Quantitative and diversity analysis of DNA accumulation on in-use HVAC filters from tropical buildings

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2017

Irvan Luhung. (2017). Quantitative and diversity analysis of DNA accumulation on in-use HVAC filters from tropical buildings. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/69828>

<https://doi.org/10.32657/10356/69828>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**QUANTITATIVE AND DIVERSITY ANALYSIS OF
DNA ACCUMULATION ON IN-USE HVAC FILTERS
FROM TROPICAL BUILDINGS**

**IRVAN LUHUNG
SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING
2017**

**QUANTITATIVE AND DIVERSITY ANALYSIS OF
DNA ACCUMULATION ON IN-USE HVAC FILTERS
FROM TROPICAL BUILDINGS**

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A thesis submitted to the Nanyang Technological University in
fulfillment of the requirement for the degree of Doctor of Philosophy

2017

ACKNOWLEDGEMENT

I would like to first thank my family, especially my father who encourages me to take up this wonderful learning opportunity as a PhD researcher. I would like to also express my gratitude to Prof. Victor Chang from the school of CEE, NTU and Prof. William W Nazaroff from the University of California, Berkeley for their invaluable insights and feedbacks during my journey as a graduate student researcher. I would like to also thank all my colleagues from the SinBerBEST program, researchers from the Advanced Environmental Biotechnology Centre (AEBC) in the Nanyang Environment and Water Research Institute (NEWRI), Singapore and the Singapore Centre on Environmental Life Sciences Engineering (SCELSE) in Nanyang Technological University (NTU), Singapore for providing all the support and lab facilities required for in this study.

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SUMMARY

Buildings are built for human activities. The development of a smart building system should pay equal attention to the well-beings of the human occupants. Ventilation/Heating Ventilation and Air Conditioning (HVAC) system filter is the first line of defence in occupant protection which keeps the indoor environments safe from potential airborne pollutants. This thesis focuses on the analysis of biological material accumulated on functioning HVAC filters and attempts to discern whether the results can be a good representatives of indoor air quality. This thesis consists of three major parts; the method optimization, the cross-sectional HVAC filter study and the longitudinal HVAC filter study.

The method optimization part focuses on maximizing the biomass yield from a given environmental samples, namely our HVAC filter samples. We found that adding additional cell lysis, particularly high temperature water bath sonication could increase the biomass yield considerably. Our work has also proposed a concentration approach in the middle of the extraction step which can be used in series with the additional sonication, minimizing time and possible DNA loss.

With our optimized protocol, we analysed a set of HVAC filters serving separate, well-characterized indoor environments (cross-sectional study). Our findings confirm that different indoor conditions such as occupancy level and the function of the indoor environment are well reflected in both the absolute and relative abundances of certain bacteria and fungi on HVAC filter. While there are still factors to be considered, these findings support the application of HVAC filters as an indicator of indoor environmental quality.

The final part (longitudinal study) focused on one air handling unit filter. We investigated how the biomass accumulate on the filter during its operation time. Based on the microbial community analysis, fungal DNA on the filters was found to mainly originate from the outdoor air intake, while the bacterial DNA originate from the indoor sources. In addition, the biomass accumulation profile revealed that there is a loss rate for certain biomass already trapped on the filter. This finding indicates that an interpretation of biomass concentration based on HVAC filter sampling must be completed with its corresponding meta-data.

LIST OF PUBLICATIONS

Published Journal Articles

1. **Luhung, I.**, Yan, W., Miller, D., Ng, C.K., Cao, B., Chang, V.W.C. 2015. Protocol Improvements for Low Concentration DNA-Based Bioaerosol Sampling and Analysis. PLoSOne. e0141158.
2. Gall, E.T., Cheung, T., **Luhung, I.**, Schiavon, S., Nazaroff, W.W. 2016. Real-time Monitoring of Personal Exposures to Carbon Dioxide. Building and Environment. (104). 59-67.

Journal articles in-review (submitted)

1. **Luhung, I.**, Yan, W., Xu, S., Yamamoto, N., Chang, V.W.C., Nazaroff, W.W. DNA Accumulation on Ventilation System Filters in University Buildings in Singapore. Submitted to Building and Environment.
2. Yan, W., Chen, A., **Luhung, I.**, Gall, E.T., Chang, V.W.C., Nazaroff, W.W. Deposition of Particles and Bioaerosol on Air-conditioning Cooling Coil. Submitted to Atmospheric Environment.

Journal articles in-preparation (draft manuscript completed)

1. **Luhung, I.**, Yan, W., Chang, V.W.C., Nazaroff, W.W. Time-Series Profile of Bioaccumulation on Ventilation System Filter in a Library, to be submitted.

Conferences Proceedings

1. **Luhung, I.**, Yan, W., Nazaroff, W.W., Chang, V. Time-Series Profile of Bioaccumulation on an HVAC Filter in a Singapore University Library. Poster ID 724, Indoor Air 2016 Hong Kong, 3-8 July 2016
2. **Luhung, I.**, Yan, W., Chang, V.W.C., Nazaroff, W.W. 2015. Bioaccumulation on HVAC filters in University Buildings in Singapore. Paper #157, Healthy Building America 2015. Boulder, CO. 19-22 July 2015.

3. **Luhung, I.**, Yan, W., Chang, V.W.C., Nazaroff, W.W. Indoor Bioaerosol in Different Ventilation System. AF0737, 2014 International Aerosol Conference, Busan, South Korea.
4. **Luhung, I.**, Yan, W., Nazaroff, W.W., Chang, V. DNA Based Protocol Optimization for Bioaerosol Sampling in Urban Tropical Environment. HP0654, Indoor Air 2014 Hong Kong, 7-12 July 2014.
5. Yan, W., **Luhung, I.**, Chang, V., Nazaroff, W.W. Characterizing the Indoor Microbiome in an Office in Singapore Before and After Cleaning to Address a Mold Problem. HP0658, Indoor Air 2014 Hong Kong, 7-12 July 2014, Hong Kong.

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LIST OF SYMBOLS

HVAC = Heating Ventilation and Air Conditioning

AHU = Air Handling Unit

PSI = Pollutant Standards Index

Ct = Cycle threshold value from qPCR assays

Df = weekly accumulation rate of DNA mass on the actual HVAC filter panel

De = weekly accumulation rate of DNA mass on the extracted filter area

Af = The surface area of the actual HVAC filter panel

Ae = The surface area of the extracted filter piece

Q = Volumetric flow rate

To = Operating hours of the AHU

Vs = Total air processed by the AHU

C = Estimated conditional bioaerosol concentration based on HVAC filter sampling

E = Estimated conditional human emission factor based on HVAC filter sampling

N = Occupancy level in terms of person-hr/wk

Nd = Occupant intensity, the ventilation rate normalized occupancy

CHAPTER 1

INTRODUCTION

1.1 Background: Bioaerosols in Building's Ventilation System

As most humans are known to spend more than 90% of their time indoor, interaction between human and building become an important factor in establishing numerous building applications and operations. There is a need to ensure that indoor environmental quality which favours comfort and health of building occupants is not compromised in the attempt of achieving other standards, such as choosing the optimum building materials or gaining energy efficiency. The economic benefit of addressing such matter has been well documented in the literature[1-4]. The advantages gained from increase in work performances, reduced sick leaves or health bills far outweighs the cost needed to achieve the standard indoor air quality.

One of the main focus in maintaining indoor environmental quality standard is understanding the dynamics of bioaerosols in indoor environments. Bioaerosols refer to the biological portion of airborne particles (aerosols). It can originate from outdoor or indoor sources, mainly from the occupants themselves. Bioaerosols include microorganisms such as bacteria, fungi and their by-products. Interest in bioaerosol research has grown rapidly over the past few decades, spurred by the advent of analytical methods based on DNA. There are numerous reasons for interest in measuring bioaerosols. Some research has been motivated by potential human health effects [5,6]. While airborne microorganisms can be benign or even beneficial to humans, [7,8], bioaerosols are also known to cause infections, allergenic responses and toxic effects ranging from relatively common asthma and sick building syndrome to epidemic diseases (such as SARS) and bioweapon use (such as Anthrax) in extreme cases [9-12]. Indirect impacts of bioaerosols, such as crops infection [13], biodegradation of building materials [14,15] or how it can be transported widely by the wind, affecting life along the way [16-18], have also attracted equal attention to understand the dynamics of bioaerosols around us. Such

interest to measure or identify bioaerosols in different environments, however, is seldom coupled with satisfactorily robust technical capabilities given the limitations of current culture-based and DNA-based sampling and analysis methods for filter and liquid samples. Researchers are often faced with problems such as collecting enough biomass in a relatively short time (low time resolution)[19] or low sampling efficiency.

In addition to the lack of reliable methods, the efforts put to understand the role of bioaerosols in a building's ventilation system is still far from adequate. It is very likely that the way ventilation system operates and controls air distribution between rooms determines the presence of bioaerosols in building premises. More than 90% of office buildings in Singapore are equipped with a centralized ventilation and air conditioning system. In tropical climate, such systems are commonly referred to as air conditioning and mechanical ventilation system (ACMV), because heating is not needed. However, to be consistent with common international usage, we will refer to these systems using the common HVAC acronym. Current regulations of HVAC system operation and practices (SS554, SS553) were developed without detailed knowledge about how accumulation of biological material can happen in various parts of the HVAC system, predominantly on the air processing filter as it treats the air prior to supplying to the indoor environment. HVAC filters are integrated into ventilation systems as the first line of defence, to protect both downstream equipment and building occupants in indoor environments. Our filter of interest is the indoor primary HVAC filter. This filter processes a mix of recirculated indoor air and the outdoor air. In Singapore, owing to the consistently high latent heat load, central air conditioning systems generally operate with a very high proportion of recirculated air and a correspondingly small proportion of outside air. For the buildings studied here, the sampled air-handling filters processed air that was a blend of >90% recirculated air and <10% outdoor air. In accordance with Singapore regulations, ventilation system filters must be replaced within six months of installation.

HVAC filter assumes two very important roles in the distributions of various indoor or outdoor pollutants, which includes bioaerosols, in a functioning building with a centralized air handling unit (AHU). The first role is to prevent airborne

pollutants from reaching the downstream equipment and ultimately the building occupants. In a typical AHU system, HVAC filters precede a cooling coil which adjusts the temperature and humidity of the supply air. The wet surface of cooling coil is ideal for microbial colonization, especially when the system is off during night time which causes the temperature of the coils to rise. It is feasible that microbial by-products such as endotoxins[20,21] or re-aerosolization of the accumulated biomass back to the indoor environments contribute to the adverse impacts of bioaerosols mentioned previously. Studying the particulate matters (PM) which are inevitably trapped on the filters can help us understand the major characteristics of the pollutants in the specific building and subsequently make operational decisions that best suit the building occupant's need, such as choice of filter grade or filter replacement frequency.

The second role is related to bioaerosol study in general. In the context of indoor bioaerosol study, direct indoor sampling with a vacuum pump in an actual in-use location such as classrooms, offices or libraries often invites challenges such as space availability, long duration or noise issues. Several recent studies [22] have started to propose an idea it is possible to consider HVAC filters as a time-averaged, long term indoor air sampler. As it primarily processes re-circulated indoor air (>90%) in most ventilation system, HVAC filter has the potential to act as an indicator for indoor environmental quality, which can be collected for off-site analysis with relatively incremental effort. There is still a need, however, to thoroughly evaluate the implication of HVAC filter as a proper air sampler. Information about HVAC filters utilized as an air sampler is still scarce in the literature. It is necessary to first investigate whether dynamics in indoor environment such as occupancy or activity level is well reflected in a study based on HVAC filter sampling. Other factors such as different filter grade/material's efficiency, transport losses and the outdoor influence must also be considered to accurately interpret the final result.

1.2 Research Scope and Focus

This project has two major Focus. The first focus is method development. We restrict our method development study to culture-independent DNA-based analysis

which is best suited for both biomass quantification and microbial community identification. Processing relatively new environmental samples often comes with their unique challenges, such as new inhibitors or extraction impurities. Based on the existing bioaerosols analysis methods in the literature, a protocol best-suited for our environmental samples from different parts of an HVAC system must first be developed.

The second focus of this project is analysing bioaerosols in the HVAC system, particularly on the HVAC filter. We aim to achieve a thorough characterization of biomass accumulated in a building's HVAC filter to identify the sources and influencing factors of biological matters which deposit on the filter. This analysis includes both absolute quantification and microbial diversity analysis as well as correlating these two pieces of information with indoor operation parameters, e.g. air flow speed or occupancy levels. These two key findings are expected to be able to make a meaningful contribution in understanding the role of our ventilation system in the dynamic correlation between bioaerosols in indoor environments and the occupants.

CHAPTER 2

LITERATURE REVIEW

2.1 Protocol Optimization of Bioaerosols Analysis Methods

The road to developing the much needed standardized methods for bioaerosol research has been challenging mostly due to major technical limitations. First, bioaerosol concentrations are naturally dilute in the environment [17,23-26]. The low concentrations of interest lead to detection limits and sensitivity problems in subsequent analysis. The second issue is the fact that bioaerosols are highly dynamic in time and space [17,27-30]. This large variability makes it difficult to establish and compare protocols from various studies.

Culture-based and microscopic methods dominated early bioaerosol research [6,27,31] and are still used today [32-35] because of their practicality and specificity. However, microscopic methods are generally labour intensive, while the culture based methods might carry a certain level of bias particularly when a broader range of microorganisms are targeted. The culture based methods require the captured organisms to be both viable and culturable on the selected medium, which only accounts for less than 5% of total organisms in the air [25,36,37]. Furthermore, some sampling approaches may cause some stress to the targeted microorganisms and lead them, to completely or partially, lose their viability during or after collection [23,38,39].

To complement culture-based assessments, culture-independent methods have been developed. Several bioaerosol analysis protocols were built based on biological entities such as endotoxin [40,41] or glucans [42]. While these methods have merit, DNA-based analysis, which uses PCR (polymerase chain reaction) as the core technology has emerged to assume the main role [23,24,43,44] in recent bioaerosol studies. Unlike other biological entities, DNA is ubiquitous in all living things. This advantage has driven a variety of DNA-based technology, such as time-saving commercial DNA extraction kits (MOBIO, Qiagen, etc.), DNA quantification devices (Nanodrop, Qubit, qPCR), and advanced DNA sequencing

technologies [45-47] to flourish with a trend towards increased technical capabilities and rapidly decreasing unit costs.

The focus of this method development part is to deal with challenges associated with low bioaerosol concentration from complex environmental samples, utilizing culture-independent, DNA-based analysis methods. Previous studies have commonly applied three approaches to optimize DNA yield from low biomass samples, which include: (1) extended sampling with high flowrate or long duration [23,48]; (2) improving the sample extraction process [18,22,49]; and (3) attempting to concentrate samples [12]. However, the use of these approaches is often not complemented with details on to what extent or how these efforts directly affect final results. This study aims to provide a thorough investigation of how the following three factors may improve DNA yield from our targeted bioaerosol environmental samples: (i) the effect of thermal sonication on DNA yield; (ii) the effect of long-duration filter sampling on nonviable DNA based analysis; and (iii) an alternative method to pool several low concentration samples; may improve DNA yield from bioaerosol environmental samples.

1.2 Investigation of bioaccumulation on HVAC filter: cross-sectional and longitudinal studies

The majority of past indoor bioaerosol studies have chosen to collect samples directly from the indoor environment of interests (indoor dust, air impaction, air filtration)[48,50-53]. While direct indoor air sampling has its merits, problems such as loud noise and the long duration needed to gather the amount of biomass required for various analysis methods often makes it difficult for researchers to sample an actual functioning indoor spaces. HVAC system filter as an alternative integrated indoor air sampler can be collected with much less effort and would typically contain enough biomass for numerous analysis purposes after months of operation period.

Appreciating the attractive potential as an air sampler and the actual importance of ventilation system filters in processing both recirculated indoor and outdoor air for building occupants, researchers have begun to analyse the particulate matter collected on these filters. One of the earliest such studies involving HVAC filter

dust was performed in two shopping centres in Singapore [54]. In that study, the microbiota measured on HVAC filters was primarily bacteria which could originate from indoor sources. Additional studies of the dynamics of biological materials on ventilation system filters followed. Noris et al. [22] used culture based methods to quantify biomass recovered from both indoor and HVAC filter dust, completed by their diversity analysis. Pigeot-Remy et al. [55] studied the survivability of certain bacteria on HVAC filters. Hoisington et al. [56] attempted to characterize bacterial communities on the filters across different retail stores in the US and Liu et al. [57] quantified and analysed the diversity of culturable bacteria and fungi in offices across Beijing. Most recently, Haaland and Siegel [58] have suggested that analysis of accumulated material on AHU filters could serve a “quantitative filter forensics” function in building investigations. Quantitative analysis on biological pollutants deposited on HVAC filters is still very limited in the literature. Among the aforementioned studies, only 3 of them [22,54,57] considered the HVAC filters as a quantitative sampler of indoor pollutants and none of them applied culture independent methods in their analysis.

At current state, the amount of specific evidence also remains limited regarding factors affecting the presence, abundance and dynamics of biological materials on AHU filters. For example, as human occupants are known to be a major source of bacteria and fungi indoor [48,49,52,59,60], it is still not clear whether occupancy level of the served indoor environment would also affect the diversity or quantity of microbial content on the filters. Azimi et al. [61] has reported that the efficiency of the typically used MERV 7 or 8 rated filters (for commercial and residential buildings) is limited for certain size of particles. It is also unknown whether the biomass that is collected at any point during the filter operation is well conserved until the sample collection point. It is possible that DNA is physically dislodged or even degrade due to high face velocity, higher temperature during off time or passively during the long operation period. It is important to study these factors to better understand the strengths and limitations of AHU filters as part of the mechanical ventilation system and to possibly gain some insight into the levels and/or diversity of indoor bioaerosols in occupied spaces. To our knowledge, there is still no study which provides both culture-independent quantitative and diversity

analysis of biomass accumulated on a set of AHU filters serving separate, distinguishable indoor environments.

As we aim to contribute in understanding the aforementioned possible influencing factors, we carried out two parts of an in-depth study targeting the HVAC filter. The first part reports quantitative results and interpretive analysis of baseline measurements of biological materials accumulated on used ventilation system filters from several locations (cross-sectional study). We also provide information regarding how different indoor conditions of the chosen locations affect bioaccumulation on these filters. Based on the results of the cross sectional study, the second part focuses on one locations with the most volatility during the operation period. The goal of the second part is to evaluate the time-series profile of the bioaccumulation on the filters and how the microbial communities change over time (longitudinal study).

All measurements are made using culture-independent DNA-based analysis techniques. We assessed the biomass concentration recovered from normally used ventilation-system filters and investigated the influence of the surrounding factors on bioaccumulation on these filters in different indoor environments. The chosen indoor environments are characterized based on their general functions, size, interior design, and occupancy levels. The operating hours, supply air flow rate and total filter area of each air handling unit were also specified. Outdoor reference was measured for the longitudinal study to possibly gain insight on how the outdoor air intake contribute to the microbial communities on the filter over time. The microbial diversity and speciation was done by undertaking the high-throughput sequencing targeting bacterial 16S rDNA and fungal internal transcribed spacer (ITS) sequences to analyse the microbial composition on the filter samples.

2.3 Research Objectives

Based on current state of research in this literature review, a set of objectives were set to potentially fill some research gaps and questions identified previously;

- Develop an optimized analysis protocol for actual HVAC filter samples deployed in functioning buildings

- Provide baseline concentration of DNA and microbial communities accumulated on HVAC filters from tropical buildings
- Assess the feasibility of considering HVAC filters as a probe of the served indoor air quality
- Possibly advise on building operational parameters such as choice of HVAC filter types or filter replacement frequency based on our research findings.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bioaerosol Samples Collection

We collected mainly two types of environmental samples with different biomass loadings for this study. For the method optimization, the approaches were tested both on the targeted used-mechanical-ventilation-system (HVAC) filters which represents high biomass samples and filter-based ambient air samples which represent low biomass samples.

All ambient air samples were collected by means of filtering air onto a polyethersulphone (PES) filter membrane (47mm, 0.2 μm pore size, Pall Corporation, USA) using a diaphragm vacuum pump (HCS Scientific, Singapore). The PES filter membrane is loaded onto an in-line filter holder (no size cut, Pall Corporation, USA) with one side connected to the vacuum pump. The pumps typically run at a flow rate of 16 L/min (with membrane filter resistance) for up to 20 hours at the desired locations. After sampling, the filter holder is sealed with paraffin and brought back to the lab in an ice box for analysis. The membrane filter is taken off the in-line holder in a bio-safety hood with sterilized tools such as scissors and tweezers to prepare for DNA extraction. Unprocessed filters are all stored in -20°C .

The ventilation system filters were obtained during regular air-handling unit (HVAC) filter replacement schedule in buildings of the Nanyang Technological University (NTU), Singapore. All HVAC filter samples were collected with permission from NTU facilities management office and the servicing company (SMM Pte. Ltd., Singapore). Except for the longitudinal time-series study, the collected filters were typically in service for three months prior to acquisition. The filters are indoor secondary units, which come after the coarse primary filter that treats only outdoor air. The secondary filter served as a filter for a mixture of both recirculated indoor air plus make-up outdoor air. During sampling, right before they are taken down for replacement, the filters are directly cut on-site with sterilized tools into smaller pieces (2×5 cm) and put into a 50 mL falcon tube. Six to seven

pieces of filters are usually collected in one sampling session for backup and replication purposes. The falcon tubes (with filter samples) are transported back to the lab in an icebox for analysis. Filter cutting and extraction preparation are done in a bio-safety hood. Leftover filters are also stored in -20°C.

3.2 DNA Extraction

MOBIO Power Water (PW, MOBIO Carlsbad, USA) DNA extraction kits were used for all extractions. MOBIO PW kit extracts genomic DNA and it is designed to facilitate filter samples. The kit consists of 5-ml tubes with assorted bead size for its bead beating step and pre-set solutions (PW1-PW6) for its cell lysis, DNA purification and final elution step. In our application, the ambient air membrane filters (PES) were directly put into the 5-ml bead beating tube of the PW kits using flame sterilized tweezers and working under a bio-safety hood. The HVAC filter pieces (2 × 5 cm per piece) were also directly placed in the bead beating tube for extraction. For maximum particle removal from the filters, both types of filters were inserted into the tubes with its front size facing the inside of the tube.

Cell lysis is a process of breaking a cell or parts of a cell in order to get the intra-cellular matter out, including DNA. MOBIO PW kit utilizes the bead beating process combined with a lysing agent (solution PW1) to lyse the cell. Briefly, 1 mL of solution PW1 is added to the bead tube (with the filter inserted) and the bead beating step was carried out by vortexing the sample tubes at maximum speed for 5 minutes. After vortexing, the 5-mL bead beating tubes are centrifuged at 4000 rcf for 2 minutes to settle the liquid. The liquid is then transferred to a clean 2 mL tube. It is common to still have some visible impurities carried over at this step. The 2 mL tubes are then centrifuged again with 13000 rcf for 1 minute to separate the impurities from the liquid. Avoiding the settled impurities, the supernatant is moved to a clean 2 mL sample tubes.

DNA purification follows after the cell lysis step. Soluble impurities such as leftover cell debris or humic acid from the particulate matters collected during sampling could compromise the integrity of subsequent DNA analysis steps, especially those that involve polymerase chain reaction (PCR). MOBIO PW kit uses solution PW2 to precipitate PCR inhibitory substances which could be carried over

from the lysis step. 200 μ L of solution PW2 is added to the solution. After brief mixing, the solution is incubated at 4°C for 5 minutes. The precipitation is separated from the solution by centrifuging at 13000 rcf for 1 minute. While avoiding the precipitation at the bottom, the supernatant is transferred to the next clean 2 mL tubes.

The DNA is further purified through the spin filter binding step. Similar to other commercial DNA extraction kits, MOBIO PW kit uses a silica membrane spin filter to bind and separate the DNA from possible leftover inhibitors. The DNA solution is first conditioned by adding high concentration salt solution (PW3) with 1:1 ratio to maximize its binding factor to the silica membrane spin filter. After quick mixing, the solution is loaded to the spin filter column and the column is centrifuged at 13000 rcf for 1 minute to force the liquid through the membrane. It is expected that after this step, all DNA is bound to the silica membrane while other possible impurities flow through the filter with the liquid. The flow through liquid can be discarded. Final cleaning step of the spin filter is done by introducing alcohol based solution PW4 and PW5 to the spin filter. After adding 650 μ L of solution PW4 to the spin filter column, the tube is again centrifuged at 13000 rcf for 1 minute. The same step is repeated for solution PW5. The flow through liquid can be discarded.

The final elution step re-elutes the purified DNA which is still bound to the silica membrane into a Tris-buffer solution PW6. While it is possible to elute DNA in nuclease free water, using tris buffer helps prevent the DNA from degrading of common causes such as long term storage or freeze thaw cycle which comes prior to re-using the DNA after storage. In our application, 60 μ L of solution PW6 is used for the final elution of all extractions. Upon loading solution PW6 to the spin filter column, the column is first incubated for 5 minutes in room temperature. After incubation, the column is centrifuged at 13000 for 1 minute to force the elution buffer through. The spin filter column can now be discarded. The flow through liquid of this step is the final DNA solution.

3.3 DNA Quantification: fluorometry and qPCR

DNA fluorometry (Qubit, Life Technologies, USA) and quantitative PCR (Roche LC 480, Basel, Switzerland) were applied to quantify and verify the quality of the extracted DNA solutions. Qubit fluorometer measures total DNA concentration by introducing a specific dye that turns highly fluorescent when it binds to double stranded (ds) DNA. The ds DNA high sensitivity kit was used for all of the Qubit assays. Qubit buffer solution was prepared by mixing the HS DNA buffer with HS DNA dye with a ratio of 1:200. For each measurement in this study, 190 μL of this buffer mix solution was mixed with 10 μL of the DNA sample template before it is loaded to instrument for measurement.

The measured DNA concentration is presented in terms of ng of DNA per μL of elution liquid (ng/ μL). The total mass of DNA (ng) is then calculated by multiplying the concentration with the amount of elution liquid used during extraction. For low concentration air samples, the derived DNA concentration is expressed in terms of ng of DNA per volume of air sampled through the 47-mm PES filter (ng/ m^3 of air). For the higher biomass AHU filter samples, the final total DNA concentration is expressed in terms of total extracted DNA per area of filter segmented for extraction (pg or ng/ cm^2 of filter).

Quantitative PCR was also carried out to estimate concentrations of DNA associated with specific microbiological targets. Total bacteria and total fungi were the primary targets for all environmental samples analysed in this study. Specific to the sampling duration experiment in chapter 4, a custom GFP gene was targeted in the qPCR assay. Quantitative PCR is based on the concept of DNA replication which doubles the number of targeted DNA sequences on every thermal cycle.

The main components needed for a qPCR assay are the master mix which contains free nucleotides, polymerase enzyme and MgCl_2 as catalyst, the forward and reverse primers which aim only at the targeted DNA sequence, the Taqman probe which emits fluorescent signal detected by the instrument and the DNA sample template. The Taqman probe and primers would bind to the specific targeted sequences from our DNA samples at a temperature set by the instrument. The master mix enables the polymerase reactions which elongate and duplicate DNA starting from the sequences where the primers are bound to, also at a fixed temperature. As DNA duplicates, the elongated chain breaks the bond between the

Taqman probe and DNA, which would in-turn make it fluorescent. The qPCR instrument sets a repeating thermal/temperature cycle facilitating all the binding and elongation reactions in order to detect the increasing fluorescent signal emitted by the released Taqman probe. To avoid over-duplication, all qPCR assay in this study was carried out within 35 thermal cycles. The qPCR mix consists of 10 μ L of master mix with x2 concentration, 2 μ L each of forward and reverse primers with 10 mM concentration, 2 μ L of Taqman probe with 2 mM concentration and 4 μ L of DNA sample, forming a total of 20 μ L reaction volume for each well.

The number of cycles needed for each sample to cross a pre-determined intensity point of fluorescent signal is recorded as the Ct (cycle threshold) value. Recent qPCR instruments use second derivative maximum analysis method which records the point where acceleration of the fluorescence signal is at its highest as its set point to determine the Ct value. The lower the Ct value, the higher the amount of targeted DNA sequence in the sample as fewer cycles of DNA duplication are needed to reach the set point. In addition, at 100% qPCR efficiency, each Ct unit reflects a doubling of DNA abundance, approximately a doubling of mass.

Standard curves (Ct value vs. DNA mass concentration) were established for general bacteria, fungi and GFP based on Qubit measurements of extracted pure bacteria (*E. coli*), fungus (*Aspergillus fumigatus*) and GFP-tagged *Shewanella oneidensis* [62] suspensions in water, which were chosen as equivalent representatives. Ct values were converted to DNA concentration based on standard curves which plot log Ct values vs log DNA concentration values. These DNA concentration values were obtained by extracting a known amount of pure suspensions of the specific targeted group of microbes. For example, for bacteria's standard curve, a known amount of *E. coli* (based on spectrophotometric measurement) suspensions in water was diluted 10, 100, 1000, and 10000 times and DNA was extracted from each of the diluted suspensions. The final DNA solution from each dilution were both measured by Qubit and qPCR in order to have a linear plot between the two variables, hence forming the standard curve. Same methods were applied for both Fungi (with *Aspergillus fumigatus*) and GFP genes *Shewanella oneidensis*. Unlike general bacteria and the specifically targeted *Shewanella oneidensis*, it is not possible to have an average number of 18S genes for general fungi as it may vary even between different strains of the same species. Therefore, fungi concentration was at times expressed in

terms of spores equivalent (SE) instead of the regular DNA concentration. Similar to the Qubit result calculations, the partial DNA concentrations were finally converted to pg of DNA per m³ of air (pg/m³) for ambient air samples and to pg of DNA per area of filter (pg or SE/cm²) for AHU filter samples.

Three sets of primers and probes were used for the qPCR assay. These are sequences from 16S region for general bacteria, 18S region [63] for general fungi and the GFP gene for the experiments involving *Shewanella oneidensis*. Table 3.1 lists the detailed sequences of the aforementioned primers and probes, each of which was synthesized by TIB Molbiol (Berlin, Germany).

Table 3. 1 Probe and primer sets for qPCR assays

Function	Sequence
Forward primer, 16S (bacteria)	5'–ACTCCTACGGGAGGCAG–3' BAC338F
Reverse primer, 16S (bacteria)	5'–GACTACCAGGGTATCTAATC–3' BAC805R
Taqman Probe, 16S (bacteria)	6FAM–TGCCAGCAGCCGCGGTAATAC–3'–BBQ BAC516F
Forward primer, 18S (fungi)	5'–GGRAAACTCACCAGGTCCAG–3' FungiQuant-F
Reverse primer, 18S (fungi)	5'–GSWCTATCCCCAKCACGA–3' FungiQuant-R
Taqman Probe, 18S (fungi)	6FAM–TGGTGCATGGCCGTT–3'–BBQ FungiQuant-PrbLNA
Forward primer, GFP gene	5'–ATGGAAACATTCTTGGACACAAATTG–3' GFP-S
Reverse primer, GFP gene	5'–GTTGATAATGGTCTGCTAGTTGAACG–3' GFP-R
Taqman Probe, GFP gene	6FAM– TCCATTCTTTTGTCTGCCATGATGT–BBQ GFP-TM

Specific to the DNA pooling performance test and the impact of sampling duration on the Gram-negative GFP-tagged bacteria test in chapter 4, part of the data is reported in terms of total recovered DNA (ng or pg) as the purpose of the experiments was to investigate the amount of DNA recovered regardless of concentration. To obtain these measures, the DNA concentrations measured by both Qubit and qPCR (ng/ μ L and pg/ μ L) were multiplied by the elution volume used during extraction to obtain the final extracted DNA mass for each sample (ng for Qubit and pg for qPCR). Lastly, potential inhibitor in the DNA solutions was checked by performing a series of dilutions on the samples and subsequently qPCR.

PCR inhibitors such as humic acid which could originate from dusts could compromise the actual Ct values produced by the qPCR run. In the case of no inhibition in the final DNA solution, the difference in Ct values among the dilutions were expected to match the magnitude of the dilutions performed.

3.4 Microbial Diversity Analysis: High-throughput DNA Sequencing

DNA sequencing technologies have grown rapidly over the past few years. Illumina sequencing platform was chosen for this study mainly due to cost and its suitability to sequence microbial genomes[47] with different sizes. Illumina Miseq is deemed suitable for the size of the targeted bacterial and fungal genome as compared to Hiseq. We performed amplicon sequencing in which the targeted bacterial and fungal DNA sequence is first amplified through a series of PCR and the amplicons (results of DNA replications) are then sequenced on a certain base pair (bp) read length with the barcodes adapter attached.

There are two main PCRs required for amplicon sequencing. The first PCR amplification targeted the 16S V3 and V4 region for bacteria and ITS3 and ITS4 region for fungus. The main purpose for this PCR is to amplify only the targeted DNA sequence within the DNA sample which would typically contain a plethora of DNA sequences from various sources. The primers for bacteria 16S are 341f forward 5'-

*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG***CCTACGGGNBGCASCAG**

-3' and 805r reverse 5'-

*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG***GACTACNVGGGTATCT**

AATCC-3' [64]. The primers for ITS are ITS3 forward 5'-

*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG***GCATCGATGAAGAACG**

CAGC-3' and ITS4 reverse 5'-

*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG***TCCTCCGCTTATTGAT**

ATGC-3' [65]. These primers include adapter sequences on the 5' end (in italic).

The second PCR is termed Index PCR. The main purpose for this PCR is to attach the index barcode to the amplicons from the first PCR. It is necessary for the sequencer (Illumina Miseq) to detect the barcode and start reading the sequences of

the amplicons. Index PCR was subsequently performed with Nextera XT dual index barcodes. Amplicon purification follows after each PCR with AMPure XP beads to remove primers and dimers. The final library size was assessed on Agilent BioAnalyser DNA1000 chip and concentration of each library was determined by KAPA quantification kit. The libraries were finally pooled together in equimolar amounts before loading on the sequencer. The pooled library was sequenced using the Illumina Miseq nano v2 reagents on paired 250-bp reads.

After sequencing, the resulted raw DNA sequences must first go through a few quality control steps before taxonomic assignment and diversity analysis can be done. The steps are poly N tails trimming, length trimming and chimeric sequences removal. In the poly N tail trimming step, sequences with poly N tails were trimmed by the Trimmomatic version 0.33 [66]. The read 1 and 2 sequences were joined with a minimum allowed overlap of 10 bp in QIIME software [67]. Length trimming was conducted by the Galaxy Tool version 1.1 with a minimum threshold length of 100 bp [68,69]. The chimeric sequences were removed in mothur v.1.25.0 [70] against the fungalITSdatabase database containing named fungal ITS sequences [71] for fungal ITS, and against a reference Ribosomal Database Project (RDP) database [72] for bacterial 16S rDNA.

For the fungal ITS sequences, taxonomic assignments were performed using BLASTN version 2.2.28+ [73] against the fungalITSdatabaseID database containing named fungal ITS sequences [71] and classified based on each taxonomic rank using FHiTINGS [74]. For the bacterial 16S rDNA sequences, taxonomic assignments were performed using the RDP Naïve Bayesian Classifier [72] with 0.8 as a confidence cutoff value. The Shannon and Chao1 indices (α diversity) were calculated based on the lowest number of sequences from the sample subsampled from each library and clustered into 97% OTUs. The β diversity analysis was done by plotting the principal coordinate analysis (PCoA) diagram based on the distance matrix between the compared samples using the Jaccard and ThetaYC algorithm.

CHAPTER 4

PROTOCOL OPTIMIZATION FOR LOW CONCENTRATION BIOAEROSOL ANALYSIS METHODS

**The work in this chapter has been published with the following detail:*

Luhung et al. (2015). Protocol Improvements for Low Concentration DNA-Based Bioaerosol Sampling and Analysis. PloS one 10: e0141158.

4.1 Objectives

The main objective of the method development project is to optimize our existing DNA analysis workflow to better deal with challenges associated with low bioaerosol concentration and inhibitors from our environmental samples. Specifically, we are looking to investigate: (i) the effect of thermal sonication on DNA yield. Thermal sonication step is an additional cell lysis step incorporated to our DNA extraction protocol to help lyse cells which could be harder to break such as fungal cell with rigid cell wall or gram-positive bacteria with thicker membrane. (ii) the effect of long-duration filter sampling on nonviable DNA based analysis. On this investigation, we are looking to see whether the DNA of microbial cells that are captured during sampling is conserved and (iii) an alternative method to pool several low concentration samples is explored to facilitate DNA analysis methods which typically requires higher DNA concentration as a template such as DNA sequencing.

4.2 Experimental design

4.2.1. Determining the optimum temperature for the thermal sonication.

We first conducted an experiment to determine the optimum temperature for water-bath sonication. The DNA extraction followed the original protocol with an additional 30 minutes of water bath sonication and thermal incubation at different

temperatures (Elmasonic, SH250EL, Germany). The thermal sonication step was added after the addition of solution PW1, before the bead beating step. The sample tubes were vortexed shortly (1 min) to mix the PW1 and then put into the water bath sonicator for sonication at 37 kHz in sweep mode. The temperature settings tested include no-treatment (original MOBIO PW protocol), water-bath sonication only (no heating), 55 °C, 60 °C, 65 °C, 70 °C and 75 °C sonication. Total extracted DNA was then directly measured by fluorometry (Qubit, Life Technologies, USA) and specific targets of bacterial (16S) and fungal (18S) marker genes were estimated by qPCR (LC480, Roche Scientific, Switzerland).

We further examined the effect of sonication and heat incubation using the temperature from the previous experiment on two additional sets of environmental samples: ambient air samples (low biomass) and AHU filter samples (high biomass). The DNA extraction of one set of samples followed the original MOBIO PW protocol, while the other used additional high temperature sonication. The final DNA yields were analysed with Qubit and qPCR.

4.2.2. Sampling duration experiment

Two experiments shown in Fig. 4.1 were carried out to assess the effect of sampling duration on extracted DNA results. Fig. 4.1a illustrates the first experiment, which employed a pair of identical sampling trains operated in parallel. With one train, the air was sampled continuously for 24 hours using a single filter. The second train also operated for 24 hours, but the filters were replaced with new ones at eight-hour intervals so that three sequential filters were collected in all. All four filters were then processed. The one 24-hour filter was extracted alone, while the DNA extracted from three 8-hour filters was pooled into a single DNA solution. Qubit measurements and qPCR were then applied to the final DNA solutions. The purpose of this investigation was to assess whether there is any DNA loss associated with long-duration filter-based sampling. If DNA degrades with extended sampling, then the yield recovered from three separate filters would be higher in sum than the single 24-h filter, since these sequential filters are each exposed to a lesser duration of air sampling.

Figure 4.1 Sampling duration experiments for microorganisms collected on filters. (a) – Comparison of 1×24 hour versus 3×8 hour parallel sampling. Two sets of sampling trains collected air at the same time and location for 24 hours. The first set sampled the air continuously for 24 hours on a single filter, while the other set’s filter was replaced every 8 hours. DNA extracted from the three 8-h filters was then pooled and the result is compared with the first set which was extracted alone. **(b) – Testing loss of GFP-tagged bacteria during sampling.** A set of filters sampled air at the same time and location for a total duration of 20 hours. Known quanta of GFP-tagged *S. oneidensis* cells were spiked onto the filters at staggered timepoints, exposing them to different durations of air sampling.

Fig. 4.1b illustrates another experiment, which was performed utilizing a species of Gram-negative bacteria which belongs to the Phylum *Proteobacteria*. Green-fluorescent protein (GFP)-tagged Gram-negative bacteria *Shewanella oneidensis* [62] was chosen for this experiment as this species is unlikely to present in the ambient air. Quanta of these bacteria were spiked onto filter samples at different times during ambient-air bioaerosol sampling, exposing the *Shewanella* cells to airstreams for different sampling durations. Filter 1 was spiked at 0 h (beginning of sampling), filter 2 at 8 h, filter 3 at 14 h, filter 4 at 20 h (i.e., after completion of sampling) and one set of filters was processed without any spike as the blank. Utilizing information from a preliminary experiment, we spiked the filters with an amount of cells that is comparable to the total DNA mass normally

collected during 20 hours air sampling at this particular location, i.e., 10^6 cells counts. After sampling, DNA was extracted using the same protocol described previously. A qPCR analysis was also performed using custom designed GFP-sequence primers and probes.

4.2.3. DNA concentration / pooling approach

A concentration approach is proposed in this study to investigate whether more biomass could be gathered from several low concentration samples without necessitating amplification either by PCR or via culturing. Fig. 4.2 illustrates an approach to concentrate DNA in the middle of DNA extraction process.

Figure 4. 2 Concentration approach during extraction. The DNA from three filters is pooled during the spin-filter binding step of DNA extraction. The total DNA yield is then compared to the one filter extracted alone to investigate whether the expected 3-to-1 ratio is obtained.

To test the concept, four sets of AHU filters and ambient air samples, consisting of four replicates each (32 in total) were prepared and extracted in this manner. For each

set of four filters, the DNA solutions from three filters were introduced onto the same spin filter after the lysis and inhibitor removal step, whereas the remaining one filter was extracted alone. The extracted DNA concentrations were used to verify whether the expected 3-to-1 ratios were obtained for both high and low biomass samples.

4.3 Results

4.3.1. Thermal sonication incubation.

The effects of sonication and thermal incubation on the final DNA yield are reported in terms of total DNA per cm² area of filter or per m³ of air (as determined by Qubit), and as targeted DNA sequence per cm² area of filter or per m³ of air (as determined by qPCR). Fig. 3 shows that the DNA yields of the tested AHU filter samples increases gradually as the incubation temperature rises. The total DNA yield shown in Fig. 4.3a was 1.48 ng/cm² with no treatment, increasing to 4.56 ng/cm² and 5.22 ng/cm² with incubation treatment at 65 °C and 70 °C, respectively. The qPCR result on the same set of samples (Fig. 4.3b) shows a similar trend. The bacterial DNA concentration rose from 51 pg/cm² to 320 pg/cm² with incubation at 65 °C. The bacterial DNA yield then declined to 262 pg/cm² with incubation at 70 °C and 168 pg/cm² with incubation at 75 °C. Similarly, the highest concentration of fungal DNA (1290 pg/cm²) was also obtained from samples incubated at 65 °C. The concentration of fungal DNA remained relatively constant for samples incubated at 70 °C before decreasing to 914 pg/cm² at 75 °C.

Figure 4.3 DNA measurements of AHU filter samples for seven treatment pathways of sonication at varying temperature. (a) - Total DNA yields (Qubit). (b) – Results from bacterial and fungal qPCR. N = 4 for each case. * denotes statistically significant difference to mean of DNA yield extracted with 65 °C incubation.

Based on these results, 65 °C was chosen as the incubation temperature for the remaining investigations in this study. To further evaluate the applicability of sonication and thermal incubation to a broader range of biomass concentrations, these parameters were applied to two sets of environmental samples: a set of AHU filters collected from a different building (high biomass samples) and a set of ambient air filters (low biomass samples).

The total DNA extracted from both sets of environmental samples increased substantially with the addition of sonication at 65 °C, as shown in Fig. 4.4a and 4.4b. For the AHU filter samples, the additional sonication and heat incubation boosts the mean of total DNA yield from 14 to 31 ng/cm², more than a two-fold increase (Fig. 4.4a, left bar). The DNA yield of the ambient air samples increases from 0.03 ng/m³ to 0.44 ng/m³ (Fig. 4.5b, left bar), more than a ten-fold increase.

Figure 4. 4 Improving DNA yield with additional heat and sonication lysis.

Additional sonication and thermal lysis show improved DNA yield for (a) - **AHU filter samples** and (b) – **ambient air samples** as measured by the Qubit fluorometer for total DNA (left bar, left axis) and by qPCR for bacterial (middle bar, right axis) and fungal (right bar, right axis) DNA (N = 4).

As also seen in Fig. 4.4a and 4.4b (middle and right bar), the qPCR results show a similar trend to the Qubit fluorometry data, displaying a substantial jump in DNA concentration after sonication and thermal incubation for both fungi and bacteria. Fungal DNA concentrations increased from 5.5 pg/m^3 to 187 pg/m^3 (34 $\times$) and from 557 pg/cm^2 to 3280 pg/cm^2 (6 $\times$) for ambient air and AHU filter samples, respectively. Bacterial DNA concentrations went from 1750 to 4490 pg/cm^2 (2.6 $\times$) for the AHU filters samples and from 1.9 to 10.3 pg/m^3 (5.5 $\times$) for the ambient air samples. Thus, sonication and thermal incubation was seen to be very effective for enhancing the amount of DNA extracted from both fungi and bacteria from air. Generally, the results show that such an additional processing step might be crucial for analysing low concentration bioaerosol samples.

4.3.2. Effect of sampling duration on DNA yield

Two sets of experiments were conducted to investigate the effect of filter sampling on the extracted DNA yield. The first set (Fig. 1a) compares the DNA yield from one filter sampled continuously for 24 hours with the yield from three separate filters sampled for 8 hours in series, the DNA from which was then combined into one concentrated sample. Similar to previous experiment, the DNA concentration of the samples are presented in terms of ng of DNA per volume of air sampled (ng/m^3 of air) for total DNA measurement by the Qubit and for partial DNA concentration (pg/m^3 of air) by qPCR.

Fig. 4.5. (left bar) shows that — based on DNA fluorometry — there is no distinct difference in total DNA yield between the 1×24 hour samples ($0.14 \text{ ng}/\text{m}^3$ of air) and the 3×8 hour samples ($0.13 \text{ ng}/\text{m}^3$ of air) (Paired t-test, p value = 0.18). This result led us to further analyze the DNA by performing fungal and bacterial qPCR analysis to investigate whether different yield can be seen for different microbiological targets using these two sampling approaches.

Figure 4. 5 Comparison of two sampling approaches. Comparison of a sampling approach utilizing a single filter continuously sampled for 24 h (grey bar) and a combined series of three filters, each operated for 8 h (black bar) expressed in terms of total DNA (left bar, left axis) measured by Qubit and in terms of bacterial (middle bar, right axis) and fungal (right bar, right axis) DNA measured by qPCR (N = 3).

Bacterial and fungal DNA analysis (Fig. 4.5, middle and right bar) indicates that there is no significant difference in fungal DNA concentration with 77 pg/m³ and 80 pg/m³ for the 1×24 and 3×8 hour samples, respectively (Paired t-test, p value = 0.32). However, there is a clear gap between the two sampling techniques for bacterial DNA (Paired t-test, p value = 0.001). The 1×24 hour samples yield 5.4 pg/m³ of air, whereas the 3×8 hour samples yield 8.2 pg/m³, an increase of more than 50%. This result suggests that bacterial DNA may be more sensitive to sampling duration than fungal DNA. If so, then shorter sampling time might be particularly important for preserving bacterial DNA. Fungal DNA appears insensitive to sampling duration on filter media.

The second experiment on the influence of sampling duration was performed by spiking the same amount of GFP-tagged *S. oneidensis* cells on four different filters at different times. These parallel samplings were conducted simultaneously at same location for a period of 20 hours while the DNA spiking was applied at $t = 0, 8, 14,$ and 20 h, as illustrated in Fig. 1b. As the main goal of this experiment was to understand the effect of long sampling duration on cells of a known bacterial species, the result in Table 2 is only presented in terms of total DNA mass estimated (pg) by GFP-specific qPCR.

Table 4.1 indicates that there is a diminishing trend of recoverable DNA with longer sampling duration. Up to 98% of DNA loss is shown upon exposure of the spiked filter to 20 hours of subsequent sampling. For the filters spiked at the end of air sampling, 10.2 ng of GFP gene DNA was recovered, whereas only 0.13 ng (1.3%) of GFP gene DNA was recovered from filters spiked at $t = 0$. The DNA recovered from the filters spiked at 8 h and 14 h were intermediate between these two limiting values: 0.73 ng (7.1%) and 1.13 ng (11%) respectively. This result suggests that much of the biomass collected at the beginning of an extended sampling period could be lost throughout subsequent sampling, especially for species more susceptible to stress from air sampling.

Table 4. 1 qPCR results from a series of 20-h samples illustrating the decay of GFP-tagged *Shewanella oneidensis* cells.

Sample treatment ^a	No. of replicates	Estimated mass of GFP DNA recovered (ng) ^b	Exposure to air sampling (hours)	% of DNA retained (relative to t=0)
No spike	3	0.02 ± 0.001	-	-
Spike at $t = 0$	6	0.13 ± 0.02	20	1.3%
Spike at $t = 8$ h	3	0.73 ± 0.01	12	7.1%
Spike at $t = 14$ h	3	1.13 ± 0.02	6	11%
Spike at $t = 20$ h	6	10.2 ± 0.3	0	100%

^a Time (t) during the 20-h sampling period when a spike of *S. oneidensis* cells was applied to the filter.

^b Mean ± standard deviation reading from qPCR analysis using GFP probe and primers

4.3.3. Mid extraction pooling or concentration approach

The performance of DNA pooling during the extraction step was analysed by means of Qubit measurements and qPCR in terms of total DNA mass extracted (ng or pg). The result compares the DNA yield of one filter and the yield of three replicate filters combined.

The DNA is first extracted to a certain extent and combined into one concentrated sample during the spin filter binding step of the MOBIO PW protocol (Fig. 4.2). Table 4.2 displays the ratios of biomass extracted from one and three filters which range from 1:2.5 to 1:3.9. These ratios are close to the theoretical value for lossless concentration of 1:3.

Table 4. 2 qPCR cycle threshold value data for mid-concentration approach considering both 16S bacterial and 18S fungal probe-and-primer sets.

Ambient Air Samples	Mean Total DNA ^a (ng)	Mean DNA Concentration ^b (ng/m ³)	Mean Bacterial DNA ^a (pg)	Mean DNA Concentration ^b (pg/m ³)	Mean Fungal DNA ^a (pg)	Mean DNA Concentration ^b (pg/m ³)
1 Filter	3.1 ± 0.9 (29%)	0.3	167 ± 37 (22%)	17.4	1481 ± 142 (10%)	154
3 Filters	11.9 ± 1.9 (16%)	0.4	414 ± 114 (28%)	14.4	3780 ± 440 (12%)	131
Ratio	1 : 3.9		1 : 2.5		1 : 2.6	
Concentration Deviation (%)	28%		21%		18%	
AHU Filter Samples	Mean Total DNA (ng)	Mean DNA Concentration (ng/cm ²)	Mean Bacterial DNA (pg)	Mean DNA Concentration (pg/cm ²)	Mean Fungal DNA (pg)	Mean DNA Concentration (pg/cm ²)
1 Filter	44 ± 6 (14%)	4.4	570 ± 46 (8%)	57	2970 ± 410 (14%)	297
3 Filters	148 ± 12 (8%)	4.9	1798 ± 124 (7%)	60	10100 ± 1420 (14%)	338
Ratio	1 : 3.4		1 : 3.2		1 : 3.4	
Concentration Deviation (%)	12%		5%		14%	

^a Mean ± standard deviation for $N = 4$ samples in each case

^b Concentrations calculated from total DNA extracted divided by total amount of air sampled through one and three filters (for ambient air samples) or total area of AHU filter extracted (for AHU filter samples).

The calculated concentrations per unit volume of air sampled (ng/m^3) and per unit area of AHU filters (ng/cm^2) in Table 4.2 are expected to be the same (between one and three filters) as these comparisons were made from replicates of the same samples. The difference of concentrations calculated from one and three filters are relatively small with a margin of error of $\leq 28\%$ (using the results from one filter as the reference). The high concentration AHU filter samples display smaller deviations, ranging from 5 to 14%. Conversely, the low concentration ambient air samples have higher deviation ranging from 17 to 28%.

4.4 *Discussions*

4.4.1. *Additional Cell Lysis: Thermal sonication incubation.*

The extraction step plays a big role in the effectiveness of DNA-based analysis of environmental samples. As DNA-related technology grows, a variety of extraction kits are continuously improving the efficiency in both DNA yield and extraction time. The MOBIO Power Water (PW) kit was selected in this study mainly due to its frequent use in previous bioaerosol studies [18,49], as well as compatibility with the size of our filter samples. The PW kit uses 5 ml sample tube which allows filter samples to be directly placed inside the tubes (47 mm PES and the 5 cm^2 cut AHU filters) without having to scrape, grind, vacuum or dissolve the filters before proceeding with extraction steps. This feature minimizes extraction time and biomass loss. Choosing the appropriate kits design and size is important. Other bioaerosol studies have chosen other extraction kits such as the MOBIO Power Max Soil kit for processing larger sample volumes in 50 ml tubes [75] or MOBIO Power Soil for more rigorous inhibitor removal when smaller starting tubes (2 ml) can be used [76].

DNA extraction consists of three main steps: 1) cell lysis to expose the intracellular material; 2) isolation of DNA from contaminants; and 3) final elution. For environmental samples, the challenge in DNA extraction often comes from the lysis and isolation steps. Environmental samples start with a mixture of species with different characteristics, which may require different approaches to effectively lyse all the cells. Moreover, if inhibitors such as humic acid from floor dust or

particulate matter suspended in the air remains in the final DNA solution, the integrity of subsequent analysis steps, such as qPCR, can be compromised.

As a quality control measure, a dilution series was performed on a separate set of DNA samples extracted from AHU filter and PES ambient air filter to verify potential inhibitor effects. The results suggested no lowered inhibition from the diluted samples. Furthermore, most of our qPCR results correspond well with the Qubit measurements and all of our final DNA samples are clear in colour. Given the fact that some of the samples had high dust contents (e.g. the extracts from AHU filters), it seems the MOBIO PW kit was able to effectively handle the inhibitors in all environmental samples collected in this study.

Final DNA yield of an extraction is often related to its lysis. The complex matrix of bioaerosol samples has forced several studies to apply some improvements to their lysis protocols, such as sonication [18], thermal incubation [49], and chemical addition [22,24]. Three commonly used cell lysis methods are physical disruption, chemical and enzymatic lysis. This study focused on the impact of a combination of sonication and thermal incubation (physical disruption) because this approach, combined with the physical bead beating in the MOBIO protocol, is known to be effective in lysing cells with tough cell wall/membrane (spores, Gram-positive bacteria) and small bacteria cells[77]. In addition, it is relatively more flexible to be applied to almost all DNA extraction protocols. The impact of chemical and enzymatic lysis on various forms of bioaerosol samples, however, also warrants future investigations.

Choosing the optimum temperature for high temperature sonication can be challenging when applied to environmental samples with unknown quantity and composition of biomass. Ideally, one wants the temperature to be high enough to help lyse the cells, but also not too high to avoid DNA denaturation or lysis buffer compatibility issues. Some studies have chosen to incubate their samples at 60 °C[78] or 65 °C [18,49] without elaborating the underlying reasons for the choice. The results from Fig. 4.3a and Fig. 4.3b agree that the highest DNA yields came from filter samples incubated at either 65 °C or 70 °C. It is likely that DNA was damaged or denatured, causing the concentration to decrease when we applied too much energy at 75 °C. Incubation at 65 °C over 70 °C was chosen for the rest of

the study as it preserves more bacterial DNA (Fig. 4.3b) and displays more consistency among the replicates (Fig. 4.3a and Fig. 4.3b).

In order to verify the validity of this temperature choice, ANOVA single factor followed by Tukey honest significant difference (HSD) analysis for pairwise comparisons was performed comparing results from samples incubated at 65 °C to the other temperatures (65 °C to 55 °C, 60 °C, 70 °C, and 75 °C) for total, fungal and bacterial DNA. The means of total DNA for all 5 temperatures showed no significant difference from that of 65 °C ($p\text{-value} > 0.05$). However, the analysis results of fungal and bacterial DNA provided further insights. For fungal DNA, only comparisons of 65 °C to 60 °C and 65 °C to 70 °C have $p\text{-values} > 0.05$. As for bacterial DNA, only comparison between 65 °C and 70 °C results in $p\text{-value} > 0.05$. The results may explain why different studies mentioned above incubated their samples at different temperatures to obtain the required DNA yield. It is likely that in the complex matrix of an environmental sample, different target biomass would behave differently under varying temperatures. Therefore, depending on the purpose of a study, various incubation temperatures may be considered. For example, when only rough estimation of total DNA via Qubit is required, any temperature from 55 °C to 75 °C may all produce comparable results. If the focus is on fungal DNA, temperatures from 60 °C to 70 °C may be chosen. Lastly, when bacterial DNA is targeted, temperatures at 65 °C or 70 °C could be the options as they have equally high yields.

In the present study, our focus is to preserve as much DNA as possible for further analysis of all three DNA types. The statistical analysis above shows that there are only two temperatures (65 °C and 70 °C) that are within the range of temperatures that produces the highest yield for all DNA types (total, bacterial and fungal). Between these two temperatures, the lower one (65 °C) is chosen as the final incubation temperature because in addition to the higher consistency and better preservation of bacterial DNA, lower temperature is also preferred to save energy and further reduce the chance of DNA denaturation during processing.

During sonication, the additional force provided by heating [79] and cavitation [80] help disrupt the cell walls and subsequently release more intracellular matter, including DNA. The impact of sonication alone on DNA yield can be seen from the

difference between no treatment and sonication only bars in Fig. 3a and 3b. The total DNA yield goes up by 23% (Fig. 3a) with additional sonication alone while bacterial and fungal DNA yield went up by 43% and 28%, respectively (Fig. 3b). The effect of heat incubation is seen to dominate with up to 600% increase in DNA yield as the temperature is increased. The influences of the two approaches appear to be interrelated, with sonication playing a supporting role. It is also worthwhile to note that sonication itself dissipates heat in the water. We've observed that when the instrument is operated in sonication-only mode, water temperature rises to 35-40 °C within the first 5 minutes and then stays in that range for the remainder of the 30-min process.

We would like to also highlight that there are indeed limitations associated with studies utilizing environmental samples. It can be observed from the results that the impact of one improvement approach (i.e. thermal sonication) varies on different target biomass. Compared to the typical controlled experiments, the complex environmental matrix makes it very difficult to estimate the absolute extraction efficiency from environmental samples. The absolute extraction efficiency can only be calculated when artificial samples with a known starting amount (spiking a clean/PM-loaded filter with a known amount of cells or using nebulizer to mimic bioaerosol sampling) are used. These efficiencies, however, are more relevant for studies targeting a specific species or a small group of species as they only correspond to the chosen cells that are spiked, which in most cases, does not represent actual environmental conditions. We believe it is beneficial to also report the improvements of an optimization approach (physical disruptions, enzyme, etc.) tested on environmental samples to complement the more readily available studies with known samples in the literature.

We further tested the improved protocol with 65 °C sonication on two sets of environmental samples with significantly different biomass concentrations. The results displayed in Fig. 4.4a and 4.4b show that the total DNA yield from both ambient air and AHU filter samples increased significantly with the addition of a 65 °C sonication step. Note that two of four replicates for total DNA from ambient air samples without sonication (Fig. 4.4b) were below the detection limit of the Qubit. This result highlights the importance of sonication and heat incubation in

DNA extraction because it permits the direct use of DNA fluorometry to quantify total DNA mass for samples that would otherwise be undetectable. DNA fluorometry provides total DNA quantification in a relatively short time, which is an attractive feature in indoor environmental assessment. Total DNA measurement is generally not available in PCR-based techniques as their analysis is limited to the chosen primers. While DNA fluorometry (i.e. Qubit) is considered more accurate than light-absorbance-based methods, such as nanodrop [81], there are only limited reports of direct quantification of DNA in air samples using this method. This is likely due to its relatively higher detection limit (typically 0.0005 ng DNA/ μ L) compared to PCR-based instruments (capable of detecting DNA concentration up to 10 times lower than that of Qubit with enough duplication cycles).

As shown in Fig. 4.4a and 4.4b, large deficits of biomass for both bacterial and fungal DNA in both ambient air and AHU filter samples suggests that the original MOBIO protocol (with bead beating only) does not effectively lyse all the cells in environmental samples such as these. One can speculate that the higher DNA yield from additional lysis originates from cells with thicker walls, such as fungal spores or Gram-positive bacteria, which might not be effectively disrupted by means of only bead beating.

The results of this study indicate that it would be worthwhile to be attentive to lysis as a critical processing step for environmental bioaerosol sample analysis. The total DNA measurements of ambient air samples extracted with the unimproved protocol shows that one may miss detection with inadequate lysis. The influence of an improvement approach can also vary among different types of environmental samples (in this case, ambient air filters and AHU filters). More attention towards method development would be beneficial for future bioaerosol studies, including systematic cross-comparison of improvement techniques under a range of environmental sampling conditions.

4.4.2. Effect of sampling duration on DNA yield

To better understand how the sampling duration affects the DNA yield from the filter sample, we have utilized an open (no size-cut) filter-based sampling protocol for its relatively higher collection efficiency (as compared to liquid impinger)

[37,82], flexibility (in choosing sample flowrate and duration)[25] and cost. The naturally low bioaerosol concentrations in ambient air often lead to issues of inadequate measurement sensitivity. Therefore, to collect enough biomass, long sampling durations are sometimes inevitable for non-viable based analysis. Many studies have utilized relatively long sampling times such as eight hours [49], 20+ hours [60], or even several days for collection on individual filters [23,48].

Concerns about time-dependent decreases of the viability of captured cells during air sampling have been raised over the years. Depending on the sampling medium, microorganisms may partially or completely lose their viability owing to stress from different parameters such as shear-stress induced by air flow, osmotic pressure or lack of moisture [23,33]. Of course, DNA-based analyses do not rely on viability as the DNA can be extracted and analysed regardless of whether the captured microorganisms are viable or culturable. Nevertheless, the general concern remains: do extended sampling periods lead to degradation of previously collected DNA? The purpose of our investigation on this point was to further assess whether long sampling duration has a significant adverse impact on DNA analysis yield.

The total DNA measurement in Fig. 4.5 (left bar) suggests that culture-independent DNA based analysis is not impeded by long sampling duration on a filter media. The qPCR result (Fig. 4.5, middle and right bar), however, reveals that although there is no notable difference on the fungal DNA, 50% more bacterial DNA was preserved in the DNA solution extracted from the combined 3×8-h filters. As the amount of bacterial DNA is only a small portion of total DNA, which also includes fungal and other non-microbial DNA, the 50% reduction in bacterial DNA does not generate a notable change in total DNA. However, this result does suggest that some more vulnerable species, such as Gram-negative bacteria, may be prone to DNA loss from long duration sampling with filters.

The second experiment, illustrated in Fig. 4.1b, was conducted to confirm the above hypothesis. There are two major reasons for selecting *Shewanella oneidensis* [62] for this experiment. First, this species is embodied as Gram-negative bacteria, which are relatively less-protected from air sampling due to their thinner cell membrane. We suspect that if DNA degradation were to happen during air sampling, then less protected cells would be more vulnerable. The second reason is

because this GFP-tagged species is rarely found in ambient air. The ability to control the timing of the presence of this species on air filters through the spiking process permits us to isolate the impact of air sampling on DNA recoverability.

The qPCR result reported in Table 4.1 indicates a progressive degradation of DNA recoverability for this specific species in relation to sampling duration. This occurrence leads us to believe that without properly testing the compatibility of our target species and our chosen sampling protocol, the DNA of the cells captured at the beginning of sampling may be falsely undetected in the subsequent analysis steps. The degree of DNA recoverability loss is likely to be species dependent, as the previous results (Fig. 4.5, middle and right bar) show that fungal DNA is less sensitive to air sampling on filter media than bacterial DNA.

The two experiments indicate that the duration of filter-based air sampling might have some effects on the recoverability of DNA. Depending on the chosen sampling medium and the characteristics of the targeted species, it would be beneficial to have a preliminary investigation to assess how durable is the biomass DNA during sampling. For instance, in the case where certain Gram-negative bacteria or other more vulnerable species are targeted, shorter sampling duration would be recommended to preserve more DNA. Conversely, in the case when tougher species like fungal spores or Gram-positive bacteria are targeted, longer-term sampling can be utilized as the DNA collected from these species is less likely to degrade from sampling stress. In addition to sampling duration, storage duration may also play a part in DNA preservation. It is recommended to extract the DNA as early as possible after sampling since some DNA on the filter may continue to degrade during storage [77].

4.4.3. Mid extraction pooling or concentration approach

Various efforts to concentrate bioaerosol samples have been reported in previous studies to circumvent detection limit issues. For example, Boreson et al. [12] concentrated 5 mL of liquid from a Biosampler by filtering the collected liquid media onto a section of filter membrane before proceeding with extraction. As commercial DNA extraction kits continue to improve, it is now possible to combine DNA recovered from separate samples in the middle of extraction steps, which

saves time and, in some cases, reagents. In the case of MOBIO PW kit, the DNA can be pooled by means of introducing extraction liquid (after PW3 addition) from different samples on to the same spin filter.

One concern that arises for this concentration approach is the DNA binding capacity of the spin filter itself. According to MOBIO, the PW kit spin filter can bind up to 20000 ng of DNA, which is ample for the level of biomass processed in this study (up to 300-400 ng of DNA from 30 cm² of AHU filters following 3 months of in-use service).

The ratios resulted in Table 4.2 are all relatively close to 3:1, confirming the suitability of this pooling approach to the range of biomass concentration engaged in this study. As discussed previously, it is sometimes necessary to combine several parallel filters into one concentrated sample to overcome instrument detection limits owing to the low airborne DNA concentration. The approach of combining DNA from three samples onto one spin filter has a margin of error below 30% for the experiments reported here. We consider this outcome to be satisfactory because these deviations are not only caused by the pooling method, but also would result from natural variability of the replicate environmental samples.

In addition to the reliability, the suggested DNA pooling method is generally applicable to all extraction kits with similar spin filters. It is not limited to bioaerosol samples. Different concentration approaches generally help gather enough biomass with shorter sampling duration, which could be beneficial to DNA recoverability. Furthermore, this concentration approach allows more flexibility in designing a suitable sampling plan, because other concerns such as noise, sampler durability or site availability often arise when intense sampling activity is needed.

4.5 Chapter conclusion

Collecting enough biomass during for desired analysis methods a short time period is a well-known challenge for the typically low concentrations of bioaerosol levels found in indoor and outdoor air. This study has shown that one can improve DNA recoverability and yield in bioaerosol samples by fine-tuning several parameters such as applying more rigorous cell lysis during DNA extraction or pooling composite samples to obtain enough biomass.

In the cases reported here, outdoor air and building HVAC filter samples were analyzed using DNA-based measurement methods. Adding enhanced lysis by means of high temperature water bath sonication was proven to be effective with up to 600% increase in DNA yield at the optimum temperature of 65 °C. We found that for some more vulnerable species, up to 98% of the captured species' DNA could degrade over the course of 20 hours of filter sampling. Combining DNA from separate samples during the spin filter binding step of the DNA extraction protocol showed promising results with a margin of error below 30%.

We examined the impact of the aforementioned parameters using a commercially available DNA extraction kit, which generally demonstrates good capacity for urban environmental samples. The chosen kit seems to be able to effectively deal with inhibitors of both high biomass (AHU filters used for three months) and low biomass (PES filters applied to sample ambient air for 8-20 h) samples. Incorporating the proposed modifications saved both time and reagents in addition to achieving improvements in DNA yield.

Dealing with highly variable bioaerosol environmental samples, researchers are often faced with uncertainty on which approaches may best improve their analytical methods. Therefore, we believe that it is beneficial to have more method development-based bioaerosol studies in the future that assess how efficient different improvement techniques (e.g., enhanced chemical or enzyme lysis) are in dealing with other forms of environmental samples (liquid impinger samples, dust-wipe samples). More such efforts will help reach the goal of more standardized analysis methods for studies of aerosol microbiology.

CHAPTER 5

CROSS SECTIONAL HVAC FILTER STUDY: INVESTIGATING BIOACCUMULATION ON HVAC FILTERS IN SINGAPORE UNIVERSITY BUILDINGS

1.1 Objectives

Here, we present baseline DNA quantitative results and interpretive analysis of biological materials accumulated on used ventilation system filters from several normally functioning indoor environments, such as offices, classroom and library in a university setting. High-throughput sequencing targeting bacterial 16S rDNA and fungal internal transcribed spacer (ITS) sequences were also carried out to analyse the microbial composition on the ventilation system filters. We also aspire to provide information regarding how indoor conditions affect bioaccumulation (diversity and quantity) on these filters. To satisfy these goals, we assessed the biomass concentration recovered from normally used ventilation-system filters and attempted to correlate them with occupancy level from a set of well-characterized indoor environments. The specifications of each air handling unit hosting these filters (air flow rate, total filter area, operating hours) are also documented. This information allows us to estimate and evaluate the approach of utilizing HVAC filter sampling as a probe of indoor air quality.

1.2 Experimental design

5.2.1. The indoor environments

Ventilation system filters from nine air handling units (AHU) at Nanyang Technological University, Singapore, were acquired for this study. The nine indoor environments were three libraries (Library 1, Library 2 and Library 3), five university staff office environments (Office C, Office 1, Office 2, Office 3 and Office 4) and one classroom (Classroom). All filters were indoor secondary filters (pleated polyester, MERV 8).

Figure 5. 1 From left to right: HVAC filter collected from the AHU room, the office environment, the classroom environment and the library environment.

As mentioned previously, due to high latent heat load in Singapore, central air conditioning systems generally operate with a very high proportion of recirculated air and a correspondingly small proportion of outside air. For the buildings studied here, the sampled air-handling filters processed air that was a blend of >90% recirculated air and <10% outdoor air. In accordance with Singapore regulations, filters in mechanical ventilation systems are replaced within six months of installation. The filters in this study are routinely replaced every three months. All filters analyzed in this study were collected after the customary full three-month period of service.

Several recent studies have documented that human occupants can act as major sources of indoor bioaerosols, through shedding and resuspension [22,48,49,60,83]. To assess the influence of occupancy on the accumulation of biological material on air-handling system filters, we conducted population counts in each of the nine indoor environments studied. The total occupancy levels were estimated by means of an hourly count at each site via direct observation. These counts were undertaken each hour that the space was normally open for three weeks each, with the sampled weeks selected during the three months of filter operation.

The operating hours of the AHUs followed the opening hours of the indoor spaces. The offices followed a fixed schedule with Office C and Office 1 operated on average 60 h/wk, while the students and staff offices (Offices 2, 3 and 4) were open with an average of 91 h/wk. The classroom's AHU operated on average 26 h/wk. The three libraries operated on different schedules with average durations of 73 h/wk, 56 h/wk and 73 h/wk for Library 1, Library 2 and Library 3 respectively. The total numbers of occupants observed each day were tallied and the weekly average value is reported as an integral measure in units of person-hours per week,

N. Note that each observation of one occupant was estimated to contribute one person-hour to the cumulative weekly occupancy for that space.

In addition to occupancy, indoor environments were also characterized based on several major features, such as a description of general usage, activities observed during occupancy counts, interior furnishings, and size. We also measured total filter area (A_f , m^2) and the respective airflow speed (m/s) to estimate the volumetric supply air flow rate (Q , m^3/s). Detailed descriptions of the indoor environments and the population counts are available in the Supporting Information (Appendix B).

5.2.2. *Sampling, DNA extraction and analysis*

As described in chapter 3, Samples were obtained by cutting small pieces ($\sim 2 \times 5$ cm) from the used filter on site with sterilized scissors immediately before the filter was removed from its mount for replacement. The sampled filter pieces were transferred into a sterile 50 mL falcon tube and transported to the lab on ice for DNA extraction. At least seven filter samples were collected from each location to provide material for backup and replication. All unprocessed filters were stored at -20°C .

Each of the 10-cm^2 filter pieces was placed into the 5 mL bead beating tube of MOBIO PW for DNA extraction. The DNA extraction steps followed the original protocol with some modifications to improve DNA yield based on our optimization in chapter 4 [84]. Briefly, the modifications included a two-minute vortex after the addition of preheated solution PW1 (the lysing agent), followed by water-bath sonication at 65°C for 30 minutes. After sonication, the samples were vortexed again for 5 minutes as recommended. The rest of the extraction steps followed the protocol described in chapter 3. Extraction replicates were separate 10-cm^2 filter pieces that were cut from the same filter panel. As a negative control, clean (unused) AHU filters were briefly installed in each AHU and immediately extracted using the same protocol.

After extraction, the total DNA in each sample was measured by Qubit. The same Qubit high sensitivity (HS) dsDNA kit was used for processing all samples. Bacterial and fungal DNA levels were also quantified by means of qPCR, complemented with a standard curve to convert the C_t values to DNA

concentration. The qPCR was run using the same protocol and universal primers and probe listed in Table 3.1. DNA sequencing run on Illumina Miseq and its data analysis also follow the protocol in chapter 3.

The DNA concentrations from blank samples were all below the detection limits of both Qubit and qPCR with 35 thermal cycles, suggesting that there was no significant contribution of biomass from the maintenance company personnel during filter replacement, from the researchers during sampling and analysis of the filter pieces, or from the AHU filter materials themselves.

5.2.3. *Airborne DNA concentration and emission factor estimation*

Estimating the total DNA accumulation rate and its apportionment (total, bacterial and fungal) on the ventilation system filter (Df , in units of mass per week) was achieved by upscaling the amount of DNA extracted from the small filter area (Ae , m²) to the actual filter area (including pleats) in the AHUs (Af , m²). The DNA concentrations measured by Qubit fluorometry (ng/μL) and qPCR (pg/μL) were first converted to extracted DNA mass (De , mass units) based on the elution volume used during extractions. Total rates of accumulated DNA masses were then obtained from the following equation:

$$Df = \frac{De \times \frac{Af}{Ae}}{12} \quad (1)$$

The appearance of the factor 12 in the denominator of equation (1) converts total DNA mass accumulated to the average weekly mass accumulation rate for the 12-week filter deployment periods. This conversion was applied to facilitate comparison of Df to the occupancy counts, which were also expressed on a rate basis, in units of person-hours per week.

The DNA accumulation rate on the ventilation system filter (Df) was normalized by the average quantity of air estimated to have passed through the filter during each week (Vs , m³/wk). The volumetric air-sampling rate was estimated by multiplying the determined volumetric air-flow rate (Q , m³/s) by its weekly cumulative weekly operating duration (To , s/wk) for each environment. Upon normalization, the resulting ratio (equations 2 and 3) represents the time-average

airborne DNA concentration transferred to the filter and accumulating throughout the deployment period.

$$V_s = Q \times T_o \quad (2)$$

$$C = \frac{Df}{V_s} \quad (3)$$

In addition to determining effective time-averaged DNA concentrations, C , we also explored the occupant-induced contribution to DNA accumulation on the ventilation system filters. The premises on which this analysis is based are two: (a) that occupants are important contributors to the airborne DNA accumulating on ventilation system filters that process recirculated air; and (b) for a given category of indoor environment, the total effective occupancy-associated emission rate of DNA should scale with the occupancy level.

The exploration of the occupancy-induced contribution to DNA accumulation on the filter was achieved by regressing the inferred concentration, C , against a measure of ventilation rate normalized occupancy, Nd (person-h per m³). Equation (4) was applied to determine the normalized occupancy as the ratio of the person-h per week of occupancy (N) to the airflow volume per week flowing through the AHU filter (V_s).

$$Nd = \frac{N}{V_s} \quad (4)$$

For each category of indoor environment, we regressed the inferred DNA concentration, C , against the normalized occupancy, Nd . The slope, E (DNA mass per person-h of occupancy) represents an estimate of the occupancy-associated emission factor for DNA. We applied this analysis for total DNA as well as for bacterial and fungal DNA.

Spearman rank correlation was applied to determine the statistical significance of the association between C and Nd . In the Spearman's rank correlation analysis, both Spearman's rank coefficient (ρ) and its significance were calculated.

For additional insight, we converted DNA mass values for C and E to number of cells for bacterial DNA and to number of spores equivalent (SE) for fungal DNA. For bacterial DNA, the conversion took into account that *E. coli* (our standard) has seven 16S RNA operon copies for each genome and that the average number of 16S RNA gene operon copies in a general bacterial genome is estimated to be four [85]. For

fungal DNA, the conversion was done assuming that *A. fumigatus* has fifty-five operon copies of 18S rRNA gene per genome [86]. As the number of operon copies of rRNA is known to be highly variable in fungal genomes (even within the same species), it is difficult to determine one average number for general fungus. Therefore, we converted our fungal DNA to *A. fumigatus* spores equivalent (SE). On average, 0.0027 pg and 0.030 pg [24] of DNA is equivalent to one bacterial cell and one fungal SE, respectively, for these assumptions and approximations.

1.3 Results

5.3.1. DNA mass accumulated on HVAC filter

Table 5.1 lists the concentration of extracted DNA mass per filter surface area for each of the AHU filters. The quantities of DNA per filter area spanned these respective ranges: 1.1 to 41 ng per cm² for total DNA, 0.02 to 3.3 ng per cm² for bacterial DNA, and 0.2 to 2.0 ng DNA per cm² for fungal DNA. On average, the microbial DNA contributed approximately 17% (1.5 out of 8.9 ng/cm²) of total DNA mass accumulated on AHU filters. In the classroom and offices, the fungal contribution was consistently and substantially larger than the bacterial contribution. In the libraries, the average contribution from bacterial DNA represented a larger proportion of the total and was also modestly higher than the average contribution from fungal DNA.

Table 5. 1 DNA concentration per AHU filter surface area for total, bacterial and fungal DNA from the nine indoor environments.

Indoor environment	Occupancy (person-h/wk)	Total DNA/filter area ^a (ng/cm ²)	Bacterial DNA/filter area ^a (pg/cm ²)	Estimated bacterial cell density ^b (10 ³ cells/cm ²)	Fungal DNA/filter area ^a (pg/cm ²)	Estimated fungal density ^b (10 ³ SE/cm ²)
Office C	190	1.1 ± 0.2	23 ± 2	8.5	422 ± 15	14.1
Office 1	979	3.3 ± 0.3	79 ± 6	29.3	1200 ± 90	40.0
Office 2	1518	5.9 ± 0.8	80 ± 14	29.6	951 ± 99	31.7
Office 3	1005	11.0 ± 0.5	171 ± 10	63.3	1764 ± 99	58.8
Office 4	1938	5.9 ± 0.9	165 ± 55	61.1	777 ± 85	25.9
Classroom	2736	1.5 ± 0.5	68 ± 14	25.2	193 ± 36	6.4
Library 1	946	4.5 ± 1.1	150 ± 20	55.6	857 ± 137	28.6
Library 2	639	5.8 ± 0.5	945 ± 33	350	797 ± 31	26.6

Library 3	7232	41.3 ± 3.1	3250 ± 280	1200	1988 ± 228	66.3
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^aAverage values of total, bacterial and fungal DNA abundance per AHU filter

surface area ± standard deviation from the extraction replicates.

^b Estimated cell (bacteria) or spore equivalent (SE, fungus) abundance per filter surface area from each location; only the average value is displayed.

The estimated bacterial cell and fungal spore equivalent (SE) densities in Table 1 present analogous results to the DNA concentration per filter area. Across the nine sampled sites, estimated bacterial cell concentrations on AHU filters ranged from 8.5 thousand to 1200 thousand cells/cm²; for fungi, the corresponding range was 6.4 thousand to 66 thousand SE/cm².

5.3.2. Microbial diversity on HVAC filter

In total, 385 bacterial and 783 fungal genera were detected from five DNA samples submitted for sequencing, i.e., Office C, Office 3, Office 4, Classroom and Library 3. Figure 5.2 displays the compositions of bacterial and fungal phyla. The three most abundant bacterial phyla were *Firmicutes*, *Proteobacteria* and *Actinobacteria* with average relative abundances of 30%, 24% and 13% respectively. *Firmicutes* was detected more abundantly in the three offices, with relative abundances of 36% for the Office C, 74% for the Office 3, and 21% for the Office 4, than in the other locations, with relative abundances of 8% for the Classroom and 9.1% for the Library 3. In contrast, *Actinobacteria* was more abundant in the Classroom (22%) and the Library 3 (21%) than in the offices, i.e., 9.6% for the Office C, 5% for the Office 3, and 9.5% for the Office 4. *Proteobacteria* was abundant in the locations with high occupancy levels, i.e., 22% for the Classroom, 36% for the Office 4 and 45% for the Library 3. *Basidiomycota* was a dominant fungal phylum in the office environments, with relative abundances of 52% for the Office C, 63% for the Office 3, and 58% for the Office 4. *Ascomycota* was dominated in the Classroom and Library 3 with respective relative abundances of 70% and 85%.

Figure 5. 2 Proportional distribution of bacterial (A) and fungal (B) phyla extracted from AHU filter samples from five locations.

Figure 5.3 illustrates the relative abundances of the 40 most abundant bacterial and fungal genera detected from HVAC filters analyzed in this study. The five most abundant bacterial genera and their mean relative abundances were *Clostridium XI* (11%), *Streptophyta* (10%), *Bacillus* (6.7%), *Acinetobacter* (5.0%) and *Ktedonobacter* (3.6%). We focused our analyses on the abundances of a group of genera associated with indoor conditions such as ventilation system and human occupancy levels. *Deinococcus*, *Achromobacter* and *Roseomonas* are bacterial genera known to be found in mechanically ventilated indoor spaces [87]. These bacteria were found in this study, with a mean relative abundance of 4.1% for the three genera combined across all locations. Meanwhile, *Methylobacterium*, *Sphingomonas* and *Streptococcus* are known to be found in window-ventilated

rooms [87]. These bacteria were less abundant than the bacteria associated with mechanical ventilations, with a mean relative abundance of 1.3% for the three genera combined across all locations.

Figure 5.3 Relative abundances of the 40 most abundant bacterial (A) and fungal (B) genera. Taxa displayed represent 67% of all sequences for bacteria and 71% of all sequences for fungi.

We selected human-associated genera (gut and skin microflora) and genera known to be frequently detected in highly occupied spaces to evaluate how their

communities on HVAC filters change in accordance with the change in levels of human occupancy. Table 5.2 lists the Spearman's rank coefficients (ρ) correlating occupant densities to relative abundances of selected groups of bacterial and fungal genera and the α diversity index in the five locations. The group of human-related bacterial genera consists of *Corynebacterium*, *Propionibacterium*, *Enterobacter*, *Staphylococcus*, *Streptococcus*, *Fusobacterium* and *Veillonella* as skin or gut microflora [48,60]. A positive correlation ($\rho=0.5$), though not significant, was found between relative abundances of the group of human-related bacterial genera and human occupancy levels. Additionally, we analyze a correlation between human occupancy levels and relative abundances of the group of bacterial genera known to be frequently detected in highly occupied spaces [87]. These genera are *Lactococcus*, *Pseudomonas* and *Streptococcus* [87]. We found a strong positive correlation between relative abundances of this bacterial group and human occupancy levels ($\rho=1.00$, $p<0.05$).

Table 5. 2 Spearman's rank correlation coefficients between occupant intensity in five locations and the relative abundances of the selected groups of genera.

Groups of genera	Bacterial DNA (Relative abundance vs occupant intensity)
	Spearman's rank coefficient
Human related genera	0.50
Genera found in more occupied spaces	1.00*
Shannon diversity index	0.60
Groups of genera	Fungal DNA (Relative abundance vs occupant intensity)
	Spearman's rank coefficient
Human related genera	0.90
Shannon diversity index	-0.10

*Denotes statistical significance at $p < 0.05$ with $n = 5$.

^a Positive (negative) correlation coefficient denotes a positive (negative) correlation between the relative abundances of genera and occupancy.

The five most abundant fungal genera and their mean relative abundances were *Aspergillus* (11%), *Cladosporium* (7.4%), *Nigrospora* (4.9%), *Rigidoporus* (3.7%) and *Lentinus* (2.9%). A positive correlation ($\rho=0.90$), though not significant, was found between human occupancy levels and relative abundances of a group of human-related fungal genera of *Malassezia*, *Trichosporon*, *Rhodotorula*, *Cryptococcus*, *Pichia* and *Candida* [59]. The group of fungal genera commonly found in the environment, i.e., *Aspergillus*, *Penicillium* and *Cladosporium* [88-90] was more abundant, with the mean relative abundance of 20% for the three genera combined, than the group of the human-related fungal genera with the mean relative abundance of 0.4% for the six genera combined.

A positive correlation, though not significant, was found between human occupancy levels and the Shannon's diversity index of bacterial communities ($\rho=0.60$), but not of fungal communities ($\rho=-0.10$).

5.3.3. Estimated airborne DNA concentration and correlation to normalized occupancy level

Table 5.3 presents the conditional airborne DNA concentrations (C) based on the analysis of ventilation-system filters. For total DNA, airborne concentrations in

the nine locations varied between 2.6 pg/m³ and 107 pg/m³. There was a tendency for higher DNA concentrations to be found in the libraries, with these sites exhibiting three of the highest four individual levels, the highest overall level, and an average (48 pg/m³) that was more than 4× the corresponding mean for offices and the classroom (11 pg/m³). Average airborne bacterial DNA concentrations were in the range 0.05–8.42 pg/m³ with an overall average across all indoor environments of 1.6 pg/m³. For fungal DNA, the concentrations span a narrower range, 1.0–5.1 pg/m³ and exhibit an average, 2.5 pg/m³, which is comparable in magnitude yet somewhat larger than bacterial DNA. The bacterial and fungal DNA concentrations were also converted to bacterial cells and fungal spore equivalents to provide additional perspective. The time-averaged conditional airborne bacteria and fungi concentrations ranged from 20 to 3100 cells/m³ (average = 580) and from 33 to 173 SE/m³ (average = 83), respectively.

Table 5. 3 Conditional airborne concentrations for total, bacterial and fungal DNA extracted from AHU filters serving nine indoor environments.

Indoor environment	Occupant intensity (person-h/m ³)	Total DNA concentration ^a (pg/m ³)	Bacterial DNA concentration ^a (pg/m ³)	Estimated bacterial cell concentration (cells/m ³)	Fungal DNA concentration ^a (pg/m ³)	Estimated fungal spore concentration (SE/m ³)
Office C	0.10×10^{-3}	2.6 ± 0.4	0.05 ± 0.01	20	1.0 ± 0.1	33
Office 1	0.58×10^{-3}	6.3 ± 0.6	0.15 ± 0.01	56	2.3 ± 0.2	77
Office 2	0.62×10^{-3}	7.2 ± 1.0	0.10 ± 0.02	40	1.2 ± 0.1	40
Office 3	0.79×10^{-3}	12.9 ± 0.6	0.20 ± 0.01	75	2.1 ± 0.1	70
Office 4	2.80×10^{-3}	28.5 ± 4.2	0.80 ± 0.30	300	3.7 ± 0.4	123
Classroom	1.40×10^{-3}	8.5 ± 3.1	0.40 ± 0.10	150	1.1 ± 0.2	36
Office + Classroom Avg ^c		11.0 ± 7.8	0.28 ± 0.26	110	1.9 ± 0.9	70
Library 1	0.50×10^{-3}	14.5 ± 3.5	0.50 ± 0.10	190	2.8 ± 0.4	93
Library 2	1.20×10^{-3}	21.6 ± 1.9	3.50 ± 0.10	1300	3.0 ± 0.1	100
Library 3	3.50×10^{-3}	106.9 ± 8.1	8.42 ± 0.71	3100	5.1 ± 0.6	173
Library Avg ^c		47.7 ± 42.0	4.14 ± 3.26	1500	3.7 ± 1.1	122
Overall Average ^c		23.2 ± 30.6	1.60 ± 2.62	580	2.5 ± 1.3	83

^aConditional DNA concentration: average $\pm$ standard deviation from biological replicates.

^bConditional airborne cell (bacteria) and SE (fungus) concentration derived from AHU filter sampling; only the average value from each location is displayed.

^cLocation-based averages calculated based on average values of each location $\pm$ standard deviation from the averages of each location.

Figure 5.3. illustrates the correlation between conditional airborne DNA concentrations (C) and the ventilation-rate-normalized occupancy level (Nd) plotted across two groups of locations, the offices + classroom and the libraries, separately for total, bacterial and fungal DNA. The classroom was grouped together with the offices based on the observed activity level during the manual occupancy count. Overall, the values of both R^2 and ρ indicate moderate to high positive correlations across all six plots, indicating that the accumulation of the three categories of DNA

are all positively related to this measure of occupancy in both types of indoor locations.

Figure 5. 4 Correlation between airborne biomass concentration and occupant intensity (Nd) for total (frames A and B), bacterial (frames C and D) and fungal DNA (frames E and F) grouped for two different environment categories, Offices + Classroom and Libraries. The symbol ρ denotes the Spearman's rank correlation coefficient and * indicates statistical significance of the correlation ($p < 0.05$). The slope indicates a conditional emission factor of DNA associated with human occupancy.

1.4 Discussions

5.4.1. Bacterial and fungal communities of HVAC filters serving different environments.

Two major aims of the reported microbial community analyses are (i) to provide baseline information on bacterial and fungal compositions on HVAC filters, and (ii) to evaluate a feasibility of HVAC filters to assess indoor air quality and operational conditions of buildings such as occupancy level, ventilation system or interior settings.

Our results were consistent in terms of bacterial compositions reported by previous studies, in which *Proteobacteria*, *Actinobacteria* and *Firmicutes* were dominant phyla and *Corynebacterium*, *Pseudomonas*, *Bacillus*, *Streptophyta* and *Acinetobacter* were dominant genera detected from both indoor samples [53,56,60] and ventilation system filter samples [22,56,60]. Nonetheless, the group of the human-related bacterial genera was less abundant in our HVAC filter samples, with relative abundances ranging from 2.7% to 6.0%, than in air filter samples collected from an indoor environment, with reported relative abundances ranging from 17% to 20% [48,60]. The difference might be attributable to inability of absolute quantifications by sequencing-based methods and/or differences in sampling methods and conditions. Future studies are warranted to understand the causes of the observed difference and elaborate a sampling protocol based on HVAC filters. For instance, microbial compositions of HVAC filters need to be compared with those of air filter samples collected from indoor environments. Additionally, impacts of HVAC filter ratings and materials on collected microbial compositions need to be characterized.

Our fungal community analyses provide further insights into microbial compositions on HVAC filters. Yamamoto et al. [59] reported *Cryptococcus*, *Alternaria*, *Cladosporium* and *Epicoccum* as abundant fungal genera detected from indoor air samples collected from classrooms across different continents. Remarkably, none of these genera were among the 10 most abundant genera detected from our HVAC filters, except for *Cladosporium* which is ubiquitously found in indoor and outdoor environments [89,90]. *Alternaria* and *Epicoccum*

produce large multicellular spores, which create large settling velocities [88]. Large particles, including *Alternaria* and *Epicoccum* spores, might settle out before reaching HVAC filters, resulting in the lower abundances observed for these multicellular taxa.

The microbial compositions on HVAC filters appear to reflect the indoor conditions. First, the group of the bacterial genera of *Deinococcus*, *Achromobacter* and *Roseomonas* that are known to be detected in mechanically ventilated rooms [87] were more abundant than the group of the bacterial genera of *Methylobacterium*, *Sphingomonas* and *Streptococcus* that are known to be found in window-ventilated rooms [87]. The finding is reasonable as all of our buildings are mechanically ventilated with very few manual windows. Second, the relative abundances of the groups of human-related bacterial genera and bacterial genera that are known to be found in more occupied spaces had positive correlations with human occupancy levels. The Shannon diversity index of bacterial communities also showed a positive correlation with human occupancy levels. These findings are consistent with our understanding of human presence as a major source of bacterial emissions indoors [49,60,87,91]. Third, despite the fact that there was no substantial correlation found between the fungal α diversity index and human occupancy levels, a positive correlation was still observed between occupancy levels and human-associated fungal genera as well as common environmental fungi. One possible explanation of the low correlation between occupancy and fungal diversity is the substantially lower diversity of fungal species in tropical region as reported by Amend et al [92].

Human occupants have been reported as both primary[59] and secondary carrier [48] of fungi in indoor environment. The much higher combined abundance of environmental fungi (20%) as compared to human-related genera (0.4%) seems to indicate that building occupants mainly assume the latter role (secondary carrier) in contributing to fungi which end up depositing on HVAC filter. These correlations found on both bacterial and fungal abundances substantiate the role of HVAC filters as a potential surrogate to assess indoor air quality and operational conditions of buildings such as occupancy level, ventilation system or interior settings.

5.4.2. Conditional airborne DNA concentration and occupancy related emission factor

Utilizing AHU filters as indoor air samplers is an attractive approach for indoor environmental quality studies [58]. As shown by Noris et al. [22], it is possible to consider an AHU filter as an integrated indoor air sampler, which can be acquired for off-line analysis with relatively little incremental effort. Analyzing a building's used ventilation system filters can also help to evaluate operational parameters that best suit a building's needs, such as choice of filter grade or filter replacement frequency. In this section, we estimate airborne DNA concentration (C) based on AHU filter sampling. In addition, correlations between C and occupant intensity (Nd) are studied as a basis for estimating occupancy-associated emission factors for indoor bioaerosols that accumulate on AHU filters (E).

In presenting these results, we would first state some cautions in interpreting C as the time-averaged (for the AHU's operating hours) indoor air concentration of DNA. Among the considerations that limit the accuracy of this interpretation are the following. (a) Although most of the air passing through the filter is recirculated indoor air, there is a small contribution ($< 10\%$) of outdoor air, so some of the accumulated DNA would have originated outdoors. (b) The ventilation system filter efficiency for capturing airborne particles is less than 100%. (c) There may be transport losses that influence the relationship between room average concentrations and those in the return air passing through the AHU filter. (d) The analysis approach assumes that DNA accumulates from air, does not degrade (or become dislodged) from the filter, and does not replicate during the relatively long filter deployment period (three months). Owing to these considerations, the reported airborne DNA concentrations and the occupancy-associated emission factors (C and E) should be considered as "conditional" parameter values.

Except figure 5.4(E) of which correlation was heavily skewed by the one point from the library, the moderate to high values of both R^2 and ρ in the other parts of Figure 5.4 generally supports the idea that human occupants are a major source of biomass indoor. The slopes and intercepts of the six plots can be further translated into elements explaining the correlation between C and Nd . The intercepts can be

regarded as factors that are non-occupancy related (environmental factors). These factors could be a source of biomass (positive intercept) or sink of biomass (negative intercept). By dividing the absolute values of these intercepts with the corresponding average values of conditional bioaerosol concentration, C , from Table 3, the relative importance of these environmental factors can be estimated for each type of DNA. Bacterial DNA had the lowest impact from the environment at 6%, followed by total DNA with 16%. Fungal DNA had the highest impact from the environment, 56%. The slope, on the other hand, would represent the conditional, occupant-associated DNA emission factor for bioaccumulation on AHU filters (E , ng DNA/(person-h)).

As illustrated in Figure 5.3, the conditional DNA emission factors for the office (+ classroom) environments are 9.0 ng/person-h for total DNA, 0.29 ng/person-h for bacterial DNA, and 0.83 ng/person-h for fungal DNA. The data indicate a systematically higher conditional emission factor for total DNA and bacterial DNA in libraries (32 and 2.5 ng/person-h, respectively). On the contrary, no notable difference in fungal DNA emission factors was found from these two environment categories, with the libraries yielding 0.76 ng/person-h.

The differences in slopes of total, bacterial and fungal DNA between the office+classroom (A, C, E) plots and the libraries (B, D, F) plots suggest that people shed and/or resuspend different quantities of bioaerosols in different indoor environments. We found that occupants resuspended and/or shed more biomass in library environments as compared with the office and classroom environments, particularly for total and bacterial DNA. We suspect that the differences were mainly caused by how the indoor environments were used. In the three library environments, we observed many occupants actively walking with carts and old books, whereas occupants were mostly seated in the offices and classroom. All library environments were carpeted, whereas all the offices had tiled floorings. The classroom was partly carpeted.

1.5 Chapter conclusion

This chapter provides culture-independent abundance and diversity information on the biomass that accumulated on AHU filters in several normally functioning office, classroom, and library environments in a university. Average DNA concentrations per AHU filter surface area across nine indoor locations were determined to be in the respective ranges 1.1 to 41 ng/cm² for total DNA, 0.02 to 3.3 ng/cm² for bacterial DNA and 0.2 to 2.0 ng/cm² for fungal DNA. The accumulation of all DNA types generally showed good correlation with occupancy level. Bacterial DNA was the most sensitive in relation indoor occupancy.

In agreement with previous studies, *Proteobacteria*, *Actinobacteria* and *Firmicutes* were the most abundant bacterial phyla detected and *Corynebacterium*, *Pseudomonas*, *Bacillus*, *Streptophyta* and *Acinetobacter* were among the most abundant bacterial genera. The most abundant fungal phylum detected on our AHU filters was *Ascomycota*, while the most abundant fungal genera were *Aspergillus*, *Cladosporium*, *Nigrospora*, *Rigidoporus* and *Lentinus*. Overall, changes in occupancy level were well reflected in the relative abundances of human-related bacterial and fungal genera.

In the context of indoor bioaerosol studies, and as recently noted by Haaland and Siegel [8], AHU filter sampling provides a convenient alternative to direct indoor air sampling for gathering enough biomass from air for numerous analysis purposes. The correlation and microbial diversity analysis showed how bioaccumulation on AHU filters corresponds well with changes in various indoor conditions, such as occupancy level. Future studies involving larger samples size, testing different filter types and grades, and a controlled effort to compare indoor air sampling to its corresponding AHU filter sampling are warranted to gain deeper understanding of the roles of air handling systems in indoor bioaerosol dynamics.

CHAPTER 6

LONGITUDINAL HVAC FILTER STUDY: TIME-SERIES PROFILE OF BIOACCUMULATION ON HVAC FILTER

6.1 Objectives

This chapter aims to trace quantitatively and over time the diversity profile of biological material accumulating on an HVAC filter. Samples were collected concurrently with monitoring indoor parameters such as occupancy to understand accumulation mechanisms throughout the duration of filter use and ultimately to evaluate the effectiveness of HVAC system operation and maintenance on airborne biological materials.

6.2 Experimental design

6.2.1. The indoor environment and sampling approach

Figure 6. 1 Illustration on how the filter samples were collected every week. Immediately after collection, the gap was replaced with a new set of filters which allowed the system to run normally.

Pieces of an HVAC filter with the same size as previous experiments (2×5 cm) was collected from an in-use filter panel in a certain weekly interval from an air-handling unit (AHU) in a library at a Singapore university. A set of brand new filter panels were installed in the AHU at the last week of November 2014 for the purpose of this experiment. For every week the filter pieces were cut from the panel, the gap is immediately replaced with a new filter which allows the AHU to maintain its service normally. For each sampling session, at least 6 pieces of filters were collected for backup and replication purposes. One panel was immediately sampled after brief installation on week 1 for negative control. The sampling was carried out for a total period of 21 weeks. For the first 4 weeks, the filter samples were collected every week. From week 4 to week 14 the samples were collected every 2 weeks. After week 14 the last 2 sampling sessions were after 4 weeks (week 18) and then finally after 3 weeks (week 21).

The AHU served the same indoor space as the Library 3 environment in chapter 5. It is a reading hall in the library with an approximate volume of 4170 m^3 ($A=1250 \text{ m}^2$, $H=3.2\text{m}$). The hall was carpeted and designed as a typical library with tables, chairs and book racks in the middle. This library environment was chosen mainly due to its relatively higher biomass loading compared to the other locations and the very different indoor conditions observed during the university's vacation and semester cycle. Office environments are typically used by university staff members who report for work both during vacation and semester season. The main visitors in the library, on the other hand, are the students. Therefore, a big difference in visitor level was seen between the vacation and normal semester period. We are looking to see how bioaccumulation rate and/or microbial diversity on the filters is affected due to this indoor condition changes.

Visitors level information was obtained through an automatic counter at the gate of the library. The sensor counts the cumulative number of persons passing through the gate towards the library every day. Number of visitors (visitors/week) were then calculated by taking the weekly average for each duration between two sample collections. Figure 6.2 shows the number of weekly average visitors during the 21 week of operation time.

Figure 6. 2 Weekly average number of visitors in the library

In addition to the filter sample and visitor level, outdoor air reference was also collected during the operating period of the filter. The outdoor sampling was carried out according to the ambient air sampling protocol in chapter 3. The only difference is that instead of using PES membrane filter, we used a new HVAC filter cloth with the same ratings (MERV 8). This change of filter was done to account for possible differences in captured organisms due to a gap in filter efficiencies between the two filter types. Outdoor sampling was performed 3 times in week 16, week 18 and week 20. One sampling session lasted 24 hours and the DNA solution from the three samples were pooled together using the DNA pooling method developed in chapter 4. The final DNA solution was then submitted for sequencing.

6.2.2. *DNA extraction and analysis*

All DNA extraction and analysis steps follow the same protocol as chapter 5. the 10-cm² filter pieces was placed into the 5 mL bead beating tube of MOBIO PW for DNA extraction. The DNA extraction steps followed the original protocol with some modifications to improve DNA yield based on our optimization in chapter 4 [84]. Briefly, the modifications included a two-minute vortex after the addition of preheated solution PW1 (the lysing agent), followed by water-bath sonication at 65 °C for 30 minutes. After sonication, the samples were vortexed again for 5

minutes as recommended. The rest of the extraction steps followed the protocol described in chapter 3. Extraction replicates were separate 10-cm² filter pieces that were cut from the same filter panel.

After extraction, the total DNA in each sample was measured by Qubit. The same Qubit high sensitivity (HS) ds DNA kit was used for processing all samples. Bacterial and fungal DNA levels were also quantified by means of qPCR, complemented with a standard curve to convert the Ct values to DNA concentration. The qPCR was run using the same protocol and universal primers and probe listed in Table 3.1. DNA sequencing run on Illumina Miseq and its data analysis also follow the protocol in chapter 3.

The DNA concentrations from blank samples were all below the detection limits of both Qubit and qPCR with 35 thermal cycles, suggesting that there was no significant contribution of biomass from the maintenance company personnel during filter replacement, from the researchers during sampling and analysis of the filter pieces, or from the AHU filter materials themselves.

6.2.3. Correlating the weekly DNA accumulation rate and occupancy level

In this section, the average weekly visitors from Figure 6.1 is correlated to the weekly DNA accumulation rate from the HVAC filter. The total DNA concentration from Qubit quantification and the Ct values from qPCR assays were first converted to DNA mass concentration per filter area as described in Chapter 3 for each sample. The net difference between each sample's DNA concentration per filter area is recorded as the DNA accumulation/loss rate in the time period between 2 sample collections. This net difference is then divided by the number of weeks between the two sampling sessions to obtain the weekly averaged DNA accumulation/loss rate for each duration between samplings. The number of week were 1 week for week 1 to 4, 2 weeks for week 4 to 14, 4 weeks for week 14 to 18 and finally 3 weeks for week 18 to 21.

In addition to total DNA, total bacteria and total fungi, we also attempted to correlate visitors level to the abundance of human-related bacterial and fungal genera. We selected a group of genera previously reported to originate from skin or

gut microflora. The groups of genera were *Propionibacteria*, *Streptococcus*, *Corynebacterium* and *Veilonella* for bacteria and *Alternaria*, *Candida*, *Rhodotorula*, *Trichosporon*, and *Cryptococcus* for fungi. We decided to exclude bacterial genus *Staphylococcus* from the equation due to the relatively high outdoor influence as shown by the DNA sequencing data analysis. To estimate the absolute quantity of human related bacteria and fungi, the relative abundances from the taxonomic assignment of each genus were multiplied by the total bacterial and fungal DNA concentration per filter area from the qPCR assays. The estimated absolute abundances were then tallied to obtain the total human related bacteria and fungi concentration per filter area. Similar to total DNA quantification, the net differences of DNA concentration between each sampling sessions were first calculated and divided by the number of weeks to obtain the weekly averaged DNA accumulation/loss rate, specific to the selected groups of genera. It is also important to note that both library preparation and the bacterial quantification would carry a certain level of PCR-related biases. While the main purpose of this section is just to discern how well they correlate to each other, the actual value of absolute quantity of these group of bacteria and fungi must be interpreted with cautions.

6.3 Results

6.3.1. Microbial diversity analysis: predicting the source of each microbial community on the HVAC filter

Overall, 759 fungal and 465 bacterial genera were detected from a total of 11 time-series HVAC filter samples and the outdoor air sample. Figure 6.3 indicates that *Basidiomycota* and *Ascomycota* are the two most abundant fungal phyla, comprising more than 99% of all samples. *Basidiomycota* was the most dominant fungal phylum in all 13 samples with relative abundances ranging from 77% on Week 10 to 92% on Week 6 for HVAC filter samples and 98% for outdoor air. Phylum *Ascomycota* was found to be more abundant in HVAC filter samples. The relative abundance of *Ascomycota* increased considerably from a combined average of 7.3% during vacation weeks to 16% ($p < 0.05$, Kruskal-Wallis test) during normal semester weeks (higher occupancy level). This finding suggests that the majority of *Ascomycota* fungi on the samples are of indoor origins.

Figure 6. 3 Fungal phyla composition of the HVAC filter over the 21-week period of operation and the outdoor air

The fungal genera heatmap on Figure 6.4 provided further insight in understanding the separate source of biomass found on AHU filters. The 5 most abundant genera found on the HVAC filters and their mean relative abundances were *Schizophyllum* (16%), *Rigidoporus* (7.9%), *Lentinus* (6.5%), *Trametes* (5.7%) and *Peniophora* (5.1%). Remarkably, the outdoor air reference shared 3 out of these 5 most abundant genera. The top genera found on the outdoor air sample were *Schizophyllum* (20%), *Peniophora* (9.6%), *Grammothele* (8.6%), *Ganoderma* (6.6%) and *Rigidoporus* (5.6%). Phylum *Trametes* (4.9%, 6th) and *Lentinus* (4.8%, 7th) followed closely right after. In addition to its similarity to outdoor air, the abundances of these dominant genera stayed relatively constant throughout the 21-week period of operation despite notable changes in occupancy level, indicating that the main contributor of fungi on HVAC filter may be the outdoor air.

Figure 6. 4 Top 40 fungal genera heat map composition of the HVAC filter during the 21-week period of operation and the outdoor air

Changes in fungal composition of several featured genera were also observed. The abundances of human related fungal genera, such as *Candida*, *Cryptococcus* and *Trichosporon* [59] and common indoor fungi, such as *Aspergillus* and *Cladosporium* [76,89,93] on HVAC filter seemed to change according to indoor occupancy level. Their mean abundances increased significantly from 0.2% to 0.6% for *Candida* (3×), 0.1% to 0.3% for *Cryptococcus* (3×), 0.3% to 1.5% (5×) for *Trichosporon*, 1.1% to 4.5% for *Aspergillus* (4×) and 0.1% to 0.6% for *Cladosporium* (6×) (all $p < 0.05$ Kruskal-Wallis test) in the semester weeks with higher occupancy. Furthermore, 4 out of these 5 genera were not detected in outdoor air. Only *Aspergillus* was detected in outdoor air with relatively lower (0.9%) relative abundance. This evidence strongly suggests that these fungi came from the indoor environment. Compared to the group of dominant genera from outdoor origins, however, these genera only accounts for a substantially smaller percentage of fungi found on HVAC filter with an average combined abundance of 1.1%.

Contrary to its fungal counterpart, notable dissimilarities were seen between the outdoor air reference and the HVAC filter samples. Figure 6.5 shows that The top 3 bacterial phyla of the HVAC filter samples and their mean relative abundances were *Proteobacteria* (33%), *Actinobacteria* (26%) and *Firmicutes* (10%). *Proteobacteria* and *Actinobacteria* were more abundant in the HVAC filter samples, with the outdoor air comprised of 16% *Proteobacteria* and 7% *Actinobacteria*, whereas *Firmicutes* was more dominant in outdoor air (23%). In addition to the top 3 phyla, phylum *Bacteroidetes* was found to be significantly higher in outdoor air sample with 35% relative abundance, as compared to the HVAC filter samples (0.7%-3%). No distinct trend/change was observed on the bacterial phyla composition of the HVAC filter samples along the 21-week period.

Figure 6. 5 Bacterial phyla composition on the HVAC filter over the 21-week period of peration and the outdoor air

Bacterial genera heatmap on Figure 6.6 reveals that some of the dominant bacterial genera and their mean relative abundance for the HVAC filter samples were *Acinetobacter* (13.7%), *Deinococcus* (9.4%), *Micrococcus* (5.9%), *Corynebacterium* (3.5%), and *Pseudomonas* (2.2%). These genera were present in the outdoor air with much lower abundance with 0.1% for *Acinetobacter*, 0.7% for *Deinococcus*, 0.5% for *Micrococcus*, 0.1% for *Corynebacterium*, while *Pseudomonas* was undetected. On the contrary, bacterial genera which were abundant outdoor such as *Ktedonobacter* (5.4%), *Streptophyta* (2.2%), *Methylobacterium* (1.0%) and *Bacillus* (2.0%) comprises only a small percentage of the HVAC filter bacteria with 1.6%, 0.6%, 0.5% and 0.3% of relative abundances respectively. *Kocuria* and *Staphylococcus* were the only genera above 1% average abundance which were present in both samples with somewhat comparable average abundances. *Kocuria* accounted for 2.7% of HVAC filter bacteria and 1.6% of outdoor air bacteria, while *Staphylococcus* had 1.4% for HVAC filter and 1.0% for outdoor. This finding indicates that although there is a small contribution from outdoor air, the main source of bacteria on the HVAC filters are the indoor contributors. The bacteria influx from indoor air seemed to enter the air handling

unit (AHU) with relatively stable composition throughout the operation period of the filter as no notable change in relative abundances were observed between vacation and semester weeks.

Figure 6. 6 Top 40 bacterial genera heatmap composition on the HVAC filter during the 21-week operation period and the outdoor air.

The α and β diversity analysis displayed in Figure 6.7 also supports the idea that most bacteria on the HVAC filter could originate from the indoor environment, while most fungi are from outdoor. The principal coordinate analysis on Figure 6.7 suggests that the distance between weekly HVAC filter samples and the outdoor air is much bigger for bacteria. For fungi, the distance between the communities on the filters are closer to the outdoor reference. The Shannon diversity index on Figure 6.7 also suggests that there is a bigger gap in species richness between the outdoor air sample and the HVAC filters for bacteria. The diversity gap between outdoor and HVAC filter samples looks to be considerably smaller for fungi. The fungal

Shannon diversity index increased from 4.4 to 4.9 after week 8, possibly due to the sudden indoor contribution after the semester started, it then gradually came back to 4.5 as the HVAC system operated until week 21.

Figure 6.7 α diversity (Shannon index) and β diversity (PCoA, based on ThetaYC logarithm) analysis of the HVAC filter during the 21-week operation period and the outdoor sample for bacteria and fungi.

6.3.2. Time-series profile of DNA accumulation on HVAC filter

The absolute quantification data (fluorometry and qPCR) on Figure 6.8 suggested that each broad category of biomass exhibits distinctive pattern of accumulation on the filter. Total DNA increased relatively linearly from 5500 pg/cm² on week 1 to 33000 pg/cm² on week 21. Bacterial DNA stayed relatively constant on the range of 500-600 pg/cm² of filter for the first six weeks. It started rising from week 8 to week 12 (1500 pg/cm²) before dropping again to 1200 pg/cm² on week 14. Bacterial DNA concentration then rose steadily from week 14, reaching a peak of 2300 pg/cm² on week 21.

Figure 6. 8 Time-series profile of DNA accumulation on HVAC filter over the 21-week period.

Fungal DNA displayed a unique trend of bioaccumulation on HVAC filter over time. The concentration of fungal DNA rose from 1000 pg/cm² on week one to a peak of 4500 pg/cm² in the first six weeks, then declined steeply to 1200 pg/cm² from week 6 to week 10, before increasing again over the next 14 weeks, reaching a concentration of 4000 pg/cm² on week 21.

6.4 Discussions: *connecting the microbial diversity analysis to the DNA mass accumulation profile on HVAC filter*

Putting together the microbial diversity analysis and the time-series accumulation profile for each type of DNA has provided us with some insights

regarding how bioaccumulation occur in an in-use centralized ventilation system filter during its operation time. One important point is confirming the main influencing factor of each of the biological entities. Bacteria has long been associated with the presence of human occupants in indoor environments[48,49,60]. Indeed, our DNA sequencing analysis revealed that the main influencing factor of bacterial DNA's presence on the filter is the indoor environment, which is dominated by the occupants. For fungal DNA, the same DNA sequencing analysis indicates that the main contributor is the outdoor air intake. Dominant fungal genera showed a lot of similarities with fungi found outdoor, whereas previously reported indoor fungi comprises only a small percentage of the total fungi detected on the HVAC filters.

Figure 6.8 displayed the unique trends followed by each type of DNA as they deposit on the filter. It is clear that the accumulation of bacterial and fungal DNA is influenced by certain influencing factors as they did not just increase linearly over time. The most notable occurrences were the relatively constant level of bacterial DNA from week 1 to week 8 and the dip observed in fungal DNA from week 8 to week 10. Total DNA, however, kept accumulating in a relatively constant rate for the entire 21-week of operation, including when the aforementioned irregularities were observed on bacterial and/or fungal DNA (week 1-10). We expected total DNA to be very broad since it consists of both microbial and non-microbial entities, such as plant or animal DNA. Since microbial DNA accounts for a relatively small portion of total DNA (20-30%), it is likely that there was a steady stream of non-microbial biomass throughout the operation period which contributed to its linearity.

To understand how the number of occupants affects the bioaccumulation of each type of DNA, the average number of weekly visitors was plotted against the weekly change in DNA concentration on HVAC filter for total DNA and total fungi (Figure 6.9) as well as total bacteria, human related bacteria and human related fungi (Figure 6.10). Based on our findings from DNA sequencing analysis, we expected total bacteria, human related bacteria and human related fungi to have a good correlation to the occupants as they are a major source of biomass from the

indoor environment. Conversely, the impact of occupancy on the accumulation rate of total fungi is expected to be small.

Figure 6. 9 Correlation between weekly visitors and weekly DNA accumulation/loss rate for total DNA (above) and total fungi (below)

Figure 6.9 confirms that the accumulation rate of total fungi is not correlated to the visitor level in the library. This result agrees with our previous analysis and prediction which indicated that the indoor influence should be small for fungi. Total DNA, on the other hand, displayed a weak correlation to occupancy level (correlation coefficient is not statistically significant at $p=0.05$). This correlation can be explained by the presence of a portion of total biomass of which the main influencing factor is human occupants. DNA extracted from bacteria in general, shed human cells, human related fungi are all a subset of total DNA which could contribute to the correlation.

Figure 6. 10 Correlation between weekly visitors and weekly DNA accumulation/loss rate for total bacteria (above), human related bacteria (middle) and human related fungi (below). *denotes statistical significance at $p=0.05$.

As also expected from Figure 6.10, correlations between total bacteria and human related bacteria to occupancy are good and statistically significant.

Interestingly, despite no correlation seen between total fungi and occupancy, the absolute abundance of human related fungi correlated reasonably well with the fluctuation of visitor level. All correlation coefficients are statistically significant at $p=0.05$.

The slopes and intercepts of the linear regressions analysis in Figure 6.9 can be further interpreted into elements which explains the bioaccumulation on HVAC filter. The slope would represent each visitor's emission factor (pg DNA/person). The positive slopes suggest that the higher the visitor level, the more DNA is likely to accumulate on the HVAC filters. The intercept would represent the factors that are not related to humans/visitors. The negative values of all the intercepts indicate that there is a certain loss rate incorporated to the accumulation process on HVAC filters. This loss rate means that biomass (or DNA) which is trapped on the filter at any point of the air sampling duration is not conserved. The possible reasons for this loss rate are physical dislodge or degradation of biomass up to its DNA level which could be due to stress from high air face velocity or high temperature in the AHU room during off time. One of our results in chapter 4 [84] has also indicated the existence of this loss rate. It is also very likely that the magnitude of this loss rate varies from species to species because different species have different endurance and unique characteristics in facing the environmental challenges.

One can also speculate that this loss rate is the main reason why we see the irregularities in the bacterial and fungal accumulation profile in Figure 6.7. For DNA concentration on the filter to increase, the gain rate must be higher than the corresponding loss rate. In the case of bacterial DNA where occupancy level is a major factor, it is possible that after week 1, as the number of visitors kept dwindling down until week 8, the gain rate is reduced, which resulted in the constant profile for the first 8 weeks. As the number of visitors increased significantly after the semester started, the gain rate far surpassed the loss rate which resulted in bacterial DNA steadily increasing until week 21.

For fungal DNA, since it was revealed that the major influencing factor is the outdoor air intake, the change in visitor level could not explain the dip on week 8 and week 10 (1 month period). We have identified two possibilities for this occurrence. The first possibility is an actual decrease in the absolute abundance of

fungi in the surrounding outdoor air for this particular month. When the outdoor fungi influx is low, the loss rate could be higher than the gain rate and resulted in the dip. However, the PM data from Singapore's national environmental agency (NEA) did not show any abnormalities in PM concentrations or Pollutant Standards Index (PSI) readings during this period. The more probable possibility is the change in outdoor air intake rate in the ventilation system. As described previously, the HVAC filters in this study process >90% of recirculated indoor air and <10% outdoor air. The amount of outdoor air drawn by the system is controlled by a hatch which adjust its opening automatically according to a certain set condition in the indoor environment. Under normal operational condition, the outdoor air intake rate is relatively fixed at a certain rate (8-10% of total supply air). We have received report from the Office of Development and Facilities Management (ODFM) in the university that at the beginning of 2015, particularly in January 2015 (week 6-10), they attempted to change the regular temperature and CO₂ set point for thermal comfort study purposes. Though there is no record on how this change affected the outdoor air intake, it is very likely that the outdoor air intake rate is significantly reduced during this period of time, which resulted in the drop (loss rate > gain rate). They reverted back to the regular operational conditions after one month. As seen in Figure 6.7, the fungal DNA accumulated normally again after the drop on week 10.

6.5 Chapter conclusion

The 5 most abundant fungal genera found on the HVAC filter over the 21 week of operation and their mean relative abundances were *Schizophyllum* (16%), *Rigidoporus* (7.9%), *Lentinus* (6.5%), *Trametes* (5.7%) and *Peniophora* (5.1%). These genera were also equally abundant in outdoor air, which seems to indicate that the main contributor of fungi on the HVAC filter is the outdoor air intake.

The most dominant bacterial genera and their mean relative abundance for the HVAC filter samples were *Acinetobacter* (13.7%), *Deinococcus* (9.4%), *Micrococcus* (5.9%), *Corynebacterium* (3.5%), and *Pseudomonas* (2.2%). These genera were present in the outdoor air with much less abundance. Conversely, bacterial genera which were abundant outdoor such as *Ktedonobacter* (5.4%), *Streptophyta* (2.2%), *Methylobacterium* (1.0%) and *Bacillus* (2.0%) were present in much lower abundance on the HVAC filter. This finding indicates that the outdoor influence for bacteria in the HVAC filter is small. The main contributor for bacterial DNA on the HVAC filter could be the indoor air.

As bacterial DNA, human-related bacterial DNA and human related fungal DNA were found to correlate well with indoor occupancy level, the correlation's intercept values indicate the possible existence of a specific loss rate for each type of biomass. This loss rate could substantially affect the accumulation rate on HVAC filter because there is a chance that the DNA mass collected on the filter at any point during the sampling is not conserved. Depending on the values of gain rate and loss rate at any particular sampling time, biomass could increase, stay constant or even reduce.

In conclusion, in utilizing HVAC filter for bioaerosols study, the unique accumulation trend shown by each DNA type suggests that depending on our target biomass, an analysis based on a one-time collection of HVAC filter samples must be performed with cautions. Specific influencing factors such as occupancy level for bacteria or outdoor air conditions for fungi must complement the biomass concentration analysis to accurately interpret the final results.

CHAPTER 7

CONCLUSIONS AND FUTURE RESEARCH DIRECTION

7.1 Research Conclusion

Buildings are ultimately built for people. With research on building science continues to strive towards energy efficiency and optimum building materials, there is a need to also make sure that the well-beings of its human occupants are not sacrificed in the process of achieving this goals. This project addresses the importance of a building's ventilation system filter as the first line of defence in protecting the indoor environment from potential airborne pollutants and as an indicator of the indoor air quality itself.

We first developed an analysis method optimized to deal with unique inhibitors and the relatively lower concentration of biomass from air samples. The key findings are as follows:

- Improving cell lysis by incorporating thermal sonication increases the DNA yield of HVAC filter samples by up to 600%.
- DNA extraction kits with incorporated inhibitor removal steps is adequate to handle the inhibitor levels carried by HVAC filter samples.
- There is an indication that DNA may degrade/dislodged in the middle of air sampling on a filter media.
- The developed mid-extraction DNA pooling approach helps with low biomass issue, especially for DNA analysis methods which requires higher minimum concentration such as Qubit fluorometer or high-throughput DNA sequencing.

With an optimized analysis method, we conducted a cross-sectional study involving HVAC filters serving several different indoor spaces. We investigated whether different indoor conditions such as interior settings, occupancy and activity levels are discernible from an analysis method based on HVAC filter sampling. Our key findings are as follows:

- The microbial community analysis of our HVAC filter samples agrees with the majority of past studies analysing both indoor air and/or ventilation filter samples across regions.
- Difference in occupancy levels are well reflected in both the absolute abundances of total and bacterial DNA as well as the relative abundances of human related bacteria and fungi found on HVAC filters.
- Human occupants look to produce/shed different amount of biomass when they are exposed to different indoor environment. We found that the DNA emission factor per occupants in the library was higher than the offices. This is likely due to the difference in activity level between the two environments.

Based on the cross-sectional study, the most dynamics AHU system was chosen for the longitudinal study of bioaccumulation on HVAC filter. The chosen AHU was one from the most occupied library environment (Library 3). Filter samples were collected on a weekly basis and the changes in DNA composition and abundance were analysed. The key findings are as follows:

- Microbial diversity analysis indicates that the major contributor of fungal DNA on HVAC filter is the outdoor air, whereas the main source of bacterial DNA on the filter is the re-circulated indoor air.
- A certain loss rate of DNA mass already captured on the filter with varying magnitudes were observed during the 21-week period of operation.
- Due to the potential loss rate, an attempt to understand the indoor air quality based on a one-time collection of HVAC filter sampling must be completed with the main influencing factor of the targeted biomass. For example, information on occupancy levels is crucial in accurately interpreting bacterial data analysis.

Overall, our study has proven that HVAC filter has the capability in analysing potential airborne pollutants both from indoor or outdoor sources. Depending on the building's usage, such analysis can help building users prepare and determine operational decisions which best suit the building's needs, such as choice of filter grade or filter replacement frequency. In the context of bioaerosols study, our

results substantiate the use of HVAC filter as a probe of indoor environmental quality. However, to accurately interpret the results, it is recommended to identify and complement the analysis with information on the main influencing factors of our targeted biomass.

7.2 Future research direction

7.2.1. Continuation of research on HVAC filter: Comparison between direct indoor sampling and HVAC filter sampling

Future direction of research utilizing AHU/HVAC filter should be focused on efforts comparing various methods of direct indoor sampling to its corresponding HVAC filter analysis.

The main motivation of this research direction is based on the comparison of our results from chapter 5 to previous studies which provided quantitative information on airborne biomass concentrations in indoor environments. We focused on studies that estimated concentration in terms of amount of cells per m³ of air, performed their sampling directly in occupied indoor environments and used non-viable methods in their analysis. Among the studies we chose, Toivola et al. [51] used microscopic method, whereas Qian et al. [48], Hospodsky et al. [52] and Deng et al. [53] used similar DNA-based methods to work out cells concentration per m³ of air. In comparison with these studies, our conditional airborne DNA concentration range displayed in Figure 7.1 is broadly lower. Our bacterial count is on the lower end of the ranges in the studies, whereas our fungal count is up to three orders of magnitude lower than the reported concentrations.

Figure 7. 1 Comparison of conditional airborne cells (bacteria) and SE (fungi) concentrations and emission rates derived from AHU filters from this study (red dot) and other relevant indoor bioaerosols studies (blue dots). The dots represent the average values across locations and the lines denote the range. The top figures display the comparison of bioaerosol concentrations for (A) bacteria and (B) fungi, while the bottom figures show the comparison of emission rates for (C) bacteria and (D) fungi.

*Qian et al. is the average value from one indoor location. The other studies have multiple locations

**Deng et al. only analysed bacteria

***Toivola et al. used microscopic count to estimate the values, while the rest of the studies use similar DNA-based analysis

We also compared our conditional emission rates against studies which incorporate occupancy count to their airborne concentrations and also use non-viable based methods. Our emission rates are also lower than human contribution per person hour values reported by Qian et al. [48] and Hospodsky et al. [52]. Our bacterial emission is averagely one order of magnitude lower than both studies and

although our fungal emission is only slightly lower than Qian et al's estimation, it is still more than two order of magnitude lower than Hospodsky et al.

Based on comparisons above, it seems like a protocol utilizing HVAC filter sampling would tend to underestimate actual indoor bioaerosols concentrations. We suspect several possible reasons for these findings.

Firstly, we would like to highlight that bioaerosols are naturally very labile in time and space. Among the studies we chose as references [19,28-30,52,92], bacterial cell concentration in an indoor environment, for example, can be as low as 37 cells/m³ in one location as indicated by Deng et al. [53] or as high as 310×10^3 cells/m³ in another location as shown by Hospodsky et al. [52]. In addition, numerous studies have also indicated significant temporal differences of bacteria and fungi in the same location [29,30]. The gaps observed in Figure 7.1 could also be partially attributable to these locations, time, conditions or methods differences among the studies.

The second possible reason might be affiliated to the efficiencies of filters used during sampling. The filters used by the aforementioned studies were nucleopore filter, track-etched polycarbonate filter or Whatman quartz microfiber filters with pore sizes of 1 µm or lower. These filters are designed to trap smaller-sized particles for the purpose of water or air pollution studies and generally not applicable for use in a typical commercial building's ventilation system due to the large pressure drop. On the contrary, this study engaged actual AHU filters with MERV-8 AHSRAE rating which is expected to be 80% effective in capturing particles sized 3 – 10 µm. Azimi et al. [61] reported that MERV 8 rated filter's efficiency in capturing PM 2.5 was below 30%. Our HVAC filter samples might not properly address particles smaller than 3 µm, which are still a substantial portion of airborne biomass indoor [48,52,53]. Similar finding was also reported by Noris et al. [22]. Using culture-based method to quantify the biological portion of HVAC filter dust, they found that biomass concentration extracted from HVAC filter with low to medium efficiency (MERV 3-8) was averagely one order of magnitude lower than those extracted from dust collected indoor.

The third reason might be related to the loss rate previously linked to our HVAC filter-based sampling. Our results from chapter 4 and chapter 6 have also

reported that microorganisms which are already captured by the filter could be physically removed or degrade up to its DNA level due to sampling stress or long duration[84]. It is possible that the endurance of cells on a filter media may depend on its specific species properties. The model organism used in our chapter 4 experiment, Luhung et al. [84] for example, was *Shewanella Oneidensis* which is a Gram-negative bacteria and up to 98% of the spiked amount degraded after one day of air sampling. In comparison with the previously mentioned studies (several hours to days of sampling duration, 0.1-0.3 m/s face velocity), our HVAC filter samples had considerably higher duration and face velocity (three months, 1-2 m/s face velocity). It might be possible that a portion of biomass which was already on the filter at any point during its operation time had been degraded/removed prior to sample collection.

We recommend two main direction of future studies. The first direction is to take aim at these series of investigations on HVAC filter. Firstly, we proposed a controlled experiment which compare the bioaerosol concentration derived from direct indoor sampling to HVAC filter sampling. Secondly, we proposed a lab-based experiment addressing the possible loss rate on the HVAC filter. After collection of a used HVAC filter, the filter panel is divided into two sets of filter pieces. The first set of filters are put in a conditioned chamber and DNA mass is quantified after every interval. The second set of filters are put in a controlled chamber with a clean air introduced to it. We expect to see the difference in loss rate between the two sets of filter, if any. The two sets of experiments above can be carried out with two different types of filter grade in order to account for the possible filter efficiency factor.

With sample availability (due to different operating schedules, temporal and spatial variabilities among AHUs) for statistical power often being an issue in this study, the second main direction should aim at continuing to develop a comprehensive database of microbial communities, both in terms of diversity and absolute quantity, found on HVAC filters in different indoor conditions from different locations around the world. Building on our contribution from tropical buildings, enough effort to compile data from various studies will ultimately warrant a point where it is possible to confidently perform other potential NGS

interpretation, such as community, clustering, ordination (CCA, NMS) or other correlation analyses in link with the corresponding environmental parameters in the indoor environment served by the filter.

7.2.2. Other potential research directions

We have also identified several possible research directions based on our results on HVAC filter analysis,

- Research on the dynamics between biological and chemical pollutants on HVAC filter such as ozone or CO₂.
- Research on other parts of the HVAC system, such as a research on the presence of biological material and the biomass removal factor on the wet cooling coil system which comes right after HVAC filter.
- Research on personal exposure level of building occupants: Some of the dominant bacterial and fungal genera found in our study contain species that are potentially pathogenic for building occupants, such as *Clostridium XI* for bacteria or *Aspergillus* for Fungi. Personal sampler can be utilized by a group of building occupants and the estimated exposure levels of these group of genera can be estimated by both qPCR and high-throughput sequencing approaches.

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APPENDIX A

Ambient air and AHU/HVAC filter sampling schedule for Chapter 4

Duration	RH (%), T (°C) and Weather	Details and Preparation for Extraction	Purpose
24 Hours	47-74%, 28-34 °C, clear	6 pumps operating at the same time <ul style="list-style-type: none"> 3 pumps for 3 × 8 h ($N = 3$) 3 pumps for 1 × 24 h ($N = 3$) 	Figure 4.5
20 Hours	Day 1 : 47-79%, 27-34 °C, clear Day 2 : 54-77%, 28-33 °C, clear Day 3 : 57-78%, 27-33 °C, clear	7 pumps operating at the same time (for each run) <ul style="list-style-type: none"> 2 pumps for spike at time 0 ($N = 6$) 1 pump for spike at 8 hour ($N = 3$) 1 pump for spike at 14 hour ($N = 3$) 2 pumps for spike at 20 hour ($N = 6$) 1 pump for no spike ($N = 3$) 	Table 4.1
20 hours	Day 1 : 64-82%, 25-31 °C, clear Day 2 : 59-82%, 27-32 °C, clear	4 pumps operating at the same time (for each run) <ul style="list-style-type: none"> 2 pumps for no-treatment ($N = 4$) 2 pumps for additional treat ($N = 4$) 	Figure 4.4a and 4.4b
8 hours	49-81%, 27-33 °C, clear	8 pumps for operating at the same time (for each run)	Table 4.2

		<ul style="list-style-type: none"> • 6 pumps for filters to be concentrated ($N = 4$) • 2 pumps for filters extracted separately ($N = 4$) 	
AHU and Collection	Indoor Environment and Filter Spec.	Details and Preparation for Extraction	Purpose
Library Office NTU January 2014	Library Office, Reading Space Polyester, MERV-8 rating, 10 cm thickness	$2 \times 5 \text{ cm}^2$ pieces of filters were cut and extracted panel <ul style="list-style-type: none"> • All replicates from the same filter • 4 pieces for no treatment ($N = 4$) • 4 pieces for with-treatment ($N = 4$) 	Figure 4.4a and 4.4b
Auditorium Hall NTU January 2014	Large auditorium hall for seminar, etc. Polyester, MERV-8 rating, 5 cm thickness	$2 \times 5 \text{ cm}^2$ pieces of filters were cut and extracted panel <ul style="list-style-type: none"> • All replicates from the same filter • 12 pieces, 3 to 1 sample ($N = 4$) • 4 pieces extracted separately ($N = 4$) 	Table 4.2
Activities Centre NTU September 2013	Studying room, game area Polyester, MERV-8 rating, 10 cm thickness	$2 \times 5 \text{ cm}^2$ pieces of filters were cut and extracted panel <ul style="list-style-type: none"> • All replicates from the same filter • 28 pieces for 7 extraction ($N = 4$) 	Figure 4.3a and 4.3b

qPCR standard Curves for GFP, General fungus and general bacteria

Standard Curve for GFP Genes			
Cell counts	Average DNA Concentration by Qubit (ng/mL)	Cp Value Average	Log Concentration
10^7	1480	18.16	3.17
10^6	105	22.62	2.02
10^5	12.5	26.81	1.10
10^4	1	30.75	0.00
10^3	0.1	33.6	-1.00

Standard Curve for Bacteria (16S)		<i>E.coli</i> was used as equivalent representative
Average DNA Concentration by Qubit (ng/mL)	Cp Value Average	Log Concentration
40000	10.52	4.60
4000	13.95	3.60
400	18.02	2.60
40	21.84	1.60
4	26.61	0.60

Standard Curve for Fungus (18S)		<i>Aspergillus.sp</i> was used as equivalent representative
Average DNA Concentration by Qubit (ng/mL)	Cp Value Average	Log Concentration
5000	14.04	3.70
500	17.83	2.70
50	21.42	1.70
5	25.52	0.70
0.5	29.97	-0.30

Potential inhibitor check

Ambient air 24 hours sampling

Cp Value average		Difference (times)	Cp Value average		Difference (times)
Bacteria	27.25	-	Fungus	24.14	-
B:10x	30.32	8.4	F:10x	27.41	9.65
B:100x	33.43	8.63	F:100x	30.71	9.85

AHU filter with high dust content

Cp Value average		Difference (times)	Cp Value average		Difference (times)
Bacteria	21.98	-	Fungus	19.21	-
B:10x	25.12	8.82	F:10x	22.42	9.25
B:100x	28.25	8.75	F:100x	25.64	9.32

APPENDIX B

Indoor environment description for chapter 5

Location	Occupancy intensity (person-h/wk)	General description	Estimated room volume (m ³)	Average air speed (m/s)	Total AHU filter area (m ²)	Volumetric Flow rate (m ³ /s)	Estimated weekly air sampled (m ³ /wk)
Office C	190	Recently renovated staff office Notable Items : a big room with new desks and chairs for staffs. General Activity: Occupancy very low, some computer works, very little walking Flooring: Tile floors	2800	1.7	5.2	8.8	1.9E+06
Office 1	979	Faculty office Notable Items : hallways with separated rooms for faculties, tables and chairs in the rooms, some book racks General Activity: Very quiet, little walkings on the hallways, small meetings in the rooms. Flooring: Tile floors	2500	2.0	4.0	7.9	1.7E+06
Office 2	1518	Research staff/students office Notable Items : a big room with separated large wooden desks for each staff/student, chairs. General Activity: some walking, mostly sitting down while doing computer works, generally empty during lunch time. Flooring: Tile floors	2000	2.1	3.6	7.5	2.4E+06

		Staff office and robotic research centre					
Office 3	1005	<p>Notable Items : Partly filled with wooden desks and chairs for staffs, partly empty spaces for robotic test activities</p> <p>General Activity: Electronic works, computer works, some walking around</p> <p>Flooring: Tile floors</p>	2000	2.2	1.8	3.9	1.3E+06
Office 4	1938	<p>Staff office and computer lab</p> <p>Notable Items : Desks with separators and chairs for staffs, long tables with computers for classes</p> <p>General Activity: Mostly computer works, little walking</p> <p>Flooring: Tile floors</p>	2500	0.5	4.0	2.1	6.9E+05
Classroom	2736	<p>Large Lecture room</p> <p>Notable Items : typical classrooms where everybody is facing the front, chairs and tables, whiteboard</p> <p>General Activity: Students are mostly seated, discussing and listening, lecturer sometimes walk around</p> <p>Flooring: partly carpeted</p>	4350	1.5	13.7	21.1	1.9E+06
Library 1	946	<p>Quiet reading room</p> <p>Notable Items : Book racks filled with books, carts to return books, long tables and chairs</p> <p>General Activity: Reading, computer work, moving books with cards, people walking in and out the room</p> <p>Flooring: Fully carpeted</p>	2000	1.0	7.3	7.2	1.9E+06
		Small reading room					

Library 2	639	<p>Notable Items : Book racks with books, carts to return books, wooden tables, chairs</p> <p>General Activity: Constant walking in and out the rooms, Books being moved with carts, taken and returned.</p> <p>Flooring: Fully carpeted</p>	530	1.1	2.3	2.6	5.1E+05
Library 3	7232	<p>Reading hall</p> <p>Notable Items : Book racks with books, carts to return books, wooden tables, chairs, public computers</p> <p>General Activity: Very heavy walking activities, books being moved with carts, taken and returned, drinking, computer work</p> <p>Flooring: Fully carpeted</p>	4000	1.2	6.4	7.8	2.0E+06

Information on the length measurement and air flow speed measurement

Filter area and room size measurement:

- Length measurement was performed using a combination of ruler, measuring tape and laser distance meter (Fluke 419D, 80m maximum, ± 2 mm maximum error, WA, USA).
- Laser distance meter was used for measuring surfaces which were hard to reach, such as high ceilings or unreachable walls.

Air velocity measurement:

- Velocalc air velocity meter 9545-A (TSI, Shoreview, MN, USA) was used to estimate the air velocity going through the AHU filters. The velocalc is calibrated annually by submitting the instrument to the local TSI branch in Singapore.
- The measurement was performed according to the company's recommendation in an online article titled "Traversing a duct to determine average air velocity or volume" (TSI online article AF-106, available online).
- All of the filters are attached in a rectangular-shaped panels. Briefly, as guided by the above article for rectangular shaped filter panel, 49 points (7 points horizontally and vertically) were determined based on a certain ratio of the filter panel size following the log-Tchebychef method.

- The instrument was set to log a measurement every second. The probe of the velocicalc is placed 1 cm above the filter in operation and a 5-s measurement was made for each of the 49 points.
- The average value for each AHU is determined as the estimated air velocity.