure that sufficient sampled material is present.

The evaluated air samplers' theoretical concentration factors relative to the reference sampler showed that while the gelatin filters had a theoretical concentration factor of 2, the other samplers had much higher theoretical concentration factors ranging from 46 to 201 (Table 1). The theoretical relative concentration factors were transformed into end-to-end relative biological concentration factors (BCFs) and physical concentration factors (PCFs), respectively, by multiplying them with the BSEs and PSEs obtained in the current work (Table S3). A consolidated summary showing the BCFs and PCFs for each air sampler and test agent is provided as SI (Table S4).

The results showed that the BCFs and PCFs for the evaluated samplers were consistently higher than for the reference sampler, except the BCFs for 4 μm SM with SASS 3100 and gelatin filters, and the PCFs for 1 μm FS with OMNI-3000 and 4 μm MS2 with gelatin filters. The highest BCFs and PCFs were observed with XMX-CV for all test aerosols ($\geq$200), except 4 μm MS2, 1 μm BG, and 1 μm FS. For 4 μm MS2, the SASS 2300 had the highest BCF ($\sim$93) and PCF ($\sim$84). For 1 μm BG and 1 μm FS, the SASS 3100 had the highest BCF (BG; $\sim$44) and PCF (BG; $\sim$50, and FS; $\sim$43). In comparison to the other evaluated samplers, low BCFs and PCFs were consistently observed for the gelatin filters ($\leq$3) and OMNI-3000 ($\leq$10).

In summary, the observed results showed that while several of the evaluated air samplers may underestimate bioaerosol concentration levels due to having lower sampling efficiencies (BSEs/PSEs) than the reference sampler (Table S3), they would generally obtain samples that contained higher concentrations of sampled material due to having higher concentration factors (BCFs/PCFs) than the reference sampler (Table S4).

Air Sampler Suitability Assessments

Based on the demonstrated performance of the evaluated air samplers (Figures 1 and 2, and Tables S3 and S4), generalized features regarding their suitability for various bioaerosol sampling applications were assessed (Table 2).

The suitability features were separated into quantitative and qualitative sampling applications because the fundamental air sampler performance requirements may be different depending on whether the objective is to accurately determine concentration levels (quantify) or to detect/identify (qualify).

Since the sampling efficiency directly impacts bioaerosol concentration level estimates (e.g., an absolute sampling efficiency of 50% results in a two-fold underestimation of the true level), it may be considered a fundamental performance requirement for quantitative bioaerosol sampling. The evaluated air samplers' BSEs and PSEs were therefore used to assess their suitability for quantitative sampling applications. An air sampler was considered suitable if the relative sampling efficiency was $\geq$0.5, thus implying less than twofold underestimation of the bioaerosol level compared to the reference sampler (Table 2).

While a direct quantitative relationship between air and sample is essential for accurate quantification, qualitative sampling applications are, however, not dependent on the existence of such a direct relationship.

For qualitative applications, obtaining sufficient material (i.e., above the analysis method's detection limit) may be considered the primary sampling objective, and the sampler's ability to concentrate bioaerosols from the air and into a sample may therefore be considered a fundamental performance requirement. The evaluated air samplers' BCFs and PCFs were therefore used to assess their suitability for qualitative sampling applications. An air sampler was considered suitable if the relative concentration factor was $\geq$1.0, thus implying similar or higher concentration levels of sampled material compared to the reference sampler (Table 2).

It should however be noted that because the T&E results obtained in the current work were relative to the BioSampler, the employed suitability criteria were also inherently relative. For the purpose of this study, the PSEs and BSEs and concentration factors of the BioSampler were therefore considered as "performance benchmarks," although this should not be taken to suggest that the BioSampler has 100% absolute PSEs and BSEs for all bioaerosols or that its concentration factors will be suitable for all bioaerosol sampling purposes.

The BioSampler was chosen as a reference sampler because it has been shown to have sampling efficiencies $\geq$90% for the particle sizes involved in this study (Willeke et al. 1998; Kesavan et al. 2011). The BioSampler has also been shown to inflict limited sampling stress allowing retained cultivability of stress-sensitive airborne microorganisms (Lin et al. 1999, 2000). However, some of the T&E results obtained in the current study, and especially the BSEs for 4 μm SM aerosols observed with XMX-CV and SASS 2300 which both were $>$1.5, could suggest that the BioSampler inflicted sampling stress that reduced the

TABLE 2

Generalized features regarding the evaluated air samplers' suitability for various bioaerosol sampling applications

Bioaerosol sampling applications		Gelatin filters	SASS 2300	SASS 3100	Coriolis FR	OMNI-3000	BioCapture 650	ESP prototype	XMX-CV
Quantitative sampling (suitability requirement: relative sampling efficiency $\geq$0.5 compared to the BioSampler)[a]	Particle size (1 μm and 4 μm) (determined for FS and BG aerosols)	Both sizes	None	Both sizes	Both sizes ($\sim$0.5 for 1 μm)	None	4 μm only	4 μm only ($\sim$0.5 for 4 μm)	4 μm only
	PSE-dependent analysis method (e.g., qPCR) (determined for 4 μm FS, BG, KR, SM, and MS2 aerosols)	FS, BG, KR and SM	SM and MS2	All agents	All agents ($\sim$0.5 for MS2)	None[b]	All agents ($\sim$0.5 for SM)	FS, BG and KR ($\sim$0.5 for FS)	FS, BG, KR and SM
	BSE-dependent analysis method (e.g., cultivation) (determined for 4 μm BG, KR, SM, and MS2 aerosols)	BG, KR, and MS2	KR, SM, and MS2	BG and KR	All agents	None[b]	BG, KR, and MS2	BG and KR ($\sim$0.5 for both)	BG, KR, and SM

(Continued on next page)

TABLE 2
Generalized features regarding the evaluated air samplers' suitability for various bioaerosol sampling applications *(Continued)*

Bioaerosol sampling applications		Gelatin filters	SASS 2300	SASS 3100	Coriolis FR	OMNI-3000	BioCapture 650	ESP prototype	XMX-CV
Qualitative sampling (suitability requirement: relative concentration factor $\geq$1.0 compared to the BioSampler)[c]	Particle size (1 μm and 4 μm) (determined for FS and BG aerosols)	Both sizes	Both sizes	Both sizes	Both sizes	Both sizes based on BG, only 4 μm based on FS	Both sizes	Both sizes	Both sizes
	PCF-dependent analysis method (e.g., PCR-based detection) (determined for 4 μm FS, BG, KR, SM, and MS2 aerosols)	FS, BG, KR, and SM	All agents	All agents	All agents	BG and FS[b]	All agents	All agents	All agents
	BCF-dependent analysis method (e.g., cultivation-based detection) (determined for 4 μm BG, KR, SM, and MS2 aerosols)	BG, KR, and MS2	All agents	BG, KR, and MS2 ($\sim$1.0 for MS2)	All agents	BG[b]	All agents	All agents	All agents

BSE: Biological sampling efficiency; PSE: physical sampling efficiency; BCF: biological concentration factor; PCF: physical concentration factor; FS: FluoSpheres; BG: *Bacillus atrophaeus* spores; KR: *Kocuria rhizophila*; SM: *Serratia marcescens*; MS2: Bacteriophage MS2.

[a]An air sampler was assessed suitable for quantitative applications when its relative sampling efficiency was $\geq$0.5 (i.e., less than two-fold underestimation of the concentration level compared to the BioSampler).

[b]The OMNI-3000 was not tested with 4 μm KR, SM, and MS2 aerosols.

[c]An air sampler was assessed suitable for qualitative applications when its relative concentration factor was $\geq$1.0 (i.e., similar or higher concentration of sampled material available for the downstream analysis compared to the BioSampler).

cultivability of SM more than some of the evaluated samplers. While these are interesting observations, further studies will be needed to verify and investigate these results. Still, they may be seen as an interesting reminder about that in all likelihood no single air sampler will be universally optimal, or even suitable, for all bioaerosols and bioaerosol sampling purposes.

CONCLUSION AND IMPLICATIONS

Selecting an air sampler for bioaerosol sampling applications is a nontrivial task that must be performed with great care to ensure that the obtained results accurately and reliably describe the studied bioaerosol. The current work contributes to increase user awareness about important factors that should be considered during air sampler selection. Our results highlight the importance of harmonizing air sampler performance to the targeted bioaerosol and the downstream analysis method, and provide several generalized features regarding the evaluated air samplers' suitability for various bioaerosol sampling applications (Table 2).

Although this study involved a diverse selection of test agents, several other types of commonly studied bioaerosols were not specifically addressed (e.g., fungi, fungal spores, protists, pollen, and microbial fragments such as endotoxins and mycotoxins). However, since we established PSEs and BSEs for aerosol test agents with highly variable morphologies and sensitivities to sampling stress, practitioners interested in other types of bioaerosols may still be able to extract relevant performance information, at least within the evaluated particle size range. Extrapolations of the results to include other types of bioaerosols should in any case only be carefully attempted and mindfully considered, even within the microorganism classes that were specifically evaluated.

We believe that the performance measurements presented in this work could contribute to a more well-harmonized selection of air sampling equipment, and thereby lead to more accurate, reliable, comparable, and relevant exposure assessments for airborne pathogens and other bioaerosols of interest.

FUNDING

This work was funded by the Norwegian Defence Research Establishment.

SUPPLEMENTAL MATERIAL

Supplemental data for this article can be accessed on the publisher's website.

REFERENCES

Ahmed, M. F., Schulz, J., and Hartung, J. (2013). Air Samplings in a Campylobacter Jejuni Positive Laying Hen Flock. *Ann. Agr. Env. Med.*, 20:16–20.

Alvarez, A. J., Buttner, M. P., and Stetzenbach, L. D. (1995). PCR for Bioaerosol Monitoring: Sensitivity and Environmental Interference. *Appl. Environ Microbiol.*, 61:3639–3644.

An, H. R., Mainelis, G., and Yao, M. (2004). Evaluation of a High-Volume Portable Bioaerosol Sampler in Laboratory and Field Environments. *Indoor Air*, 14:385–393.

Baron, P. A. (1998). Personal Aerosol Sampler Design: A Review. *Appl. Occupat. Environ. Hyg.*, 13:313–320.

Bartlett, K. H., Lee, K. S., Stephens, G., Black, W., Brauer, M., and Copes, R. (2002). *Evaluating Indoor Air Quality: Test Standards for Bioaerosols, School of Occupational and Environmental Hygiene*. University of British Columbia, Vancouver, Canada.

Bergman, W., Shinn, J., Lochner, R., Sawyer, S., Milanovich, F., and Jr, R. M. (2004). High Volume, Low Pressure Drop, Bioaerosol Collector Using a Multi-slit Virtual Impactor. *J. Aerosol Sci.*, 36:619–638.

Black, J. E. (2011). *Evaluation of XMX/2L-MIL Virtual Impactor Performance and Capture and Retention of Aerosol Particles in Two Different Collection Media*. DTIC Document.

Black, J. E., and Cooper, C. W. (2012). *Interinstrument Variability and Validation Study for the XMX/2L-MIL Biological Air Sampler*. DTIC Document.

Brixey, L. A., Paik, S. Y., Evans, D. E., and Vincent, J. H. (2002). New Experimental Methods for the Development and Evaluation of Aerosol Samplers. *J.Environ. Monitor.*, 4:633–641.

Burge, H. A., and Solomon, W. R. (1987). Sampling and Analysis of Biological Aerosols. *Atmos. Environ. (1967)*, 21:451–456.

Burton, N. C., Grinshpun, S. A., and Reponen, T. (2007). Physical Collection Efficiency of Filter Materials for Bacteria and Viruses. *Ann. Occup. Hyg.*, 51:143–151.

Buttner, M. P., Cruz, P., Stetzenbach, L. D., Klima-Comba, A. K., Stevens, V. L., and Cronin, T. D. (2004). Determination of the Efficacy of Two Building Decontamination Strategies by Surface Sampling with Culture and Quantitative PCR Analysis. *App. Environ. Microbiol.*, 70:4740–4747.

Buttner, M. P., and Stetzenbach, L. D. (1991). Evaluation of Four Aerobiological Sampling Methods for the Retrieval of Aerosolized Pseudomonas Syringae. *Appl. Environ. Microbiol.*, 57:1268–1270.

Buttner, M., Willeke, K., and Grinshpun, S. (2002). Sampling and Analysis of Airborne Microorganisms, in *Manual of Environmental Microbiolgy*, C. J. Hurst, R. L. Crawford, G. Knudsen, M. McInerney and L. D. Stetzenbach, eds., ASM Press, Washington, DC, pp. 814–826.

Carvalho, E., Sindt, C., Verdier, A., Galan, C., O'Donoghue, L., Parks, S., et al. (2008). Performance of the Coriolis Air Sampler, a High-Volume Aerosol-Collection System for Quantification of Airborne Spores and Pollen Grains. *Aerobiologia*, 24:191–201.

Chang, C.-W., and Chou, F. C. (2011). Methodologies for Quantifying Culturable, Viable, and Total Legionella Pneumophila in Indoor Air. *Indoor Air*, 21:291–299.

Chang, C.-W., Chou, F.-C., and Hung, P.-Y. (2010). Evaluation of Bioaerosol Sampling Techniques for Legionella Pneumophila Coupled with Culture Assay and Quantitative PCR. *J. Aerosol Sci.*, 41:1055–1065.

Cooper, C. W. (2010). *High Volume Air Sampling for Viral Aerosols: A Comparative Approach*. DTIC Document.

Cox, C. S., and Wathes, C. M. (1995). *Bioaerosols Handbook*. Lewis Publishers, Boca Raton, FL.

Eduard, W., and Heederik, D. (1998). Methods for Quantitative Assessment of Airborne Levels of Noninfectious Microorganisms in Highly Contaminated Work Environments. *Am. Ind. Hyg. Assoc. J.*, 59:113–127.

Enderby, J. C. (2012). *Comparative Analysis of Two Biological Warfare Air Samplers Using Live Surrogate Agents*. DTIC Document.

Estill, C. F., Baron, P. A., Beard, J. K., Hein, M. J., Larsen, L. D., Deye, G. J., et al. (2011). Comparison of Air Sampling Methods for Aerosolized Spores of B. Anthracis Sterne. *J. Occup. Environ. Hyg.*, 8:179–186.

Fabian, P., McDevitt, J. J., Houseman, E. A., and Milton, D. K. (2009). Airborne Influenza Virus Detection with Four Aerosol Samplers using Molecular and Infectivity Assays: Considerations for a New Infectious Virus Aerosol Sampler. *Indoor Air*, 19:433–441.

Georgakopoulos, D. G., Després, V., Fröhlich-Nowoisky, J., Psenner, R., Ariya, P. A., Pósfai, M., et al. (2009). Microbiology and Atmospheric Processes:

Biological, Physical and Chemical Characterization of Aerosol Particles. *Biogeosciences*, 6:721–737.

Gómez-Domenech, M., García-Mozo, H., Alcázar, P., Brandao, R., Caeiro, E., Munhoz, V., et al. (2010). Evaluation of the Efficiency of the Coriolis air Sampler for Pollen Detection in South Europe. *Aerobiologia*, 26:149–155.

Griffin, D. W., Gonzalez, C., Teigell, N., Petrosky, T., Northup, D., and Lyles, M. (2011). Observations on the Use of Membrane Filtration and Liquid Impingement to Collect Airborne Microorganisms in Various Atmospheric Environments. *Aerobiologia*, 27:25–35.

Grinshpun, S. A., Chang, C.-W., Nevalainen, A., and Willeke, K. (1994). Inlet Characteristics of Bioaerosol Samplers. *J. Aerosol Sci*., 25:1503–1522.

Grinshpun, S. A., and Clark, J. M. (2005). Measurement and Characterization of Bioaerosols. *J. Aerosol Sci*., 36:553–555.

Grinshpun, S. A., Willeke, K., Ulevicius, V., Donnelly, J., Lin, X., and Mainelis, G. (1996). Collection of Airborne Microorganisms: Advantages and Disadvantages of Different Methods. *J. Aerosol Sci*., 27, Supplement 1:S247–S248.

Henningson, E. W., Lundquist, M., Larsson, E., Sandström, G., and Forsman, M. (1997). A Comparative Study of Different Methods to Determine the Total Number and the Survival Ratio of Bacteria in Aerobiological Samples. *J. Aerosol Sci*., 28:459–469.

Hermann, J. R., Hoff, S. J., Yoon, K. J., Burkhardt, A. C., Evans, R. B., and Zimmerman, J. J. (2006). Optimization of a Sampling System for Recovery and Detection of Airborne Porcine Reproductive and Respiratory Syndrome Virus and Swine Influenza Virus. *Appl. Environ. Microbiol*., 72:4811–4818.

Kesavan, J.S., Bottiger, J. R., and McFarland, A. R. (2008). Bioaerosol Concentrator Performance: Comparative Tests with Viable and with Solid and Liquid Nonviable Particles. *J. Appl. Microbiol*., 104:285–295.

Kesavan, J. S., Doherty, R. W., and Bottiger, J. R. (2003). *Performance Characterization Methods of Aerosol Samplers, Edgewood Chemical Biological Center (ECBC)*. Aberdeen Proving Ground, MD.

Kesavan, J. S., Schepers, D., and Bottiger, J. (2011). *Characteristics of Twenty-Nine Aerosol Samplers Tested at US Army Edgewood Chemical Biological Center (2000–2006)*. DTIC Document.

Kesavan, J., Schepers, D., and McFarland, A. R. (2010a). Sampling and Retention Efficiencies of Batch-Type Liquid-Based Bioaerosol Samplers. *Aerosol Sci. Technol*., 44:817–829.

Kesavan, J., Schepers, D., Sutton, T., Deluca, P., Williamson, M., and Wise, D. (2010b). *Characteristics Sampling Efficiency and Battery Life of Smart Air Sampler System (SASS) 3000 and SASS 3100*. DTIC Document.

Kesavan, J., and Stuebing, E. (2009). Aerosol Sampling Efficiency Evaluation Methods at the US Army Edgewood Chemical Biological Center, in *Atmospheric and Biological Environmental Monitoring*, Y. Kim, U. Platt, M. Gu, and H. Iwahashi, eds., Springer, Netherlands, pp. 83–103.

Li, C.-S. (1999). Evaluation of Microbial Samplers for Bacterial Microorganisms. *Aerosol Sci. Technol*., 30:100–108.

Li, S. N., Lundgren, D. A., and Rovell-Rixx, D. (2000). Evaluation of Six Inhalable Aerosol Samplers. *Am. Ind. Hyg. Assoc. J*., 61:506–516.

Lin, W. H., and Li, C. S. (1999). Evaluation of Impingement and Filtration Methods for Yeast Bioaerosol Sampling. *Aerosol Sci. Technol*., 30:119–126.

Lin, X., Reponen, T., Willeke, K., Grinshpun, S. A., Foarde, K. K., and Ensor, D. S. (1999). Long-Term Sampling of Airborne Bacteria and Fungi into a Non-Evaporating Liquid. *Atmos. Environ*., 33:4291–4298.

Lin, X., Reponen, T., Willeke, K., Wang, Z., Grinshpun, S. A., and Trunov, M. (2000). Survival of Airborne Microorganisms During Swirling Aerosol Collection. *Aerosol Sci. Technol*., 32:184–196.

Macher, J., ed. (1999). *Bioaerosols: Assessment and Control*. American Conference of Governmental Industrial Hygienists (ACGIH), Cincinnati, OH.

Macher, J. M., and Willeke, K. (1992). Performance Criteria for Bioaerosol Samplers. *J. Aerosol Sci*., 23(Suppl. 1):647–650.

McFarland, A. R., Haglund, J. S., King, M. D., Hu, S., Phull, M. S., Moncla, B. W., et al. (2010). Wetted Wall Cyclones for Bioaerosol Sampling. *Aerosol Sci. Technol*., 44:241–252.

Millner, P. D. (2009). Bioaerosols Associated with Animal Production Operations. *Bioresource Technol*., 100:5379–5385.

Nevalainen, A., Pastuszka, J., Liebhaber, F., and Willeke, K. (1992). Performance of Bioaerosol Samplers: Collection Characteristics and Sampler Design Considerations. *Atmos. Environ. Part A. General Topics*, 26:531–540.

O'Connell, K. P., Bucher, J. R., Anderson, P. E., Cao, C. J., Khan, A. S., Gostomski, M. V., et al. (2006). Real-Time Fluorogenic Reverse Transcription-PCR Assays for Detection of Bacteriophage MS2. *Appl. Environ. Microbiol*., 72:478–483.

Radosevich, J. L., Wilson, W. J., Shinn, J. H., DeSantis, T. Z., and Andersen, G. L. (2002). Development of a High-Volume Aerosol Collection System for the Identification of Air-Borne Micro-Organisms. *Lett. Appl. Microbiol*., 34:162–167.

Reponen, T., Willeke, K., Grinshpun, S., and Nevalainen, A. (2011). Biological Particle Sampling, in *Aerosol Measurement*, John Wiley & Sons, Inc., New York, pp. 549–570.

Rule, A. M., Kesavan, J., Schwab, K. J., and Buckley, T. J. (2007). Application of Flow Cytometry for the Assessment of Preservation and Recovery Efficiency of Bioaerosol Samplers Spiked with Pantoea agglomerans. *Environ. Sci. Technol*., 41:2467–2472.

Saikaly, P. E., Barlaz, M. A., and de los Reyes, F. L. (2007). Development of Quantitative Real-Time PCR Assays for Detection and Quantification of Surrogate Biological Warfare Agents in Building Debris and Leachate. *Appl. Environ. Microbiol*., 73:6557–6565.

Shahamat, M., Levin, M., Rahman, I., Grim, C., Heidelberg, J., Stelma, G., and Colwell, R. (1997). Evaluation of Media for Recovery of Aerosolized Bacteria. *Aerobiologia*, 13:219–226.

Sinclair, R., Boone, S. A., Greenberg, D., Keim, P., and Gerba, C. P. (2008). Persistence of Category A Select Agents in the Environment. *Appl. Environ. Microbiol*., 74:555–563.

Tseng, C.-C., and Li, C.-S. (2005). Collection Efficiencies of Aerosol Samplers for Virus-Containing Aerosols. *J. Aerosol Sci*., 36:593–607.

Van Droogenbroeck, C., Van Risseghem, M., Braeckman, L., and Vanrompay, D. (2009). Evaluation of Bioaerosol Sampling Techniques for the Detection of Chlamydophila Psittaci in Contaminated Air. *Vet. Microbiol*., 135:31–37.

Vitko Jr., J., Franz, D., Alper, M., Biggins, P., Brandt, L., Bruckner-Lea, C., et al. (2005). Committee on Materials and Manufacturing Processing for Advanced Sensors. The National Research Council of the National Academies, The National Academic Press, Washington DC. Sensor Systems for Biological Agent Attacks: Protecting Buildings and Military Bases.

Willeke, K., Lin, X., and Grinshpun, S. A. (1998). Improved Aerosol Collection by Combined Impaction and Centrifugal Motion. *Aerosol Sci. Technol*., 28:439–456.

Xu, Z., Wu, Y., Shen, F., Chen, Q., Tan, M., and Yao, M. (2011). Bioaerosol Science, Technology, and Engineering: Past, Present, and Future. *Aerosol Sci. Technol*., 45:1337–1349.

Yao, M., and Mainelis, G. (2007). Analysis of Portable Impactor Performance for Enumeration of Viable Bioaerosols. *J. Occup. Environ. Hyg*., 4:514–524.

Zhao, Y., Aarnink, A. J. A., Doornenbal, P., Huynh, T. T. T., Koerkamp, P. W. G. G., de Jong, M. C. M., et al. (2011). Investigation of the Efficiencies of Bioaerosol Samplers for Collecting Aerosolized Bacteria Using a Fluorescent Tracer. I: Effects of Non-Sampling Processes on Bacterial Culturability. *Aerosol Sci. Technol*., 45:423–431.

Supplemental Information

Comparative Testing and Evaluation of Nine Different Air Samplers: End-to-End Sampling Efficiencies as Specific Performance Measurements for Bioaerosol Applications

Marius Dybwad, Gunnar Skogan, Janet Martha Blatny

MATERIALS AND METHODS

Cultivation analysis

The collected samples were diluted as needed with PBSTA before triplicate plating on appropriate growth medium plates. NA plates were used for BG and SM, while TSA plates were used for KR. MS2-containing samples were analyzed on NA plates by a pour-plate method using 1.0 x 10^7 cfu of log-phase *Escherichia coli* (DSM 4230) cells in soft NA (0.7% agar). The cultivation plates were incubated (18 hr) at 30°C (BG and SM) or 37°C (KR and MS2) and plates containing between 30 and 300 cfu or pfu were manually counted.

Molecular analysis

The qPCR assay for KR was designed using Primer-Blast with standard parameters for primer search and specificity checking (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The query sequence was *gyrB* from the genome sequence of *Kocuria rhizophila* DC2201 (NC_010617). Specificity checking was performed towards the GenBank nr nucleotide collection database.

Nucleic acids were purified using NucliSENS isolation kits (bioMérieux, Marcy l'Etoile, France) and the EasyMAG instrument (bioMérieux). Samples containing SM, KR and MS2 were purified directly, while BG samples were homogenized (1 ml sample, 60 s, max intensity) with a Mini Beadbeater-8 (BioSpec, Bartlesville, OK) using bead beating tubes (2ml, Sarstedt, Nümbrecht, Germany) containing 0.5 g 100 µm and 0.5 g 500 µm zirconium-silica beads (BioSpec). Purified nucleic acids were analyzed as triplicate reactions in 96-well PCR plates (Roche Diagnostics, Indianapolis, IN) using a LightCycler 480 instrument (Roche Diagnostics). The primers and probes were purchased from Invitrogen (Table S1).

Each qPCR reaction (20 µl) contained; 2X SYBR Green master mix (10 µl, Roche Diagnostics), PCR grade water (6 µl, Roche Diagnostics), 10 mM forward and reverse primers (1 µl each) and template (2 µl). The qPCR program consisted of denaturation (95°C, 5 min) and 40 cycles of denaturation (95°C, 20 s), annealing (60°C, 20 s) and extension (72°C, 20 s).

Each qRT-PCR reaction (20 µl) contained; 5X Reaction buffer (4 µl) and 50X Enzyme blend (0.4 µl) from the Real-time Ready RNA Virus Master kit (Roche Diagnostics), PCR grade water (10.6 µl), 10 mM forward and reverse primers (1 µl each), 10 mM probe (1 µl) and template (2 µl). The qRT-PCR program consisted of reverse transcription (50 °C, 8 min), denaturation (95°C, 5 min) and 40 cycles of denaturation (95°C, 20 s), annealing (60°C, 20 s) and extension (72°C, 20 s).

Standard curves were constructed for each test agent by purifying nucleic acids from serial dilutions of the respective spray solutions. PCR grade water was used as negative

amplification controls, while standards from the standard curves were used as a positive amplification controls and internal calibrators. Melting curves were constructed to verify specific amplifications when SYBR Green was used. Possible PCR inhibition due to differences in the collection liquids were investigated by analyzing nucleic acids purified from collection liquids spiked with standards from the standard curves. No inhibition was observed for any of the collection liquids. Possible false positive PCR amplification was addressed by testing each PCR assay to all test agents, and no cross-reactivity was observed.

Direct count analysis

The collected samples were diluted as needed with PBSTA and filtered onto black polycarbonate membrane filters (Isopore, 25mm diameter, 0.2 µm pore-size, Millipore) housed in polypropylene filter holders (Swinnex-25, Millipore). The membrane filters were mounted on microscopy slides and analyzed using a fluorescence microscope (AxioSkop 2, Carl Zeiss, Thornwood, NY) fitted with a digital camera (AxioCam HRc, Carl Zeiss). Yellow-green FS (1 µm) were viewed using 488nm and 525nm band pass filters (filter set 52, Carl Zeiss), while red Fluospheres (4 µm) were viewed using 546nm and 575-640nm band pass filters (filter set 20, Carl Zeiss). Photomicrographs were captured (AxioVision, Carl Zeiss) from random field-of-views (>20 fields or >1000 FS) and processed using image analysis software (ImageJ, http://rsbweb.nih.gov/ij/index.html).

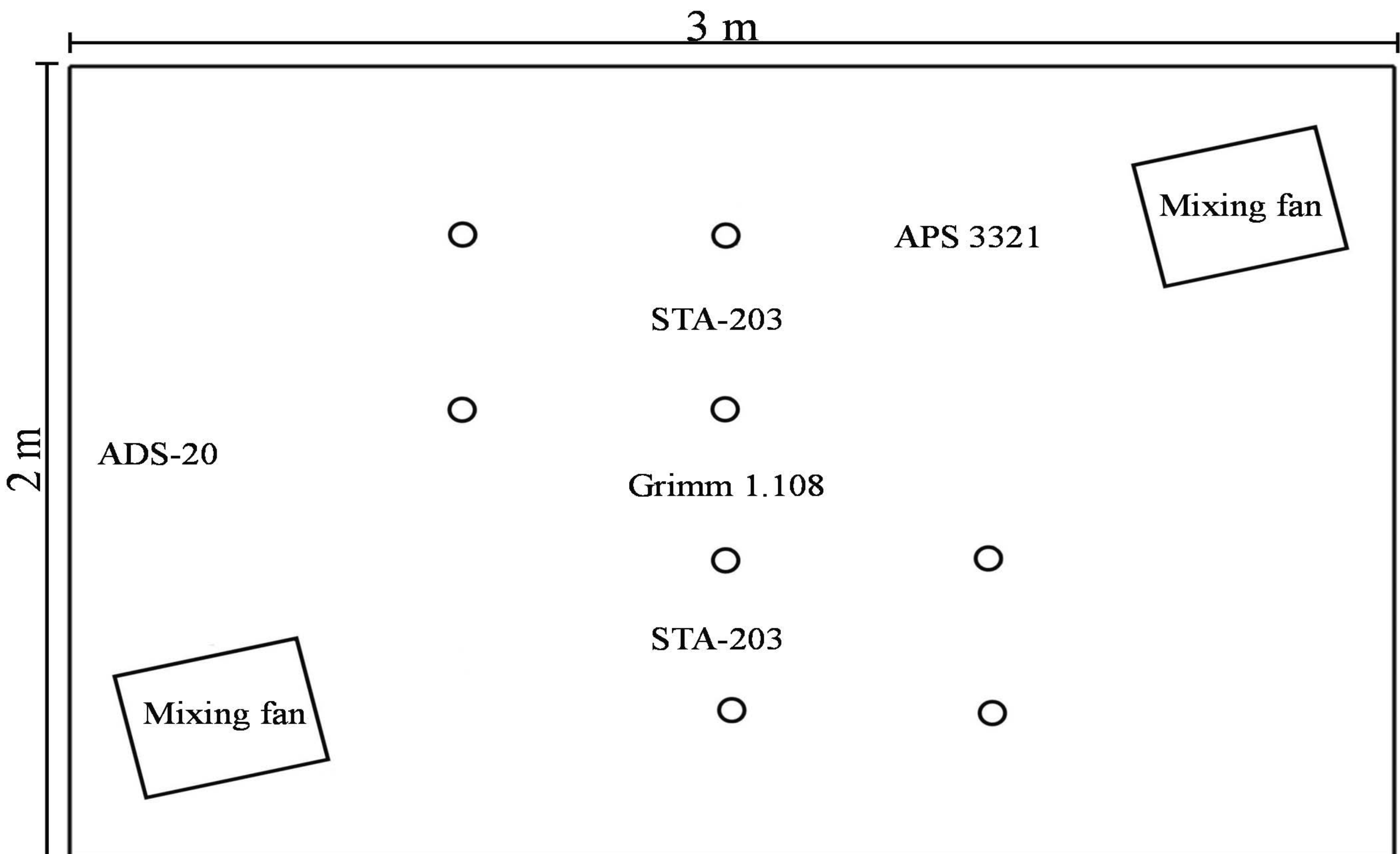

Figure S1. Top-down schematic layout of the 12 m^3 aerosol test chamber (ATC).The sampling positions used for air sampler testing are designated with open circles. Mixing fan; two chamber-mounted mixing fans (120 mm), ADS-20; injection port for test aerosols from the ADS-20 aerosol dilution system, STA-203; two chamber-mounted slit-to-agar samplers, APS3321; aerodynamic particle sizer, Grimm 1.108; optical particle counter.

Figure S2. Air sampling equipment subjected to testing and evaluation (T&E) in this study. From the left: OMNI-3000, SASS 2300, Coriolis FR, BioSampler (reference sampler), BioCapture 650, SASS 3100, XMX-CV, and ESP (prototype). The gelatin filters are not shown in this picture.

Table S1. Primers and probes.

Organism	Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Detection probe (5'-3')	Product size (bp)	References
Serratia marcescens	gyrB	AGTGCACGAACAAACTTACAG	GTCGTACTCGAAATCGGTCACA	n.a.	138	Saikaly et al. 2007
Bacillus atrophaeus spores	recA	ACCAGACAATGCTCGACGTT	CCCTCTTGAAATTCCCGAAT	n.a.	131	Buttner et al. 2004
Kocuria rhizophila	gyrB	CCGTGGACATGCACCCCACC	CGGGACAGGGCGTTGACCAC	n.a.	148	This study
Bacteriophage MS2	A protein	GTCGCGGTAATTGGCGC	GGCCACGTGTTTTGATCGA	FAM-AGGCGCTCCGCTACCTTGCCCT-BBQ	77	O'Connell et al. 2006

n.a.; not applicable, bp; base pair.

Table S2. Particle size distributions for the test aerosols based on APS 3321 aerodynamic particle sizer measurements. The size distributions were calculated based on all aerosol experiments and reported as the mass median aerodynamic diameter (MMAD) with geometric standard deviation (GSD), and a MMAD size range for the individual experiments.

Aerosol test agent	**Particle size distribution based on APS 3321 measurements from all aerosol experiments**					
	1 µm MMAD targeted			**4 µm MMAD targeted**		
	MMAD (µm)	**GSD**	**Range (µm)**	**MMAD (µm)**	**GSD**	**Range (µm)**
FluoSpheres (FS)	1.04	1.09	1.02-1.20	3.84	1.10	3.67-3.96
Bacillus atrophaeus spores (BG)	1.22	1.31	1.03-1.46	4.04	1.34	3.83-4.20
Kocuria rhizophila (KR)				3.92	1.40	3.61-4.37
Serratia marcescens (SM)				4.38	1.53	4.08-4.63
Bacteriophage MS2 (MS2)				3.87	1.36	3.66-4.35

MMAD; mass median aerodynamic diamater, GSD; geometric standard deviation

0.35 ± 0.04	0.36 ± 0.13	0.47 ± 0.06	0.55 ± 0.14	0.52 ± 0.09	0.13 ± 0.09	0.39 ± 0.07	0.48 ± 0.23	0.62 ± 0.22	0.03 ±
0.10 ± 0.01	0.08 ± 0.02	0.13 ± 0.02	0.43 ± 0.10	0.39 ± 0.09	1.60 ± 0.39	0.57 ± 0.11	0.85 ± 0.20	0.39 ± 0.16	0.63 ±
0.73 ± 0.12	0.83 ± 0.15	0.63 ± 0.11	0.83 ± 0.18	0.77 ± 0.11	0.01 ± 0.01	0.52 ± 0.06	0.78 ± 0.17	0.57 ± 0.28	0.02 ±
1.28 ± 0.09	1.18 ± 0.09	1.25 ± 0.16	1.17 ± 0.30	1.03 ± 0.08	0.03 ± 0.04	1.07 ± 0.17	1.18 ± 0.32	0.92 ± 0.17	1.06 ±
0.22 ± 0.05	0.19 ± 0.06	1.07 ± 0.27	1.26 ± 0.20	1.11 ± 0.17	2.63 ± 0.56	0.98 ± 0.17	1.45 ± 0.32	1.00 ± 0.31	0.21 ±
0.22 ± 0.17	0.22 ± 0.06	0.74 ± 0.21	0.78 ± 0.13	0.60 ± 0.14	0.18 ± 0.05	0.46 ± 0.13	0.90 ± 0.27	0.57 ± 0.11	0.72 ±
0.49 ± 0.25	0.53 ± 0.08	0.88 ± 0.08	0.69 ± 0.09	0.67 ± 0.07	0.72 ± 0.18	0.53 ± 0.16	0.88 ± 0.17	0.62 ± 0.19	0.70 ±
0.16 ± 0.10	0.20 ± 0.09	0.08 ± 0.05	0.16 ± 0.05	0.21 ± 0.07	n.d.	n.d.	n.d.	n.d.	n.d.

ce microscopy-based direct count analysis, Cultivation; plate count analysis, q(RT-)PCR; quantitative (reverse transcriptase) polymerase chain reaction analysis.

and PSEs were determined relative to a reference sampler (BioSampler) and reported as an averaged value (± standard deviation) based on a minimum of five aerosol experiments.

	28.7	29.5	38.6	45.1	42.7	10.7	32.0	39.4	50.9	2.5
	14.8	11.9	19.3	63.7	57.8	237.1	84.5	126.0	57.8	93.4
	44.4	50.5	38.3	50.5	46.8	0.6	31.6	47.4	34.7	1.2
	2.9	2.7	2.9	2.7	2.3	0.1	2.4	2.7	2.1	2.4
	44.3	38.3	215.5	253.8	223.6	529.7	197.4	292.0	201.4	42.3
	13.4	13.4	45.0	47.4	36.5	10.9	28.0	54.7	34.7	43.8
	24.8	26.9	44.6	35.0	33.9	36.5	26.9	44.6	31.4	35.5
	7.3	9.1	3.6	7.3	9.6	n.d.	n.d.	n.d.	n.d.	n.d.

iorescence microscopy-based direct count analysis, Cultivation; plate count analysis, q(RT-)PCR; quantitative (reverse transcriptase) polymerase chain reaction analysis.

ical and physical concentration factors relative to the reference sampler (BioSampler) were calculated by multiplying the theoretical relative concentration factors (Table 1) with the end-to-end relative biological :ively (Table S3).

Rapid Identification of *Bacillus anthracis* Spores in Suspicious Powder Samples by Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS)

Dybwad, M, AL van der Laaken, JM Blatny, and A Paauw

Applied and Environmental Microbiology, 2013, 79(17):5372-5383

Paper IV

AEM
Journals.ASM.org

Rapid Identification of *Bacillus anthracis* Spores in Suspicious Powder Samples by Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS)

Marius Dybwad,[a,b] Anton L. van der Laaken,[c] Janet Martha Blatny,[b] Armand Paauw[c]

Norwegian Defence Research Establishment FFI, Kjeller, Norway[a]; Norwegian University of Science and Technology, Department of Biotechnology, Trondheim, Norway[b]; TNO, Department of Earth, Environmental and Life Sciences, Rijswijk, The Netherlands[c]

Rapid and reliable identification of *Bacillus anthracis* spores in suspicious powders is important to mitigate the safety risks and economic burdens associated with such incidents. The aim of this study was to develop and validate a rapid and reliable laboratory-based matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis method for identifying *B. anthracis* spores in suspicious powder samples. A reference library containing 22 different *Bacillus* sp. strains or hoax materials was constructed and coupled with a novel classification algorithm and standardized processing protocol for various powder samples. The method's limit of *B. anthracis* detection was determined to be 2.5×10^6 spores, equivalent to a 55-μg sample size of the crudest *B. anthracis*-containing powder discovered during the 2001 Amerithrax incidents. The end-to-end analysis method was able to successfully discriminate among samples containing *B. anthracis* spores, closely related *Bacillus* sp. spores, and commonly encountered hoax materials. No false-positive or -negative classifications of *B. anthracis* spores were observed, even when the analysis method was challenged with a wide range of other bacterial agents. The robustness of the method was demonstrated by analyzing samples (i) at an external facility using a different MALDI-TOF MS instrument, (ii) using an untrained operator, and (iii) using mixtures of *Bacillus* sp. spores and hoax materials. Taken together, the observed performance of the analysis method developed demonstrates its potential applicability as a rapid, specific, sensitive, robust, and cost-effective laboratory-based analysis tool for resolving incidents involving suspicious powders in less than 30 min.

Bacillus anthracis is one of the most feared biological threat agents. This Gram-positive, spore-forming bacterium, designated a category A agent, is the causative agent of anthrax (1). The infective route of *B. anthracis* is inhalation, ingestion, or contact through skin lesions, leading to the development of inhalation, gastrointestinal, or cutaneous anthrax, respectively. *B. anthracis* can enter a resting stage by producing endospores that are highly resistant to environmental influences such as temperature, radiation, and humidity extremes, illustrated by the fact that *B. anthracis* spores have been shown to persist in various harsh environments for decades (2). Historically, *B. anthracis* has played a central role as a biological warfare agent, but in 2001, the mailing of letters containing powders of *B. anthracis* spores to news media and government offices in the United States (the Amerithrax incident) killed five people, sickened an additional 17, and resulted in economic disbursements of more than one billion U.S. dollars, demonstrating *B. anthracis* as a bioterrorism agent (3, 4). The majority of incidents involving suspicious powders eventually turn out to be hoaxes (i.e., the powder does not contain *B. anthracis* spores) (5). However, even hoax incidents have economic and psychological impacts, since they cannot easily be discriminated from bioterrorism incidents and must therefore be handled as real threats until the presence of *B. anthracis* spores or other hazards can be ruled out. In the United States alone, about 3,000 incidents involving suspicious powders occur each year (6). Rapid and reliable methods capable of ruling out hoax materials and confirming the presence of *B. anthracis* spores in powder samples are important to mitigate the safety risks and economic burdens associated with both bioterrorism and hoax incidents. Such methods assist in reducing the time needed to respond to possible and real bioterrorism events, as well as normalizing the situation after a hoax is encountered.

Several commercial methods are currently available for analyzing suspicious powder samples, ranging from basic powder screening kits that can provide first responders with an initial assessment of a powder's content to sophisticated laboratory techniques that are able to identify or rule out the presence of biological threat agents (7). The methods' sensitivity and specificity vary substantially, but they also differ in other analysis-related properties, such as speed, cost, and infrastructure and user experience level requirements. More than one method is generally needed when analyzing suspicious powder samples because the methods' analysis-related properties have a tendency to be inversely related (i.e., simple and rapid methods are often not sufficiently sensitive or specific, while sophisticated laboratory methods are often time-consuming and expensive and require expert users and a specialized infrastructure).

Recent advances in mass spectrometry (MS) and the introduction of soft ionization techniques such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) that allow MS analysis of intact organic macromolecules have led

Received 27 May 2013 **Accepted** 26 June 2013
Published ahead of print 28 June 2013
Address correspondence to Marius Dybwad, marius.dybwad@ffi.no.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01724-13.
Copyright © 2013, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AEM.01724-13

to an increased interest in MS-based microorganism identification methods, especially within infectious disease diagnostics and the biodefense community (8–14). MS is not restricted to predetermined targets, which stands in contrast to most other molecular detection techniques that generally rely on molecular recognition and selective binding (e.g., antibodies and nucleic acid probes and primers). In particular, MALDI-time of flight (TOF) MS has shown potential for rapid identification of microorganisms based on whole cells or whole-cell extracts (15), exemplified by the introduction of commercial analysis platforms (e.g., MALDI Biotyper from Bruker Daltonics and SARAMIS from AnagnosTec) that are now commonly used in hospital diagnostic laboratories (16–21).

MS-based methods (e.g., MALDI-TOF MS and liquid chromatography-tandem MS) involving several different analysis concepts such as mass spectrum fingerprinting and top-down or bottom-up proteomics have been successfully used to detect and characterize small acid-soluble proteins (SASPs) in *Bacillus* sp. spores (8–11, 22–39). SASPs, originally described in *B. subtilis* spores, have been shown to confer resistance to DNA damage and to function as a source of amino acids during spore germination (40). SASP family proteins are abundantly found in *Bacillus* sp. spores, and because of their basic nature, they can be selectively solubilized in acids and easily protonated to provide strong signals when ionized by MALDI or ESI (8). SASPs have been proposed as candidate biomarkers capable of discriminating between various *Bacillus* sp. spores, including those within the *Bacillus cereus sensu lato* group (*B. anthracis*, *B. cereus sensu stricto*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, and *Bacillus pseudomycoides*). The predominant SASPs found in *B. cereus sensu lato* group spores by MALDI-TOF MS are recognized as α-, β-, α/β-, and γ-SASPs (23).These SASPs are generally highly conserved within and between the various species of the *B. cereus sensu lato* group, and their true potential as unique biomarkers of *B. anthracis* spores has therefore been discussed and shown to depend on the analysis method used (24, 26, 27, 29, 32, 38, 39).

The aim of the present study was to develop a rapid, reliable, and cost-effective laboratory-based analysis method for identifying *B. anthracis* spores in suspicious powders. The development and validation of a MALDI-TOF MS-based classification method able to discriminate between powder samples consisting of *B. anthracis* spores, spores of closely related *Bacillus* spp., and commonly encountered hoax materials are described.

MATERIALS AND METHODS

Microorganisms and hoax materials. *Bacillus* sp. strains were obtained from the American Type Culture Collection; the Deutsche Sammlung von Mikroorganismen und Zellkulturen; the U.S. Department of Agriculture Agricultural Research Service culture collection; and Alvin Fox at the Department of Pathology, Microbiology, and Immunology, University of South Carolina, Columbia, SC. All of the *Bacillus* sp. strains used belong to the *B. cereus sensu lato* group (*B. anthracis*, *B. cereus sensu stricto*, *B. thuringiensis*, and *B. weihenstephanensis*) or the *B. subtilis* group (*B. subtilis* and *B. atrophaeus* [formerly known as *B. globigii*]). Emphasis was placed on obtaining several *B. cereus sensu stricto* and *B. thuringiensis* strains closely related to *B. anthracis*, based on the University of Oslo *B. cereus* group MultiLocus and MultiData Typing website (http://mlstoslo.uio.no) (41). The *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* strains were also selected to completely cover the previously described SASP diversity found within each of these species (8–11, 22–39). A dry powder formulation of *B. atrophaeus* spores was obtained from Dugway Proving Grounds (Dugway, UT), and a dry powder pesticide formulation (Turex WP 50) containing *B. thuringiensis* spores was obtained from Certis Europe (Utrecht, The Netherlands). The spores from these powders were subsequently recultured and isolated, and the isolates obtained were designated *B. thuringiensis* Kurstaki/Aizawai and *B. atrophaeus* Dugway, respectively. Commercially available powdered substances associated with powder letter hoaxes (42, 43) and referred to here as hoax materials were purchased from local supermarkets or obtained from Sigma-Aldrich (St. Louis, MO). Table 1 provides a complete list of the *Bacillus* sp. strains and hoax materials used in this study.

Spore production. The *Bacillus* sp. strains were recovered from frozen stocks, maintained on blood agar plates, and clonally seeded into cultivation flasks containing 2×SG modified Schaeffer sporulation broth (44, 45). The cultivation flasks were incubated (30°C, 250 rpm) until more than 90% phase-bright free spores were observed by phase-contrast microscopy. The cultures were centrifuged (4,500 × *g*, 30 min), and the supernatants were discarded. The spores were washed (10,000 × *g*, 5 min) three times with cold (4°C) MilliQ water (Millipore, Billerica, MA). Between the first and second washing steps, the spore solutions were freeze-thawed (−20°C, 18 h) and incubated (room temperature, 2 h) to induce autolysis of residual endospore-containing vegetative cells. The spores were further purified by a density gradient centrifugation procedure (46). Briefly, the spores were resuspended in 20% (wt/vol) Nycodenz (Axis-Shield, Oslo, Norway) in MilliQ water and overlaid onto 50% (wt/vol) Nycodenz in MilliQ water in a centrifuge tube (2 ml). The supernatant was discarded after centrifugation (13,000 × *g*, 45 min), and the spore pellets were washed (10,000 × *g*, 5 min) three times with cold (4°C) MilliQ water to remove residual Nycodenz. The final spore preparations were quantified with a counting chamber and a phase-contrast microscope (Olympus BX41; Olympus Nederland, Zoeterwoude, The Netherlands) and confirmed to contain more than 99% phase-bright free spores. Stock solutions were made by diluting the spores to a final concentration of 1.0×10^9/ml in MilliQ water. The spore stocks were stored at 4°C for the duration of the study.

Acid-assisted protein extraction procedure. An acid-soluble protein extraction protocol suitable for powder samples containing *Bacillus* sp. spores or hoax materials was developed by modifying a previously described formic acid (FA)-based method (47). The entire protocol was completed in less than 10 min. The standard input amount for samples included in this study was 1 mg powder (Fig. 1) or $\sim 10^7$ spores (when starting directly with liquid spore stocks). The modified protein extraction protocol was as follows. MilliQ water (1.0 ml) was added to a centrifuge tube (2 ml) containing the sample and mixed by pipetting up and down. The sample tube was centrifuged (13,000 × *g*, 2 min), and the supernatant was aspirated from the center of the tube and discarded without disturbing the pellet. The pellet was resuspended in 1.0 ml MilliQ water, mixed by pipetting up and down, and centrifuged (13,000 × *g*, 2 min). The supernatant was removed, the tube was recentrifuged (13,000 × *g*, 1 min), and any residual liquid was removed. The pellet was resuspended in 15.0 μl 70% FA and mixed by pipetting up and down without introducing air bubbles. After incubation (2 min, room temperature), 15.0 μl acetonitrile (ACN; Sigma-Aldrich) was added and the solution was mixed as described above. The tube was centrifuged (13,000 × *g*, 30 s), and the supernatant was transferred without disturbing the pellet to the filter membrane of a luer-lock syringe filter (0.22-μm-pore-size, 4-mm, Millex-GV_4 hydrophilic Durapore polyvinylidene difluoride [PVDF] filters; Millipore). The liquid was filtered with a luer-lock syringe (1 ml), and the filtrate was captured in a new tube.

MALDI-TOF MS. The protein extracts were spotted (1.0 μl) onto MTP AnchorChip 600/384 T F targets (Bruker Daltonics), air dried, and overlaid with 0.5 μl matrix solution containing 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) in 50% ACN (Sigma-Aldrich) and 2.5% trifluoroacetic acid (Sigma-Aldrich). Mass spectra were acquired with FlexControl 3.0 software (Bruker Daltonics) and an AutoFlex III Smartbeam mass spectrometer (Bruker Daltonics) in positive linear

TABLE 1 *Bacillus* sp. strains and hoax materials included in this study and results concerning the MALDI-TOF MS-based classification method's specificity and robustness

				Evaluation of specificity and robustness [no. of correct classifications (total)]			Performance measurements for *B. anthracis* [no. of classifications (total)]	
Bacillus sp. strain or hoax material	Included in library?	SASGCL (classification library)	Similarity group	Specificity (blinded samples)	Robustness (operator dependence)	Robustness (instrument dependence)	False positive	False negative
B. cereus sensu lato group								
B. anthracis								
Ames(pXO1^{+}/pXO2^{+})	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. anthracis*	4 (4)	1 (1)	1 (1)	NA[a]	0 (6)
Vollum(pXO1^{+}/pXO2^{+})	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. anthracis*	4 (4)	1 (1)	1 (1)	NA	0 (6)
Sterne(pXO1^{+}/pXO2^{-})	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. anthracis*	4 (4)	1 (1)	1 (1)	NA	0 (6)
Farmer cute(pXO1^{+}/PXO2^{+})	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. anthracis*	4 (4)	1 (1)	1 (1)	NA	0 (6)
B. cereus sensu stricto								
DSM 336	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
NVH0597-99	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
DSM 31 (ATCC 14579)	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
DSM 9378 (ATCC 10876)	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
DSM 3648 (ATCC 11950, W)	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	4 (4)	1 (1)	1 (1)	0 (6)	NA
R3	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
DSM 8438	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
B. thuringiensis								
CEB97/27	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
BGSC 4AJ1	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
DSM 6102 (ATCC 33679, HD-1)	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
BGSC 4CC1	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	4 (4)	1 (1)	1 (1)	0 (6)	NA
Kurstaki/Aizawai[b]	Yes	*B. anthracis*/*B. cereus sensu stricto*-*B. thuringiensis*	*B. cereus sensu stricto*-*B. thuringiensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
B. weihenstephanensis DSM 11821	Yes	*B. weihenstephanensis*/*B. subtilis*/*B. atrophaeus*	*B. weihenstephanensis*	3 (3)	1 (1)	1 (1)	0 (5)	NA
B. subtilis group								
B. subtilis DSM 10 (ATCC 6051)	Yes	*B. weihenstephanensis*/*B. subtilis*/*B. atrophaeus*	*B. subtilis*	5 (5)	1 (1)	1 (1)	0 (7)	NA
B. atrophaeus Dugway[c]	Yes	*B. weihenstephanensis*/*B. subtilis*/*B. atrophaeus*	*B. atrophaeus*	3 (3)	1 (1)	1 (1)	0 (5)	NA
Hoax materials								
Wheat flour	Yes	Hoax materials	Wheat flour	4 (4)	1 (1)	1 (1)	0 (6)	NA
Rye flour	Yes	Hoax materials	Rye flour	4 (4)	1 (1)	1 (1)	0 (6)	NA
Dry milk	Yes	Hoax materials	Dry milk	4 (4)	1 (1)	1 (1)	0 (6)	NA
Coffee creamer				NA[f,g]	ND[i]	ND	0 (3)	NA
Washing powder				NA[f,g]	ND	ND	0 (3)	NA
Talcum powder				NA[f,g]	ND	ND	0 (3)	NA
Powdered sugar				NA[f,g]	ND	ND	0 (3)	NA
Nutrient broth				NA[f,g]	ND	ND	0 (3)	NA
Yeast extract				NA[f,g]	ND	ND	0 (3)	NA

(Continued on following page)

TABLE 1 (Continued)

Bacillus sp. strain or hoax material	Included in library?	SASGCL (classification library)	Similarity group	Evaluation of specificity and robustness [no. of correct classifications (total)]			Performance measurements for *B. anthracis* [no. of classifications (total)]	
				Specificity (blinded samples)	Robustness (operator dependence)	Robustness (instrument dependence)	False positive	False negative
Spore-containing powders								
Turex WP 50[d]				3 (3)	1 (1)	1 (1)	0 (5)	NA
Dugway *B. atrophaeus*[e]				3 (3)	1 (1)	1 (1)	0 (5)	NA
Other bacterial agents (vegetative)[h]				NA[g]	ND	ND	0 (39)	NA
Total				83 (83)	24 (24)	24 (24)	0 (164)	0 (24)

[a] NA, not applicable.
[b] Isolated from Turex WP 50.
[c] Isolated from Dugway *B. atrophaeus*.
[d] Commercial pesticide formulation containing *B. thuringiensis* Kurstaki/Aizawai spores (Certis Europe, Utrecht, The Netherlands).
[e] Freeze-dried *B. atrophaeus* Dugway spores, lot 19076-03267 (Dugway Proving Grounds, Dugway, UT).
[f] Flat-lined mass spectrum, no peaks found.
[g] No false-positive classifications observed.
[h] Vegetative cells of other bacterial agents, including various strains of *Escherichia coli* ($n = 5$), *B. anthracis* ($n = 5$), *B. cereus sensu stricto* ($n = 4$), *B. subtilis* ($n = 1$), *B. atrophaeus* ($n = 1$), *Francisella tularensis* ($n = 2$), *Clostridium botulinum* ($n = 2$), *Vibrio cholerae* ($n = 2$), *Burkholderia mallei* ($n = 2$), *Burkholderia pseudomallei* ($n = 1$), *Yersinia pestis* ($n = 2$), *Acinetobacter baumannii* ($n = 1$), *Coxiella burnetii* ($n = 1$), *Enterococcus faecalis* ($n = 1$), *Staphylococcus aureus* ($n = 1$), *Staphylococcus epidermis* ($n = 1$), *Proteus mirabilis* ($n = 1$), *Pseudomonas aeruginosa* ($n = 1$), *Serratia marcescens* ($n = 1$), *Brucella ceti* ($n = 1$), *Brucella melitensis* ($n = 1$), *Shigella flexneri* ($n = 1$), and *Shigella sonnei* ($n = 1$).
[i] ND, not determined.

mode between 2 and 20 kDa. The pulsed ion extraction time was 350 ns, the acceleration voltages were 20 kV (source 1) and 18.7 kV (source 2), the lens voltage was 8 kV, and the linear detector voltage was 1.522 kV. Each sample spot was measured by using a hexagon acquisition pattern, and the mass spectra were recorded as the sum of 2,000 laser shots with the Smartbeam Nd:YAG (355 nm) laser at 200 Hz. The instrument was externally calibrated with the Bacterial Test Standard (255343; Bruker Daltonics).

Mass spectrum data processing. The mass spectra acquired were converted into MZXML format by using a script file from Bruker Daltonics (CompassXport.exe). Matlab R2012b (MathWorks, Natick, MA) and the Bioinformatics toolbox (version 3.0) were used for data processing. Data processing involved import (mzxmlread.m), resampling (msresample.m; mass range, 3,000 to 10,000 *m/z*; 31,500 data points), smoothing (mslowess.m; Lowess smoothing; order, 2; span, 40), baseline subtraction (msbackadj.m; window and step size, 5.0 and 0.005 × *m/z*), normalization (msnorm.m; single peak max intensity, 300), and peak selection (mspeaks.m; minimum intensity threshold, 10.0 between 3,000 and 8,000 *m/z* and 3.0 between 8,000 to 10,000 *m/z*). An additional filtering step was used after peak selection to remove peak shoulders initially identified as peaks by the intensity-based algorithm (mspeaks.m). By comparing the intensities of the peaks to the intensities of the neighboring valleys (identified by using the first-, and second-order derivatives), peak shoulders were removed by applying a bidirectional peak-to-valley minimum intensity ratio of 2.0.

Reference library construction. Protein extracts were prepared by the modified FA-based method of protein extraction from samples consisting of the *Bacillus* sp. spores or hoax materials selected for library construction (Table 1). Three extractions of each sample were done on separate days, and each protein extract was measured eight times. The peak lists obtained were used to generate one consensus peak list for each *Bacillus* sp. strain or hoax material. The inclusion criteria used when binning the individual peak lists into a consensus peak list required that the peak be present in >75% (18/24) of the individual peak lists within ±300 ppm of the peak's *m/z* average. A preliminary reference library was created by binning the consensus peak lists into common library peaks by using a bin size of ±500 ppm of the peak's *m/z* average and associating each library peak with present and absent designations for each entry of *Bacillus* sp. spores or hoax materials. Three stand-alone, similarity-grouped reference

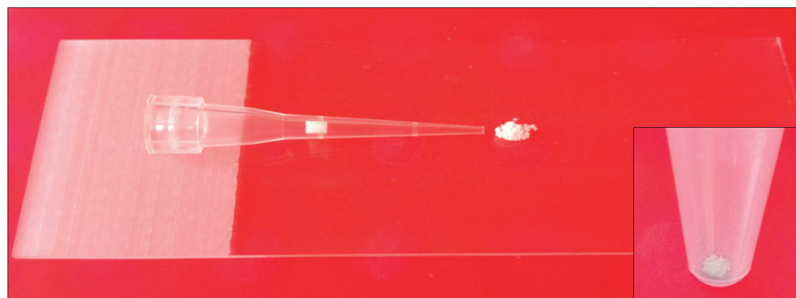

FIG 1 Powder sample (1 mg) on a microscopy slide. The inset shows the same amount of powder in a 1.5-ml centrifuge tube. A pipette tip (10 μl) is shown as a scale reference. The end facing the powder is 1 mm in diameter.

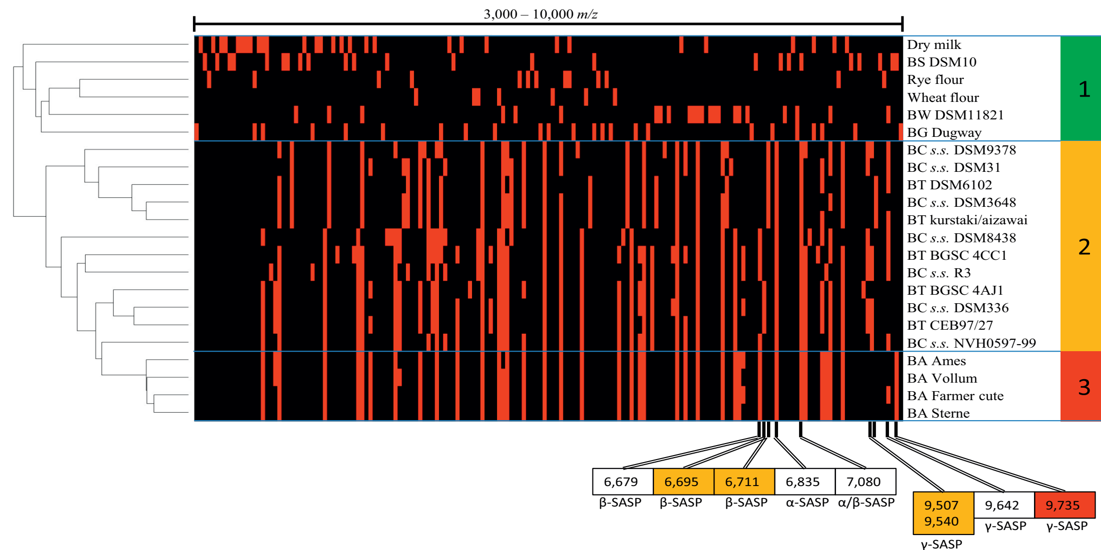

FIG 2 Dendrogram from average linkage hierarchical clustering of the preliminary reference library, including spores of 19 different *Bacillus* sp. strains and three hoax materials. The green region (region 1) corresponds to the *B. weihenstephanensis* (BW), *B. subtilis* (BS), *B. atrophaeus* (BG), dry milk, wheat flour, and rye flour entries. The orange region (region 2) corresponds to the *B. cereus sensu stricto* (BC *s.s.*) and *B. thuringiensis* (BT) entries. The red region (region 3) corresponds to the *B. anthracis* (BA) entries. The α-, β-, α/β-, and γ-SASPs observed in the *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* entries are presented below the dendrogram. SASPs found exclusively in *B. cereus sensu stricto*-*B. thuringiensis* entries (and not in *B. anthracis*) are orange, while SASPs found exclusively in *B. anthracis* entries (and not in *B. cereus sensu stricto*-*B. thuringiensis*) are red.

libraries (SASGRLs) were further constructed on the basis of the results of a hierarchical cluster analysis (average linkage) of the preliminary reference library (Fig. 2). The SASGRLs contained (i) *B. anthracis* and *B. cereus sensu stricto*-*B. thuringiensis* (merged into a composite similarity group) entries (the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library); (ii) *B. weihenstephanensis*, *B. subtilis*, and *B. atrophaeus* entries (the *B. weihenstephanensis*-*B. subtilis*-*B. atrophaeus* library); and (iii) dry milk, wheat flour, and rye flour entries (the hoax material library). In the SASGRLs, a single library entry was created to represent each similarity group by merging together all of the individual entries that were assigned to the similarity group. The peak present and absent designations in the SASGRLs were reported as percent present values (PPVs; range, 0 to 100) calculated from the number of entries used to construct the similarity group that had the peak divided by the total number of entries used to construct the similarity group. From each SASGRL, a final stand-alone similarity-grouped classification library (SASGCL) was created. Each similarity group's PPV was transformed into a PPV ratio (the similarity group's PPV divided by the sum of the PPVs of all of the similarity groups in the SASGRL) and further into an odds ratio (PPV ratio/[1 − PPV ratio]). Each SASGCL contained the same library peaks as the SASGRL it was generated from, in addition to each peak's odds ratio for each similarity group in the library. The SASGCLs also contained information about whether the library peaks could be linked to observed and/or predicted masses of SASP family proteins previously described in the literature (8, 9, 22–34, 37–39) or identified through a protein search of GenBank (http://www.ncbi.nlm.nih.gov) for SASPs. A 5× weighting correction was added to the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library to adjust the PPVs (adjusted PPV range, 0 to 500) of all of the SASP-associated library peaks observed exclusively in a single similarity group (i.e., unique similarity group SASP markers).

Sample measurement. All of the samples analyzed in this study were processed according to the procedures described for acid-assisted protein extraction, MALDI-TOF MS measurement, mass spectrum data processing, and library-based classification, unless otherwise stated. The protein extracts were measured as five spots on the MALDI target, thus generating a total of five mass spectra per analyzed sample. A consensus peak list was generated for each sample by binning peaks observed in >60% (3/5) of the individual peak lists within ±300 ppm of the peak's *m/z* average.

Classification algorithm. A custom classification algorithm was developed that matched the consensus peak list of a measured sample to library peaks (±500 ppm of the peak's *m/z* average) in each of the three SASGCLs. The classification algorithm summed the odds ratios for each similarity group in each SASGCL using matched peaks only and outputted summed size-sorted odds ratios together with the ratio of the highest to the second highest summed odds ratio. The classification algorithm also extracted the number of matched peaks in a SASGCL that could be mapped to SASP family proteins. The confidence threshold for a sample classification result was based on three different criteria that all had to be met. The first criterion was used to obtain a group classification and select one of the SASGCLs for further classification. When all of the common SASPs from the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library, α-SASP (~6,835 Da), β-SASP (~6,679, ~6,695, or ~6,711 Da), and α/β-SASP (~7,080 Da), were observed in a sample's consensus peak list, the classification algorithm used the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library for further classification and reported *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* as a confident group classification. When the common SASPs of the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library were absent, the classification algorithm selected the *B. weihenstephanensis*-*B. subtilis*-*B. atrophaeus* library when two or more peaks could be mapped to SASP family proteins found in this library and reported *B. weihenstephanensis*-*B. subtilis*-*B. atrophaeus* as a confident group classification. If fewer than two SASPs could be mapped to the *B. weihenstephanensis*-*B. subtilis*-*B. atrophaeus* library, the classification algorithm selected the hoax material library for further classification but did not report a confident group classification. For the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* or *B. weihenstephanensis*-*B. subtilis*-*B. atro-*

phaeus library, the second and third confidence criteria necessary to report a confident similarity group classification were a minimum summed odds ratio of 500 and a minimum ratio of the highest to the second highest summed odds ratio of 5, respectively. For the hoax material library, the second and third confidence criteria were more stringent to reduce the likelihood of assigning false-positive classifications to hoax materials because of random (by chance) matching of only a limited number of sample consensus peaks to the hoax material library. Specifically, a minimum summed odds ratio of 1,000 and a minimum ratio of the highest to the second highest summed odds ratio of 500 were necessary to assign a confident similarity group classification in the hoax material library. When none of the classification criteria described were met, the classification algorithm reported "no reliable classification."

LOC. The limit of classification (LOC) by the end-to-end classification method, defined here as the smallest amount of spores that allowed consistent, confident, and correct classification of a sample, was determined on 2 separate days by using spores of *B. anthracis* Sterne, *B. cereus sensu stricto* NVH0597-99, and *B. atrophaeus* Dugway. Two-fold serial dilutions of the original spore stocks were made fresh each day with MilliQ water to generate samples ranging in concentration from 4.0×10^7 to 1.25×10^6 spores ml^{-1}, as determined by phase-contrast microscopy. Initial testing was done with 10-fold serial dilutions between 1.0×10^{10} and 1.0×10^5 spores ml^{-1} to establish an appropriate concentration window for LOC testing. Each dilution was extracted in duplicate and measured as five spots on the MALDI target. The analysis was scored as successful when the sample was correctly classified. The overall mass spectrum quality (i.e., peak resolution, peak signal-to-noise, and peak-to-peak intensity ratio) was also assessed, and the mass spectra were assigned a quality indicator (excellent, good, medium, or poor) in reference to the mass spectra generated during reference library construction. These quality indicators were used to complement the classification results when determining the method's LOC.

Validation of the classification method with blinded samples. Eighty-three samples consisting of *Bacillus* sp. spores or hoax materials were created by random selection from the available *Bacillus* sp. strains and hoax materials (Table 1) and analyzed according to the classification method described. Each sample was number coded, and the operator performing the analysis was blinded to the sample content until the analysis was completed. Additionally, 18 samples containing hoax materials not included in the reference libraries (Table 1) and 39 samples containing vegetative cells of various bacterial species (Table 1) were also analyzed to investigate the method's potential for obtaining false-positive classification results.

Evaluation of the classification method's robustness. All of the *Bacillus* sp. spores or hoax materials (Table 1) were analyzed by an untrained operator (i.e., no previous experience with the method). The analysis results from these 24 samples were used to evaluate the operator-dependent robustness of the classification method. A similar set of the same 24 samples were independently processed and express shipped on dry ice to the Norwegian Defense Research Establishment FFI (Kjeller, Norway) for MS measurement on a MicroFlex LT MALDI-TOF MS instrument (Bruker Daltonics) to evaluate the instrument-dependent robustness of the classification method. To evaluate the classification method's potential for analyzing samples containing *Bacillus* sp. spores mixed with hoax materials, spores of *B. anthracis* Ames, *B. cereus sensu stricto* NVH0595-97, or *B. atrophaeus* Dugway were mixed with various hoax materials and analyzed by the method described. Each sample was analyzed on 2 separate days, and the hoax materials used were dry milk, wheat flour, rye flour, coffee creamer, talcum powder, and washing powder, for a total of seven samples (one control and six mixtures) for each *Bacillus* sp. strain per analysis round. The mass spectra generated were assigned quality indicators in accordance with the procedure described for the LOC experiments. These were used to complement the classification results when evaluating the impact of sample mixtures on the method's classification potential.

RESULTS

Spore production. High-purity spore preparations (>99% phase-bright free spores) were successfully produced for all of the 19 *Bacillus* sp. strains selected for reference library construction (Table 1). In an attempt to include at least one representative species of each member of the *B. cereus sensu lato* group, production of *B. mycoides* and *B. pseudomycoides* spores were initially attempted by using the standard sporulation protocol, but these efforts failed because of low sporulation efficiencies (<1%).

Acid-assisted protein extraction protocol. A modified FA-based protein extraction procedure was developed that includes a pre-extraction washing step to retain a high level of *Bacillus* sp. spore recovery while removing water-soluble substances (data not shown). The added sterile filtration step was shown to consistently exclude spores from the final protein extract following repeated testing with up to 1.0×10^{10} spores of *B. anthracis* Sterne, *B. cereus sensu stricto* NVH0597-99, and *B. atrophaeus* Dugway. The filter step did not have an impact on the mass spectra obtained from *Bacillus* sp. spores, consistent with observations made by Lasch et al. (48).

Reference library construction and classification algorithm. The scope of the present work was to develop a rapid method able to confidently discriminate *B. anthracis* spores from other *Bacillus* sp. spores and hoax materials in powder samples. High-quality mass spectra with consistently observed mass peaks could be generated from all of the 19 *Bacillus* sp. spore preparations produced. Of the nine hoax materials selected for the library construction (Table 1), only three (dry milk, wheat flour, and rye flour) generated mass spectra with consistent mass peaks while the rest failed to generate mass spectra with peak information (flat-lined mass spectra). Reference library construction was therefore performed with a total of 22 entries, 19 *Bacillus* sp. strains (4 *B. anthracis*, 7 *B. cereus sensu stricto*, 5 *B. thuringiensis*, 1 *B. weihenstephanensis*, 1 *B. subtilis*, and 1 *B. atrophaeus*) and three hoax materials (dry milk, wheat flour, and rye flour). A hierarchical cluster analysis (average linkage) was performed on the preliminary reference library containing all of the *Bacillus* sp. strains and hoax materials as independent library entries in order to guide the binning of the individual entries into similarity-grouped entries. The dendrogram from the cluster analysis correlated well with the known phylogenetic grouping of *Bacillus* spp. (Fig. 2). The interdispersed clustering of *B. cereus sensu stricto* and *B. thuringiensis* strains and the positioning of the *B. anthracis* strains in a homogeneous and separable cluster inside the *B. cereus sensu stricto*-*B. thuringiensis* branch also corresponded well to results of previous phylogenetic analyses of the *B. cereus sensu lato* group (41). The dendrogram also revealed that the *B. weihenstephanensis*, *B. subtilis*, *B. atrophaeus*, and hoax material entries constituted a heterogeneous cluster well separated from the *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* entries. Since the main objective of the classification method was to identify *B. anthracis* spores and because the *B. cereus sensu stricto* and *B. thuringiensis* strains were interdispersed with each other in the dendrogram, the *B. cereus sensu stricto* and *B. thuringiensis* entries were merged into a single similarity group while the *B. anthracis* entries were kept as a similarity group separate from the composite *B. cereus sensu stricto*-*B. thuringiensis* group. The preliminary similarity-grouped reference library therefore consisted of the following entries after the merging all of the individual entries into a single entry per similarity group: *B.*

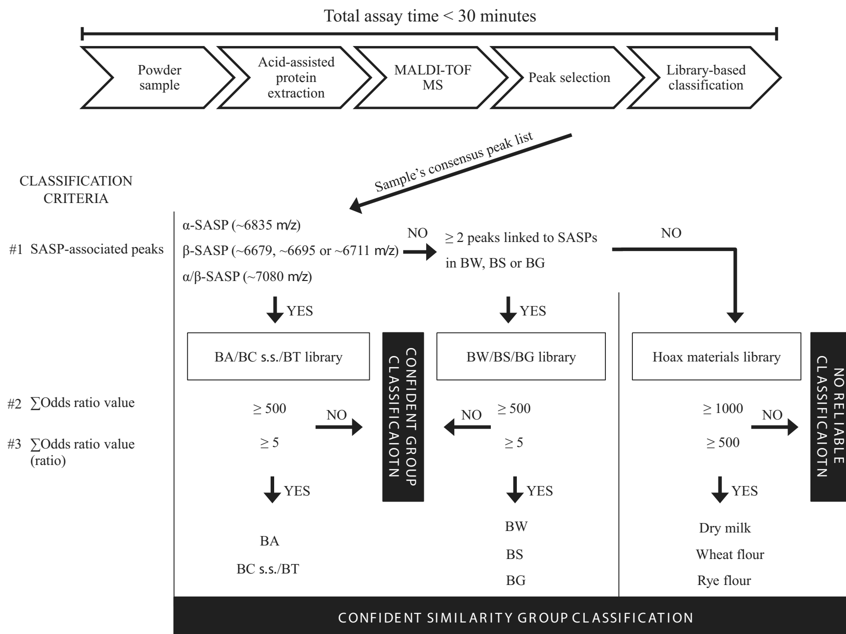

FIG 3 Flow chart summary of the proposed MALDI-TOF MS-based classification method. The library-based classification algorithm consisted of three classification criteria applied sequentially: number 1, selection of one of the three stand-alone similarity-grouped classification libraries using SASP-associated peaks in the sample's consensus peak list; number 2, similarity group summed odds ratio above a predetermined threshold; number 3, ratio of the highest to the second highest summed odds ratio above a predetermined threshold. A confident similarity group classification (*B. anthracis* [BA], *B. cereus sensu stricto-B. thuringiensis* [BC *s.s.*/BT], *B. weihenstephanensis* [BW], *B. subtilis* [BS], *B. atrophaeus* [BG], dry milk, wheat flour, or rye flour) was assigned when all of the classification criteria were met (numbers 1 to 3 for the *B. anthracis-B. cereus sensu stricto-B. thuringiensis* and *B. weihenstephanensis-B. subtilis-B. atrophaeus* libraries and numbers 2 and 3 for the hoax material library). A confident group classification (*B. anthracis-B. cereus sensu stricto-B. thuringiensis* or *B. weihenstephanensis-B. subtilis-B. atrophaeus*) was assigned when only the first classification criterion was met (not applicable to the hoax materials). "No reliable classification" was reported when the second or third classification criteria were not met for the hoax material library.

anthracis, 4 merged entries; *B. cereus sensu stricto-B. thuringiensis* composite similarity group, 12 merged entries; *B. weihenstephanensis*, 1 entry; *B. subtilis*, 1 entry; *B. atrophaeus*, 1 entry; milk powder, 1 entry; wheat flour, 1 entry; rye flour, 1 entry. The hierarchical clustering and the preliminary similarity-grouped reference library revealed that a core set of SASPs, α (~6,835 *m/z*), β (~6,679, ~6,695, or ~6,711 *m/z*), and α/β (~7,080 *m/z*), were observed in all of the *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* entries and could be used to separate them from all of the other library entries (Fig. 2). This led to the separation of these entries from the other *Bacillus* spp. and hoax materials by creating a SASGRL including only the *B. anthracis* and *B. cereus sensu stricto-B. thuringiensis* similarity groups (*B. anthracis-B. cereus sensu stricto-B. thuringiensis* library). *B. weihenstephanensis* did not have the common core set of SASP peaks found in all of the other *B. cereus sensu lato* group entries. Thus, *B. weihenstephanensis* was not included in the *B. anthracis-B. cereus sensu stricto-B. thuringiensis* library, even though it is phylogenetically recognized as a member of the *B. cereus sensu lato* group. It was found that two or more peaks corresponding to SASP family proteins were consistently observed in all of the *B. weihenstephanensis*, *B. subtilis*, and *B. atrophaeus* entries. Subsequently, this was used to separate these entries from the hoax materials by creating two SASGRLs containing (i) the *B. weihenstephanensis*, *B. subtilis*, and *B. atrophaeus* similarity groups (*B. weihenstephanensis-B. subtilis-B. atrophaeus* library) and (ii) the milk powder, wheat flour, and rye flour similarity groups (hoax material library). Figure 3 provides a flow chart summary of the proposed classification method, including the classification algorithm and final SASGCLs. Figure 4 presents representative MALDI-TOF MS spectra obtained by the analysis method described, highlighting the predominant SASPs observed in *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* spores.

LOC. The LOC experiments revealed clear differences between the *B. cereus sensu lato* group spores tested (*B. anthracis* Sterne and *B. cereus sensu stricto* NVH0595-97) and the *B. atrophaeus* Dugway spores, with a 4-fold higher LOC observed for the *B. atrophaeus* Dugway spores than for the *B. cereus sensu lato* group spores (Table 2). By employing stringent criteria for the LOC (4/4 correct classifications and good-quality mass spectra), the LOCs for *B. anthracis* Sterne, *B. cereus sensu stricto* NVH0595-97, and *B. atrophaeus* Dugway spores were 2.5×10^6, 2.5×10^6, and 1.0×10^7 spores per sample, respectively (Table 2). Nearly all of the samples containing spores corresponding to half the LOC were correctly classified but did not fulfill all of the defined quality criteria (Table 2).

Method validation and robustness evaluation. One hundred percent (83/83) of the blinded samples consisting of *Bacillus* sp. spores or hoax materials were correctly classified by the proposed

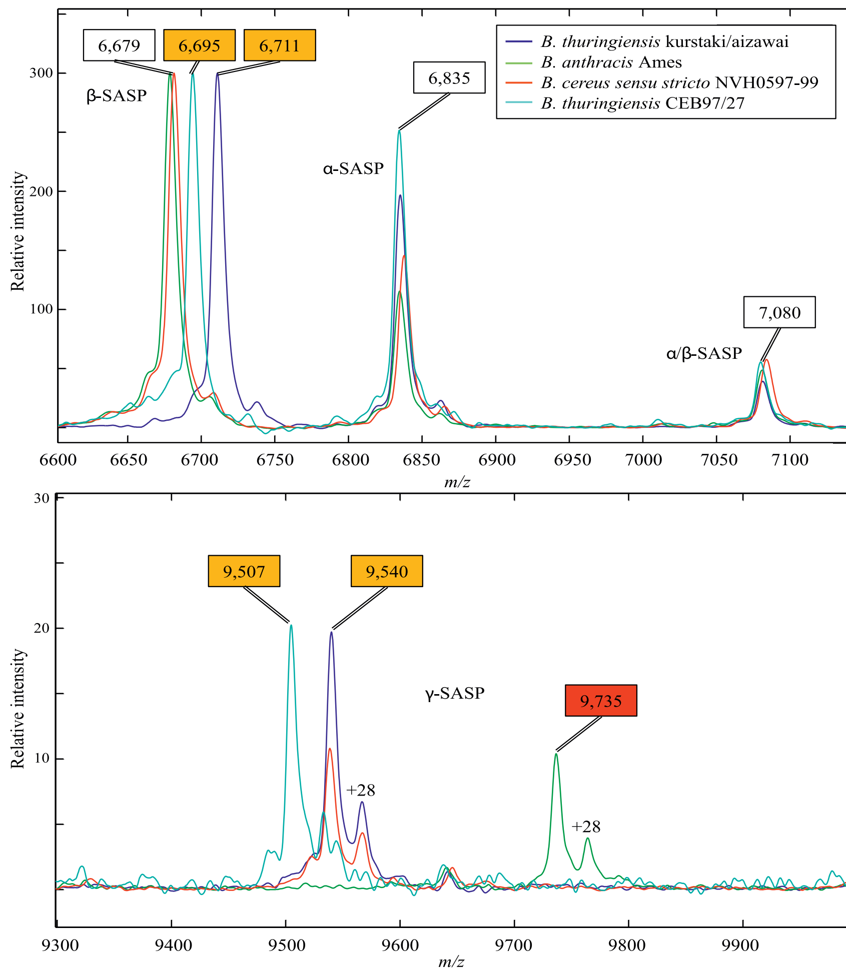

FIG 4 Representative mass spectra obtained by the MALDI-TOF MS-based analysis method described for *B. thuringiensis* Kurstaki/Aizawai, *B. anthracis* Ames, *B. cereus sensu stricto* NVH0597-99, and *B. thuringiensis* CEB97/27 spores. The upper plot presents the α-, β-, and α/β-SASPs, and the bottom plot presents the γ-SASPs. SASP-associated peaks found exclusively in *B. cereus sensu stricto* and/or *B. thuringiensis* and not *B. anthracis* are in orange, while SASP-associated peaks found exclusively in *B. anthracis* and not *B. cereus sensu stricto* and/or *B. thuringiensis* are in red. The γ-SASP peaks were commonly associated with low-intensity shoulder peaks (+28 *m/z*), probably because of FA-induced protein formylation.

classification method (Table 1). Additionally, analysis of 18 samples containing hoax materials not included in the classification library and 39 samples containing vegetative cells of various bacterial species did not lead to any (0/57) false-positive classifications (Table 1). Analysis of 24 different samples representing each of the *Bacillus* sp. strains and hoax materials by an untrained operator led to 100% (24/24) correct classification (Table 1), indicating that the performance of the classification method was not influenced by operator-dependent factors. A similar set of the 24 samples was analyzed at an external research facility (FFI, Kjeller, Norway), revealing that all of the samples (24/24) were correctly classified (Table 1). This indicated that the classification method's performance was not impacted by instrument-dependent factors (i.e., not influenced by the transport of protein extracts on dry ice or the use of a MicroFlex LT MALDI-TOF MS instrument at the external facility). In summary, the method's specificity and robustness were proven by classifying a total of 131 samples with 100% accuracy (Table 1). The performance criteria related solely to the successful discrimination of *B. anthracis* spores were demonstrated by obtaining 0% (0/164) false-positive and 0% (0/24) false-negative classification results after subjecting the classification method to samples containing a wide range of *Bacillus* sp. spores (including closely related *B. cereus sensu lato* group strains), hoax materials, and vegetative cells of other bacterial species (Table 1).

Classification potential for sample mixtures. The analysis of 42 samples containing *B. anthracis* Ames, *B. cereus sensu stricto* NVH0595-97, or *B. atrophaeus* Dugway spores mixed with hoax

TABLE 2 End-to-end detection limit (i.e., sensitivity) of the MALDI-TOF MS-based classification method

Strain and no. of spores/sample	Result	No. correct (total)	Spectrum quality
B. anthracis Sterne			
4.0×10^7	*B. anthracis*	4 (4)	Excellent
2.0×10^7	*B. anthracis*	4 (4)	Excellent
1.0×10^7	*B. anthracis*	4 (4)	Excellent
5.0×10^6	*B. anthracis*	4 (4)	Excellent
2.5×10^6	*B. anthracis*	4 (4)	Good
1.25×10^6	*B. anthracis*[b]	3 (4)	Medium
B. cereus sensu stricto NVH0595-97			
4.0×10^7	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Excellent
2.0×10^7	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Excellent
1.0×10^7	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Excellent
5.0×10^6	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Excellent
2.5×10^6	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Good
1.25×10^6	*B. cereus sensu stricto-B. thuringiensis*	4 (4)	Medium
B. atrophaeus Dugway			
4.0×10^7	*B. atrophaeus*	4 (4)	Excellent
2.0×10^7	*B. atrophaeus*	4 (4)	Good
1.0×10^7	*B. atrophaeus*	4 (4)	Good
5.0×10^6	*B. atrophaeus*[b]	3 (4)	Poor
2.5×10^6	No reliable classification[c]	0 (4)	NA[a,d]
1.25×10^6	No reliable classification[c]	0 (4)	NA[d]

[a] NA, not applicable.
[b] A single sample (1/4) led to "No reliable classification."
[c] All samples (4/4) led to "No reliable classification."
[d] Flat-lined mass spectrum, no peaks found.

materials revealed that all three types of *Bacillus* sp. spores could be successfully classified in a mixture with dry milk, wheat flour, rye flour, coffee creamer, talcum powder, or washing powder. Generally, the mass spectra obtained from *Bacillus* sp. spores mixed with hoax materials differed marginally from those of the control samples containing *Bacillus* sp. spores only. Also, all of the acquired mass spectra were of high spectrum quality. When spores were mixed with hoax materials present in the classification library, mass peaks from both the spores and the hoax materials were consistently observed without major changes in the overall mass spectrum quality or the normalized intensities of the individual mass peaks. However, one exception was seen when spores were mixed with wheat flour (see Fig. S1 in the supplemental material), since when measured alone, wheat flour generated mass spectra with two high-intensity mass peaks (~4,820 and ~4,920 *m/z*). These were the only hoax material-derived peaks that displayed higher intensities than the predominant SASPs from *Bacillus* sp. spores. Still, their intensities were only marginally stronger than those of the SASP peaks and therefore did not influence the peak selection process. However, because the first criteria of the classification algorithm (detection of two or more SASP-associated mass peaks) were achieved, only the *Bacillus* sp. spores were classified and no attempt was made to identify the hoax material by the proposed method when the sample contained *Bacillus* sp. spores.

DISCUSSION

Powder letters containing *B. anthracis* spores can represent a real threat to human health and to our society, as revealed by the 2001 Amerithrax incidents in the United States (1, 3, 4, 49). Besides the actual acts of bioterrorism, letters containing harmless powders are commonly encountered. A major challenge with these hoax incidents is that they cause anxiety, consume resources, and must be handled as a real threat until the presence of *B. anthracis* spores and/or other hazardous substances can be confidently ruled out, even if they *per se* do not represent a direct health hazard.

The present work has demonstrated the successful development of a MALDI-TOF MS-based rapid classification method for powder samples suspected of containing *B. anthracis* spores. Taken together, the observed performance of the classification method demonstrates its applicability as a rapid, reliable, and cost-effective laboratory-based analysis tool for powder samples. The end-to-end classification method achieved the sensitivity and specificity levels needed to resolve incidents involving suspicious powders and can be completed in less than 30 min.

The presence of *B. anthracis* spores is generally the first suspected hazard when encountering suspicious powders, but other threat agents could potentially be considered as well (50). The MALDI-TOF MS-based classification method developed in this work was originally aimed at suspicious powder threats involving *B. anthracis* spores but could be extended to include additional threat agents, which could increase the applicability of the proposed assay in a broader sense. This is supported by the method's observed performance, and there is reason to believe that it could be adapted and validated for other sample types (e.g., swabs and air samples), although this was outside the scope of the present study.

The mass spectra obtained from *Bacillus* sp. spores in this study were consistently dominated by mass peaks that could be linked to SASPs. Previous MS-based investigations have demonstrated the use of SASPs as discriminatory biomarkers to separate various *Bacillus* sp. spores from each other (8–11, 22–39). The challenges

associated with SASP-based discrimination increase significantly when attempting intragroup discrimination inside the *B. cereus sensu lato* group, which is not surprising considering their close genetic relationship (41). Recent studies, have pointed out that *B. weihenstephanensis*, *B. mycoides*, and *B. pseudomycoides* can be discriminated from the rest of the members of the *B. cereus sensu lato* group on the basis of their α-, and β-SASPs, except for *B. mycoides* group 1 strains, which have the same α-SASP as *B. anthracis*, *B. cereus sensu stricto*, and *B. thuringiensis* strains and the same β-SASP as some *B. cereus sensu stricto* and *B. thuringiensis* strains (22–25). It has been proposed that the mass (~6,679 Da) of the β-SASP from *B. anthracis* can be used to discriminate *B. anthracis* from *B. cereus sensu stricto* and *B. thuringiensis* on the basis of the consistent observation of one or two amino acid substitutions in the β-SASP (~6,695 or ~6,711 Da, respectively) from *B. cereus sensu stricto* and *B. thuringiensis* compared to that from *B. anthracis* (24–27, 34, 38). Recent work has revealed that some *B. cereus sensu stricto* and *B. thuringiensis* strains, although presumably rare, also have this previously assumed *B. anthracis*-specific β-SASP (22, 23, 36). To ensure the robustness of the proposed classification method when encountering such *B. cereus sensu stricto* and *B. thuringiensis* strains, several of these strains were included in the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library. Three of seven *B. cereus sensu stricto* strains (NVH0597-99, R3, and DSM8438) and one of five *B. thuringiensis* strains (BGSC 4CC1) in the *B. anthracis*-*B. cereus sensu stricto*-*B. thuringiensis* library (Table 1) have the same β-SASP (~6,679 Da) as *B. anthracis*. The γ-SASP has recently been highlighted as a potential biomarker for the discrimination of *B. anthracis* from *B. cereus sensu stricto* and *B. thuringiensis* (32). We consistently observed the γ-SASP (~9,735 Da) in all of the *B. anthracis* spores in this study. None of the *B. cereus sensu stricto* or *B. thuringiensis* strains had the same γ-SASP as *B. anthracis* but had other γ-SASP masses instead (e.g., ~9,642, ~9,540, and ~9,507 Da). By combining the collective discriminatory power of the α-, β-, α/β-, and γ-SASPs and several other non-SASP-associated peaks, we were able to develop a MALDI-TOF MS library-based classification method that could confidently discriminate *B. anthracis* spores from all other *Bacillus* sp. spores, even when challenged with closely related *B. cereus sensu stricto* and *B. thuringiensis* strains that contained the same α-, β-, and α/β-SASP (~6,835, ~6,679, and ~7,080 Da, respectively) as *B. anthracis*.

Generally, most of the MALDI-TOF MS methods in use for microorganism identification rely on cultivation before MS analysis. Thus, previous studies have not focused on the determination of exact detection limits. The proposed end-to-end classification method was found to have sensitivity comparable to that of other MALDI-TOF MS-based analysis methods for microorganism identification (51–53). Previously described MALDI-TOF MS-based methods for the direct identification and discrimination of *Bacillus* sp. spores have focused primarily on elucidating the specificity of various SASPs as biomarkers (22–25, 30, 31, 33, 34, 37, 38). To our knowledge, limited information is available on end-to-end functional assays and on sensitivity measurements (i.e., detection limits). Our proposed end-to-end classification method was shown to have stringently set LOCs for *B. anthracis* Sterne, *B. cereus sensu stricto* NVH0595-97, and *B. atrophaeus* Dugway of 2.5×10^6, 2.5×10^6, and 1.0×10^7 spores, respectively. However, it should be noted that our modified protein extraction method led to the use of constant volumes of FA and ACN (15 μl each), independently of the observed size of the spore pellet after the pre-extraction washing step, while only 1 μl of the total protein extract (30 μl) was analyzed per MALDI spot. The LOC could therefore probably be further improved by adjusting the FA and ACN volumes to the size of the pellet or, alternatively, by analyzing a larger fraction of the available protein extract. Compared to those of other sensitive detection technologies, such as PCR-based methods, the reported LOC of the proposed classification method is orders of magnitude higher. However, for applications involving the screening of suspicious powders, it can be assumed that the powder must be present in an amount discernible by the naked eye for it to be recognized as a suspicious powder threat. On the basis of the physical size of a 1-mg powder sample (Fig. 1), it seems to be unlikely that amounts far below 1 mg would warrant analysis by such methods. The powder letters involved in the 2001 Amerithrax incident in the United States contained gram quantities of *B. anthracis* spores at concentrations ranging from 2.1×10^{12} to 4.6×10^{10} spores/g (54), which would translate into 1-mg samples that contain 4.6×10^7 spores for the crudest spore preparation involved. This corresponds to more than 18 times the LOC described for *B. anthracis* spores (2.5×10^6 spores) in our study, indicating that for even the most impure powder involved in the Amerithrax incident, a sample amount as small as 55 μg would be sufficient for successful classification by the method described. In this study, the proposed classification method was successfully tested with up to 1.0×10^{10} *B. anthracis* Sterne, *B. cereus sensu stricto* NVH0595-97, and *B. atrophaeus* Dugway spores, indicating that analysis of a larger amount of powder would be possible. Still, increasing the amount of powder could compromise the robustness of the method if the powder does not primarily contain spores. A sound approach could be to analyze different amounts of the same powder samples. The results obtained in the LOC experiments showed that the LOC for *B. atrophaeus* spores was four times higher than that for *B. anthracis* and *B. cereus sensu stricto* spores. This could possibly be attributed to physical differences between *B. cereus sensu lato* and *B. subtilis* group spores, including the presence or absence of exosporium, respectively, and spore coat and cortex compositional differences. This is supported by previous reports showing lower SASP yields from *B. subtilis* group spores than from *B. cereus sensu lato* spores following extraction with ACN–5% TFA (70:30, vol/vol) (34, 55).

The results obtained from the analysis of sample mixtures demonstrated the robustness of the proposed classification method, since all of the samples containing both *Bacillus* sp. spores and hoax materials could be consistently and correctly classified. This observation also suggested that the acid-assisted protein extraction was not sensitive to the presence of additional materials in the samples, possibly helped by the pre-extraction washing step that was included in the standardized protocol to remove water-soluble substances. The proposed method classified only the spore content and not the hoax material when the sample contained a mixture of both. Since the method was developed primarily to identify *B. anthracis* spores, no attempt was made to implement hoax material classifications when *Bacillus* sp. spores were present in the sample. The classification method described was also challenged with samples consisting of *Bacillus* sp. spores generated by production methods that differed from the standard protocol described, including several solid agar production methods (e.g., Columbia blood agar, Trypticase soy agar, and nutrient agar). The classification method was also challenged with crude

spore preparations (i.e., without postproduction purification) and spore preparations that were intentionally harvested before complete sporulation (i.e., <90% phase-bright free spores). All of the samples containing *Bacillus* sp. spores produced with the alternative production protocols were correctly classified, and no discernible changes in the mass spectra obtained were seen (data not shown), which is in agreement with previous observations (31, 33, 37). The analysis of samples containing crude spore preparations generated mass spectra with several additional peaks compared to pure spores. The additional peaks were most likely derived from vegetative cells and/or medium residues, but the expected spore-associated peaks were still consistently observed. Since the additional peaks were not present in the classification library and the SASP-associated peaks were present in the mass spectra irrespective of the additional peaks, no effect on the final classification results was seen and all of the samples were correctly classified (data not shown). Taken together, these observations support the perceived robustness of the proposed classification method.

The analysis method described here is a supplement to other laboratory methodologies (e.g., cultivation-, PCR-, and sequencing-based methods). The main focus of the proposed method is to obtain a reduction of the time between the initial discovery of a suspicious powder and the first confirmed laboratory-based answer about whether it contains *B. anthracis* spores or not, in order to decide if the initiation of other countermeasures is needed. Finally, the MALDI-TOF MS method developed is a cost-effective and potentially valuable assay that can easily be deployed in response laboratories and will help to elucidate suspicious powders and substantially reduce the burden of handling them.

In conclusion, a MALDI-TOF MS-based classification method for the rapid and reliable screening of powder samples and identification of *B. anthracis* spores was developed. The results showed that the assay is specific enough to discriminate *B. anthracis* spores from genetically highly similar *B. cereus sensu lato* strains and that the method is sensitive enough to allow the successful analysis of barely visible powder samples in less than 30 min.

ACKNOWLEDGMENTS

This study was performed as collaborative work between TNO and FFI and was financially supported by the Dutch Ministry of Defense (grant V1036).

We thank Jonathan M. E. J. Heng and Ingrid A. I. Voskamp-Visser for technical assistance.

REFERENCES

1. **Centers for Disease Control and Prevention.** 2012. Bioterrorism agents/diseases A to Z by category: category A. Centers for Disease Control and Prevention, Atlanta, GA. http://www.bt.cdc.gov/agent/agentlist-category.asp.
2. **Sinclair R, Boone SA, Greenberg D, Keim P, Gerba CP.** 2008. Persistence of category A select agents in the environment. Appl. Environ. Microbiol. **74:**555–563.
3. **Jernigan DB, Raghunathan PL, Bell BP, Brechner R, Bresnitz EA, Butler JC.** 2002. Investigation of bioterrorism-related anthrax, United States, 2001. Emerg. Infect. Dis. **8:**1019–1028.
4. **Jernigan JA, Stephens DS, Ashford DA, Omenaca C, Topiel MS, Galbraith M.** 2001. Bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States. Emerg. Infect. Dis. **7:**933–944.
5. **Crighton T, Hoile R, Coleman NV.** 2012. Comparison of quantitative PCR and culture-based methods for evaluating dispersal of *Bacillus thuringiensis* endospores at a bioterrorism hoax crime scene. Forensic Sci. Int. **219:**88–95.
6. **Poore C, Clark P, Emanuel PA.** 2009. An evaluation of suspicious powder screening tools for first responders. J. Hazard. Mater. **172:**559–565.
7. **Irenge L, Gala J-L.** 2012. Rapid detection methods for *Bacillus anthracis* in environmental samples: a review. Appl. Microbiol. Biotechnol. **93:**1411–1422.
8. **Demirev PA, Fenselau C.** 2008. Mass spectrometry for rapid characterization of microorganisms. Annu. Rev. Anal. Chem. **1:**71–93.
9. **Demirev PA, Fenselau C.** 2008. Mass spectrometry in biodefense. J. Mass Spectrom. **43:**1441–1457.
10. **Fenselau C, Demirev PA.** 2001. Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom. Rev. **20:**157–171.
11. **Fox A.** 2006. Mass spectrometry for species or strain identification after culture or without culture: past, present, and future. J. Clin. Microbiol. **44:**2677–2680.
12. **Ho Y-P, Reddy PM.** 2010. Identification of pathogens by mass spectrometry. Clin. Chem. **56:**525–536.
13. **Krishnamurthy T, Rajamani U, Ross PL, Jabbour R, Nair H, Eng J, Yates J, Davis MT, Stahl DC, Lee TD.** 2000. Mass spectral investigations on microorganisms. Toxin Rev. **19:**95–117.
14. **Lista F, Reubsaet F, De Santis R, Parchen R, de Jong A, Kieboom J, van der Laaken A, Voskamp-Visser I, Fillo S, Jansen H-J, Van der Plas J, Paauw A.** 2011. Reliable identification at the species level of *Brucella* isolates with MALDI-TOF-MS. BMC Microbiol. **11:**267. doi:10.1186/1471-2180-11-267.
15. **Anhalt JP, Fenselau C.** 1975. Identification of bacteria using mass spectrometry. Anal. Chem. **47:**219–225.
16. **Carbonnelle E, Grohs P, Jacquier H, Day N, Tenza S, Dewailly A, Vissouarn O, Rottman M, Herrmann J-L, Podglajen I, Raskine L.** 2012. Robustness of two MALDI-TOF mass spectrometry systems for bacterial identification. J. Microbiol. Methods **89:**133–136.
17. **Carbonnelle E, Mesquita C, Bille E, Day N, Dauphin B, Beretti J-L, Ferroni A, Gutmann L, Nassif X.** 2011. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clin. Biochem. **44:**104–109.
18. **Cherkaoui A, Hibbs J, Emonet S, Tangomo M, Girard M, Francois P, Schrenzel J.** 2010. Comparison of two matrix-assisted laser desorption ionization–time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. J. Clin. Microbiol. **48:**1169–1175.
19. **Mellmann A, Bimet F, Bizet C, Borovskaya AD, Drake RR, Eigner U, Fahr AM, He Y, Ilina EN, Kostrzewa M, Maier T, Mancinelli L, Moussaoui W, Prévost G, Putignani L, Seachord CL, Tang YW, Harmsen D.** 2009. High interlaboratory reproducibility of matrix-assisted laser desorption ionization–time of flight mass spectrometry-based species identification of nonfermenting bacteria. J. Clin. Microbiol. **47:**3732–3734.
20. **Seng P, Drancourt M, Gouriet F, La Scola B, Fournier P-E, Rolain JM, Raoult D.** 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin. Infect. Dis. **49:**543–551.
21. **van Veen SQ, Claas ECJ, Kuijper EJ.** 2010. High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization–time of flight mass spectrometry in conventional medical microbiology laboratories. J. Clin. Microbiol. **48:**900–907.
22. **Callahan C, Castanha ER, Fox KF, Fox A.** 2008. The *Bacillus cereus* containing sub-branch most closely related to *Bacillus anthracis*, have single amino acid substitutions in small acid-soluble proteins, while remaining sub-branches are more variable. Mol. Cell. Probes **22:**207–211.
23. **Callahan C, Fox K, Fox A.** 2009. The small acid soluble proteins (SASP α and SASP β) of *Bacillus weihenstephanensis* and *Bacillus mycoides* group 2 are the most distinct among the *Bacillus cereus* group. Mol. Cell. Probes **23:**291–297.
24. **Castanha ER, Fox A, Fox KF.** 2006. Rapid discrimination of *Bacillus anthracis* from other members of the *B. cereus* group by mass and sequence of "intact" small acid soluble proteins (SASPs) using mass spectrometry. J. Microbiol. Methods **67:**230–240.
25. **Castanha ER, Vestal M, Hattan S, Fox A, Fox KF, Dickinson D.** 2007. *Bacillus cereus* strains fall into two clusters (one closely and one more distantly related) to *Bacillus anthracis* according to amino acid substitutions in small acid-soluble proteins as determined by tandem mass spectrometry. Mol. Cell. Probes **21:**190–201.
26. **Demirev PA, Feldman AB, Lin JS.** 2004. Bioinformatics-based strategies

for rapid microorganism identification by mass spectrometry, vol 25. Johns Hopkins University Applied Physics Laboratory, Laurel, MD.
27. **Demirev PA, Feldman AB, Kowalski P, Lin JS.** 2005. Top-down proteomics for rapid identification of intact microorganisms. Anal. Chem. **77:**7455–7461.
28. **Demirev PA, Ramirez J, Fenselau C.** 2001. Tandem mass spectrometry of intact proteins for characterization of biomarkers from *Bacillus cereus* T spores. Anal. Chem. **73:**5725–5731.
29. **Dickinson DN, La Duc MT, Haskins WE, Gornushkin I, Winefordner JD, Powell DH, Venkateswaran K.** 2004. Species differentiation of a diverse suite of *Bacillus* spores by mass spectrometry-based protein profiling. Appl. Environ. Microbiol. **70:**475–482.
30. **Dickinson DN, La Duc MT, Satomi M, Winefordner JD, Powell DH, Venkateswaran K.** 2004. MALDI-TOFMS compared with other polyphasic taxonomy approaches for the identification and classification of *Bacillus pumilus* spores. J. Microbiol. Methods **58:**1–12.
31. **Elhanany E, Barak R, Fisher M, Kobiler D, Altboum Z.** 2001. Detection of specific *Bacillus anthracis* spore biomarkers by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. **15:**2110–2116.
32. **Fenselau C, Russell S, Swatkoski S, Edwards N.** 2007. Proteomic strategies for rapid characterization of micro-organisms. Eur. J. Mass Spectrom. **13:**35–39.
33. **Hathout Y, Demirev PA, Ho Y-P, Bundy JL, Ryzhov V, Sapp L, Stutler J, Jackman J, Fenselau C.** 1999. Identification of *Bacillus* spores by matrix-assisted laser desorption ionization–mass spectrometry. Appl. Environ. Microbiol. **65:**4313–4319.
34. **Hathout Y, Setlow B, Cabrera-Martinez R-M, Fenselau C, Setlow P.** 2003. Small, acid-soluble proteins as biomarkers in mass spectrometry analysis of *Bacillus* spores. Appl. Environ. Microbiol. **69:**1100–1107.
35. **Krishnamurthy T, Davis MT, Stahl DC, Lee TD.** 1999. Liquid chromatography/microspray mass spectrometry for bacterial investigations. Rapid Commun. Mass Spectrom. **13:**39–49.
36. **Lasch P, Beyer W, Nattermann H, Stämmler M, Siegbrecht E, Grunow R, Naumann D.** 2009. Identification of *Bacillus anthracis* by using matrix-assisted laser desorption ionization–time of flight mass spectrometry and artificial neural networks. Appl. Environ. Microbiol. **75:**7229–7242.
37. **Ryzhov V, Hathout Y, Fenselau C.** 2000. Rapid characterization of spores of *Bacillus cereus* group bacteria by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry. Appl. Environ. Microbiol. **66:**3828–3834.
38. **Stump MJ, Black G, Fox A, Fox KF, Turick CE, Matthews M.** 2005. Identification of marker proteins for *Bacillus anthracis* using MALDI-TOF MS and ion trap MS/MS after direct extraction or electrophoretic separation. J. Sep. Sci. **28:**1642–1647.
39. **Warscheid B, Fenselau C.** 2003. Characterization of *Bacillus* spore species and their mixtures using postsource decay with a curved-field reflectron. Anal. Chem. **75:**5618–5627.
40. **Connors MJ, Mason JM, Setlow P.** 1986. Cloning and nucleotide sequencing of genes for three small, acid-soluble proteins from *Bacillus subtilis* spores. J. Bacteriol. **166:**417–425.
41. **Kolstø A-B, Tourasse NJ, Økstad OA.** 2009. What sets *Bacillus anthracis* apart from other *Bacillus* species? Annu. Rev. Microbiol. **63:**451–476.
42. **Farquharson S, Smith W.** 2004. Differentiating bacterial spores from hoax materials by Raman spectroscopy. http://www.rta.biz/images/customer-files/paperspie20035269anthraxhoax.pdf.
43. **Leask A, Delpech V, McAnulty J.** 2003. Anthrax and other suspect powders: initial responses to an outbreak of hoaxes and scares. N. S. W. Public Health Bull. **14:**218–221.
44. **Leighton TJ, Doi RH.** 1971. The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis*. J. Biol. Chem. **246:**3189–3195.
45. **Schaeffer P, Millet J, Aubert JP.** 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. U. S. A. **54:**704–711.
46. **Coleman WH, Zhang P, Li YQ, Setlow P.** 2010. Mechanism of killing of spores of *Bacillus cereus* and *Bacillus megaterium* by wet heat. Lett. Appl. Microbiol. **50:**507–514.
47. **Bruker Daltonics.** 2011. MALDIBiotyper 3.0 user manual revision 1. Bruker Daltonics, Bremen, Germany.
48. **Lasch P, Nattermann H, Erhard M, Stammler M, Grunow R, Bannert N, Appel B, Naumann D.** 2008. MALDI-TOF mass spectrometry compatible inactivation method for highly pathogenic microbial cells and spores. Anal. Chem. **80:**2026–2034.
49. **Inglesby TV, O'Toole T, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Friedlander AM, Gerberding J, Hauer J, Hughes J, McDade J, Osterholm MT, Parker G, Perl TM, Russell PK, Tonat K; Working Group on Civilian Biodefense.** 2002. Anthrax as a biological weapon, 2002: updated recommendations for management. JAMA **287:**2236–2252.
50. **Schier JG, Patel MM, Belson MG, Patel A, Schwartz M, Fitzpatrick N, Drociuk D, Deitchman S, Meyer R, Litovitz T.** 2007. Public health investigation after the discovery of ricin in a South Carolina postal facility. Am. J. Public Health **97:**S152–S157.
51. **Ahmad F, Wu H-F.** 2012. High-resolution MALDI-TOF mass spectrometry of bacterial proteins using a Tris-EDTA buffer approach. Microchim. Acta **176:**311–316.
52. **Ferreira L, Sánchez-Juanes F, González-Ávila M, Cembrero-Fuciños D, Herrero-Hernández A, González-Buitrago JM, Muñoz-Bellido JL.** 2010. Direct identification of urinary tract pathogens from urine samples by matrix-assisted laser desorption ionization–time of flight mass spectrometry. J. Clin. Microbiol. **48:**2110–2115.
53. **Hsieh S-Y, Tseng C-L, Lee Y-S, Kuo A-J, Sun C-F, Lin Y-H, Chen J-K.** 2008. Highly efficient classification and identification of human pathogenic bacteria by MALDI-TOF MS. Mol. Cell. Proteomics **7:**448–456.
54. **United States Department of Justice.** 2010. Amerithrax investigative summary released pursuant to the Freedom of Information Act. United States Department of Justice, Washington, DC. http://www.justice.gov/amerithrax/docs/amx-investigative-summary.pdf.
55. **Greenberg D, Busch J, Keim P, Wagner D.** 2010. Identifying experimental surrogates for *Bacillus anthracis* spores: a review. Invest. Genet. **1:**4. doi:10.1186/2041-2223-1-4.

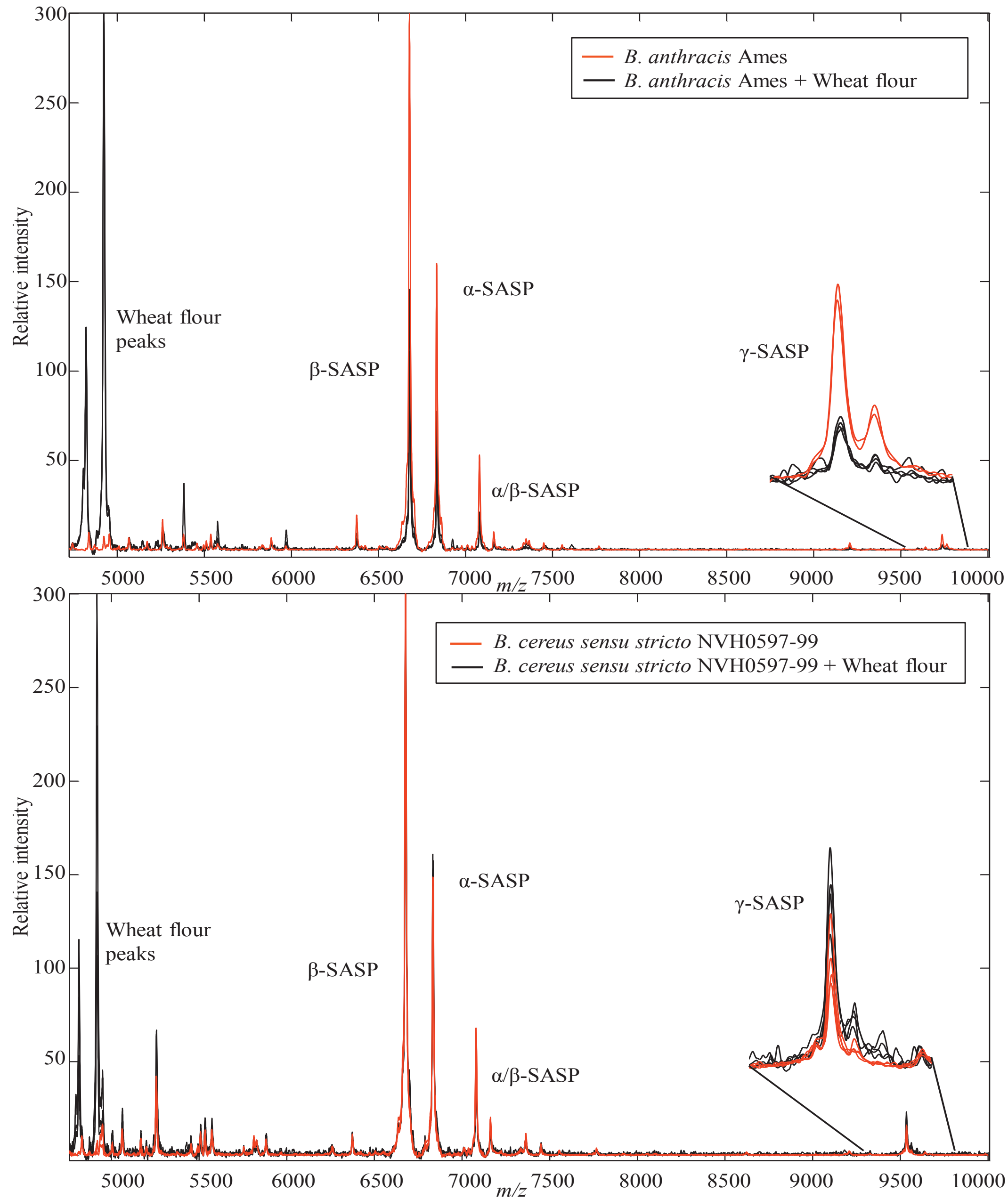

FIG S1. Representative mass spectra obtained with the described MALDI-TOF MS-based analysis method from *B. anthracis* Ames (top section) and *B. cereus sensu stricto* NVH0597-99 (bottom section) spores. The analysis was performed with pure spores (~10^7) and a similar amount of spores mixed together with 1 mg wheat flour. As can be seen the predominant SASPs (α, β, α/β and γ) were consistently observed in the mass spectra also when the spores were mixed wheat flour. The wheat flour led to two high intensity peaks (4,920 and 4,820 m/z). In some cases the relative intensities of the SASPs were altered due to the high intensity of the wheat flour peaks. However, these intensity changes had no impact on the classification results since all the predominant SASP peaks were still detected by the peak selection procedure.