Airborne Microorganisms

A methodology to examine viability of bioaerosols

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Abstract

Transmission through air is a major pathway for spreading of diseases, but much about the process and survival of airborne microorganisms is still unknown. Epidemiological studies investigating the spread of diseases can only yield information on a population level. In order to find out which parameters affect the survival of microorganisms, controlled laboratory studies are required. The aim of this thesis was to construct and validate a setup for generation of airborne *Pseudomonas syringae* and *Norovirus*. The setup consists of a sparging liquid aerosol generator (SLAG), an exposure chamber with a controlled environment and a liquid impinger (BioSampler) which samples into a liquid fluid. Bacterial samples were analyzed with flow cytometry and virus samples with polymerase chain reaction (PCR) to determine quantity and viability. *P. syringae* was aerosolized and exposed to a relative humidity of 25 % and 60 %, with a measured survivability of 58 % and 40 %, respectively. *Norovirus* was aerosolized and collected in a concentration sufficiently high to allow for quantification with PCR. Throughput of the system, i.e. final concentration versus initial concentration in the sample, was measured to be 0.2 % for both *P. syringae* and *Norovirus*. In conclusion, experimental confirmation that controlled laboratory studies on bioaerosol can be performed has been obtained. Optimization to increase the throughput of the setup has been suggested and include: parameter optimization of the generator and possible changes to instrumentation. Future research prospects with the presented method are studies on spreading of diseases and toxicological studies.

Keywords: Airborne, Microorganisms, Bioaerosol, *Norovirus*, *Pseudomonas syringae*, Viability

Sammanfattning

Smittspridning via luften är ett av de mest förekommande sätten för sjukdomsutbredning, men mycket kring processen och överlevnaden av luftburna mikroorganismer är fortfarande okänt. Epidemiologiska studier för att undersöka sjukdomars utbredning räcker inte till och kontrollerade laboratoriestudier behövs för att kunna dra slutsatser kring vilka parametrar som påverkar överlevnaden av mikroorganismer. I den här avhandlingen konstrueras och valideras en uppställning för generering av *Pseudomonas syringae* och *Norovirus* med en SLAG generator, med efterföljande exponering för en kontrollerad miljö och insamling med hjälp av en BioSampler i en vätska. För att bestämma kvantitet och viabilitet, analyseras de insamlade proverna med de mikrobiologiska tekniker flödescytometri och PCR. Analysen påvisade förekomst av mikroorganismer i de insamlade proverna och bevisade en genomströmnings-effektivitet på 0.2 % för både bakterier och virus. Uppställningen klarade dessutom av att bevara viabiliteten hos upp till 58 % av den aerosoliserade bakteriepopulationen. Sammanfattningsvis klarade uppställningen med framgång av att generera bioaerosol och, på ett analys-förberedande sätt, samla in den från luften. Ytterligare arbete med, och optimering av, uppställningen för att öka genomströmningen föreslås, såväl som förslag på passande framtida forskning.

Nyckelord: Luftburna, Mikroorganismer, Bioaerosol, *Pseudomonas syringae*, *Norovirus*, Viabilitet.

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Lund, June 2016

Tobias Svensson

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Abbreviations

(-)RNA	negative sense RNA
(+)RNA	positive sense RNA
5', 3'	five prime, three prime
AGI-impinger	Ace glass, incorporated impinger
ALI	air-liquid interface
ANOVA	analysis of variance
APS	aerodynamical particle sizer
BAD	bubbling aerosol disperser
cDNA	complementary DNA
CE	collection efficiency
Cfu	colony forming units
CPC	condensation particle counter
DMA	differential mobility analyzer
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
E. coli	*Escherichia coli*
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
FD	film drops
FRET	fluorescent resonance energy transfer
HEPA-filter	high efficiency particulate air filter
JD	jet drops
LSA	liquid sparging aerosolizer
MNV	murine *Norovirus*

mRNA	messenger RNA
NIOSH	National institute for occupational safety and health
OD	optical density
P. syringae	*Pseudomonas syringae*
PBS	phosphate buffer saline
PCR	polymerase chain reaction
Pfu	plaque forming units
PMA	propidium monoazide
PVC	polyvinyl chloride
qPCR	real-time quantitative PCR
RH	relative humidity
RNA	ribonucleic acid
RT-qPCR	reverse transcriptase qPCR
SLAG	sparging liquid aerosol generator
SMPS	scanning mobility particle sizer
ssRNA	single stranded RNA
Taq.	*Thermus aquaticus*

Populärvetenskaplig sammanfattning

Spridning av sjukdomar påverkar oss alla, men förvånansvärt lite är känt om faktorer kring spridning via luften. Därför behövs metoder för att undersöka spridning av luftburna virus, och här presenteras ny metod som har utvecklats för att undersöka spridningen av *Norovirus*, orsaken till vinterkräksjuka.

Smittspridning via luften är kanske den smittväg som är allvarligast för oss människor, eftersom det är den som är svårast att förebygga. Detta gäller särskilt för luft inomhus, där naturliga mekanismer såsom solstrålning som minskar förekomsten av mikroorganismer i luften saknas. Ett resultat av detta är att koncentrationen av smittförande ämnen ofta blir högre inomhus än utomhus, och mer långlivade. Vi människor vistas större delen av våra liv inomhus, men i de flesta fall kan vi inte kontrollera luften vi andas. I alla offentliga utrymmen saknar du som privatperson möjlighet att påverka till exempel ventilationen som direkt styr hur bra luften du andas är. Dessutom är det svårt att undvika kontakt eller närvaro av andra människor.

Vinterkräksjuka är en sjukdom som drabbar många människor, antingen direkt eller indirekt. Sjukdomen orsakas av ett virus som kallas *Norovirus* och sprids bland annat genom luften. Det har visats att viruset kan spridas effektivt, även av personer som inte visat några symptom eller redan blivit friska. Exempel på hur otäckt effektiv spridningen av kräksjuka kan vara är när en serie utbrott rapporterades på finlandsfärjan mellan Stockholm och Helsingfors i början av juni 2016. Trots upprepade ansträngningar att sanera båten insjuknade personer på nytt och det hela kulminerade med att alla leksaker på båten brändes. Fler än 200 passagerare uppskattades ha blivit smittade av sjukdomen. Kunde detta ha undvikits?

Genom att undersöka hur luftens egenskaper påverkar spridningen av till exempel *Norovirus* kan sjukdomsutbrott förebyggas. I detta syfte har jag, i samarbete med forskare vid avdelningen för aerosolteknologi vid Lunds universitet, utvecklat och testat en metod för att kunna undersöka hur luften påverkar överlevnaden av *Norovirus*, virusens så kallade viabilitet. Metoden består av att, i en kontrollerad miljö, göra *Norovirus* luftburna och utsätta dessa för olika temperaturer och luftfuktighet. Luftfuktighet och temperatur ändras för att simulera olika miljöer, till exempel vinter- eller sommarväder. Virusen samlas sedan in för att deras genetiska material, RNA, ska kunna undersökas. Analysen berättar hur mycket virus som samlats in och hur många av dessa som fortfarande är viabla. Än så länge har det enbart visats att det går att upptäcka och räkna virus i det insamlade provet, men undersökningen av viabilitet återstår att göra. Informationen om viabilitet kommer öka förståelsen för hur luftens

temperatur och luftfuktighet påverkar virusets förmåga att infektera människor. Jämförelsen kan leda till att vi får svar på frågan varför vinterkräksjuka just förekommer på vintern, genom att fylla i luckan om det är så att vädret ökar spridningen.

Studien har visat att det går att, på ett kontrollerat sätt, skapa luftburna virus och sedan samla in dem i tillräcklig koncentration för analys. Det var just att få en tillräckligt hög insamlingseffektivitet som var den största utmaningen – vilket klarades av med framgång! Den finns flera sätt att förbättra metoden på, till exempel kan uppställningens komponenter anpassas efter vilken organism som ska användas. Det betyder att uppställningen inte behöver vara stationär utan kan varieras efter behov. Metoden som har utvecklats är inte begränsad till vinterkräksjuka, utan andra luftburna smittor såsom influensa eller förkylning kan också undersökas. När mer kunskap om spridningen finns kan inomhusmiljöer anpassas för att förebygga utbrott, till exempel kan ventilationen styra temperaturen och luftfuktigheten för att göra miljön sämre för virusens överlevnad. På så vis kan kanske utbrott som det på finlandsfärjan förebyggas och både mycket lidande och kostnader för samhället undvikas.

1 Introduction

1.1 General background

Winter vomiting disease (also referred to as gastroenteritis caused by *Norovirus*) is one of the most common diseases in developed countries today and is estimated to affect 23,000,000 people in the US only each year (Mead et al., 1999). However, even though it is a common disease, much is unknown about the pathways for transmission, and not even the phenomena of seasonal increases in outbreaks have been fully investigated. A few studies (Hutson et al., 2004, Rydell et al., 2011, Nordgren et al., 2010) of *Norovirus* (formerly known as Norwalk-like virus), the virus causing winter vomiting disease, have been conducted but progress have been severely halted by the inability to cultivate human *Norovirus* (Duizer et al., 2004). Without a proper method for generating viruses experimentally, laboratory studies of spreading and environmental effects have been reliant on patient samples of human *Norovirus* or animal strains of the virus, with few studies as a result (the already mentioned (Nordgren et al., 2010, Hutson et al., 2004, Rydell et al., 2011) as examples). Instead researchers have been limited to epidemiological studies (Greer et al., 2009, Ahmed et al., 2014), an indirect measure of infectivity and survivability of the virus. A major drawback of epidemiological studies is that the causing effect of the observed results can be hard to determine as no effect can be isolated and only statistical correlation on population level can be demonstrated. To unambiguously determine the causing effects of different outcomes, structured laboratory studies are needed where every source of variation can be controlled.

Better understanding of the pathways of transmission and survivability of viruses may lead to improved infection control. The improved infection control can in turn be applied in health care wards, hospitals and other semi-closed public bodies such as schools and universities where diseases easily spread due to well populated closed areas. A lot of the outbreaks of winter vomiting disease occur in health care wards. By knowing more about the routes of transmission of the disease, effective prevention methods can be designed to reduce both the number of outbreaks and the cost for the public healthcare. In addition to decreasing the treatment costs, more knowledge can reduce the number of personnel infected every year, effectively reducing the strain on the personnel of the healthcare system. As it is now, nurses and doctors are forced to meet the infected patients on a daily basis which is why good knowledge about transmission of the disease may decrease the risk of infection and improve cleaning of risk areas.

The Swedish Public Health Authority (Folkhälsomyndigheten) have since 2003 tracked the outbreaks of individual cases of winter vomiting disease caused by *Norovirus* and concludes that it is one of the most common infections in Sweden (Folkhälsomyndigheten, 2015). They continue to establish that the outbreaks significantly increase during winter period with up to 600 new reported outbreaks per week. However, their focus was mainly on the health care system and good knowledge about the occurrence in the society is lacking. Few studies on the cost of the annual outbreaks for the health care system have been performed, but Folkhälsomyndigheten have compiled some regional studies. Eliminating winter vomiting disease would save close to 1 million SEK for any individual health care center or hospital and an estimation suggests that it would save over 100 million SEK per season in total costs.

Airborne microorganisms in general is a field partly neglected with only a few active research groups worldwide. However, the benefits from studying bioaerosols can be immense and highly applicable to society. As many diseases may spread via the air, studies on how well and how long the disease carriers survive in air are essential to create support for epidemiological studies. The spreading of diseases concerns us all and as multi resistant bacteria increase in number, methods of preventing disease rather than treating it need to be developed.

1.2 Objectives

The objective of this thesis was to construct and test an experimental setup for investigation of the effect of aerosolization (the process of making something airborne) on the viability of *Noroviruses*, as well as the bacteria *P. syringae*. This is considered to be the first step in constructing a scientific platform for viability studies of airborne microorganisms. The main components in this thesis are:

1) Creation of a reliable and reproducible method for generating airborne microorganisms.
2) Exposure of the airborne microorganisms to different environmental settings.
3) Collection of the microorganisms in a reliable and suitable manner for biological analysis.
4) Viability analysis of the microorganisms after aerosolization.

The scientific contribution of this thesis is the aerosolization and collection of *Norovirus* under controlled circumstances for investigation of their survivability in air. Ultimately, this thesis aims at answering the questions: Why winter vomiting disease has seasonal occurrence? What environmental parameters of the air affect microorganisms most? What can be done to prevent or reduce the spreading of diseases by air?

1.3 Thesis outline

This report includes a summary of the work performed during this master thesis. The **introduction** includes a general background to the topic, the objectives of the thesis as well as the outline of this report. A scientific background to the different fields and key concepts is presented in the **theory** section in the order: the model organisms (*Norovirus* and *Pseudomonas syringae*), aerosol generation, aerosol collection, viral analysis, bacterial analysis, aerosol instruments and lastly, the scientific contribution of this thesis. After the theory, a detailed description of the experiments follows in the **materials and methods** section. The experiments have been divided into three categories. First the preparative experiments with salts, polystyrene and fetal calf serum are described, then viability experiments with the bacteria *Pseudomonas syringae* and, lastly, the setup of the viability experiments with *Norovirus* are presented. The **results and discussion** section follows the same format as the material and methods section, where the work was divided into preparative experiments, bacterial experiments and viral experiments. First, the results are presented and then discussed directly after to clarify for the reader. After the results have been presented and discussed, a section follows with **reflections on the work** performed, with suggestions how it can be continued and improved. The last section presents the conclusions drawn from the work in this thesis. Additional documents (risk assessment documents, standard curve qPCR) from work during the thesis are available in **appendices A and B**.

2 Theory

2.1 Virus and in particular *Norovirus*

Viruses are the most abundant biological entity in the world and exist in great diversity (Breitbart and Rohwer, 2005). They are considered to be on the border between what can be called living organisms or not, as viruses contain genetic material but only replicates with the help of a host cell (Dimmock, 2007). A virus contains genetic material either in the shape of Ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), protected by structural proteins in the so called capsid. The proteins in the capsid is believed to, in addition to provide shell structure, aid in recognition of cell membranes and in gaining entry into the host (Zheng et al., 2006). Some viruses have an additional outer protein envelope enclosing the capsid (Dimmock, 2007). The diversity of viruses' shows in their set of genetic material, as a species may have anything from single stranded RNA to double stranded DNA. Single stranded RNA can either be complementary (negative sense, (-)RNA) or similar (positive sense, (+)RNA) to messenger RNA (mRNA). Positive sense (i.e. similar to mRNA) can be translated to proteins by the host cell directly, while negative sense (i.e. complementary to mRNA) needs to be converted in the host to positive sense before translation. An extracellular viral particle is denoted a virion.

Once inside a host cell, the virus' genetic material can start to be expressed and replication of virus takes place. Viruses often have co-evolved with their host for a long period of time resulting in high species specificity regarding which organisms a virus can infect. Therefore, transmission between species is unlikely, but if it happens can be very severe as the new host have no native resistance against the new virus. However, similar viruses for different species share a lot of traits and can thus be useful in research when the human strain cannot be examined without risk. For instance, in this thesis murine *Norovirus* (isolated from mice cells) have been used instead of the human *Norovirus* as the latter yet have no standard protocols for cultivation (Bandín and Dopazo, 2011) (though recent findings (Jones et al., 2014) present *Norovirus* infection of human B cells in the presence of enteric bacteria).

Norovirus may differ a lot in size with a diameter ranging from 26 to 35 nm (Prevention, 2009). *Norovirus'* viral genome is positive-sense, single-stranded RNA and the capsid lack an outer protein envelope. Viruses with RNA as genetic material generally experience a higher rate of mutation due to the lack of a proof-reading mechanism of RNA-polymerase (Martínez et al., 2012) and *Norovirus* is no exception (Victoria et al., 2009). Faster mutation means faster evolution which in the end renders vaccines and

host immunity inefficient as the virus constantly changes. This is a major reason behind the struggle to develop efficient vaccines against a lot of viruses in addition to the peaks observed every 2-4 years (Thorne and Goodfellow, 2014) in outbreaks of gastroenteritis (roughly the time needed for the virus to mutate enough for the immunological defense to not recognize it anymore).

The replication of *Norovirus* in a host cell occurs via a negative sense RNA intermediate, i.e. the (+)RNA of the virus is translated into (-)RNA in the host and then back to (+)RNA before amplification (Thorne and Goodfellow, 2014). The replication cycle is illustrated in Figure 1. The presence of (-)RNA can be used as an indicator of viable, infectious virus as it is essential for replication. Positive-sense RNA is very similar to mRNA and can thus be directly translated by the host cell, implying that the RNA on its own can be infectious. The human *Norovirus* strain have not yet been thoroughly studied, which mainly is a result of the inability to cultivate it in cell lines (Duizer et al., 2004). However, the murine counterpart of the *Norovirus* (murine *Norovirus*, MNV) can be cultivated in cell lines (Wobus et al., 2004) and thus it can act as a surrogate for the human strain species.

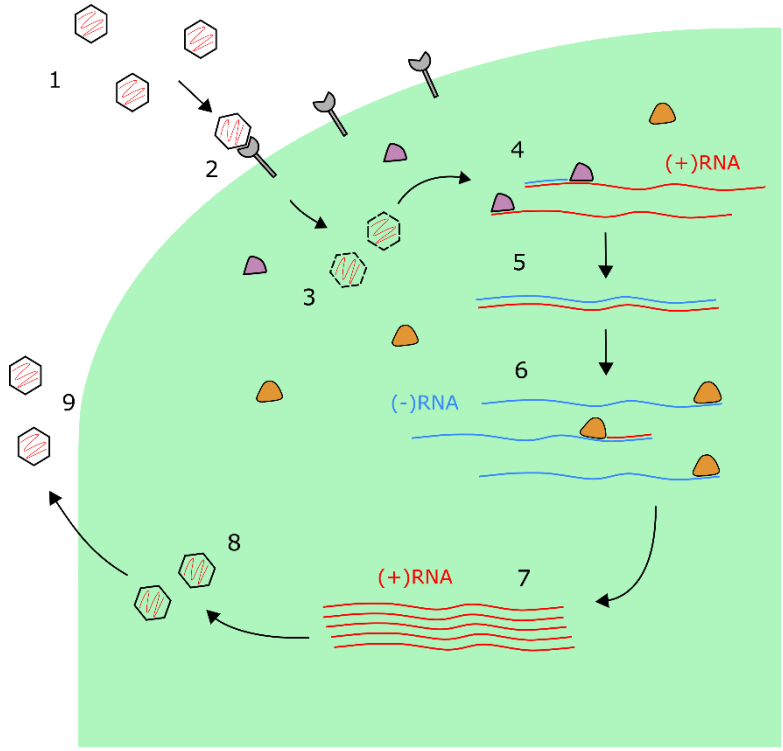

Figure 1. General overview of the replication steps of a Norovirus inside a host cell. 1) Virions target a host and triggers its receptors 2) to enter the host. Once inside, the capsid disintegrates 3) and releases the positive sense RNA. The (+)RNA gets converted into (-)RNA by proteins in the host 4), 5). 6) The (-)RNA then gets converted back into (+)RNA and 7) amplified. The free (+)RNA is 8) ordered and the capsid is re-structured into a virus. The virus exits the host to infect other cells and has now completed its replication cycle

Norovirus has been attributed to be the leading cause of outbreaks of acute gastroenteritis (in some places known as winter vomiting disease) worldwide (Patel et al., 2008), being the cause of nearly 1 out of 5 cases (Ahmed et al., 2014). Furthermore, the study of Ahmed et al. (2014) has shown the prevalence of *Norovirus* to be higher in community and outpatient settings, rather than inpatient settings. The virus have been proven to be very contagious and as few as tens of viruses (or virus aggregates) may cause infection (Teunis et al., 2008).

The main routes of infections is stated by Matthews et al. (2012) to be ingestion of contaminated water or food, or by indirect infection by contact with infected environmental surfaces. However, Marks et al. (2000) present a case where *Norovirus* has been aerosolized during vomiting and spread in the premise. The attack rate (number of people who got ill) of infection was shown to be inversely proportional to the distance to the source of aerosol (the vomiting individual), which proves capabilities of the virus to survive being airborne for at least short times. Other studies (Thornley et al., 2011, Evans et al., 2002, Cheesbrough et al., 2000) have also indirectly proven the *Norovirus* to be resilient and surviving for longer periods of time outside a host. Several studies have, although using incomplete data in most cases, been able to link an initial outbreak of gastroenteritis to sequential cases of illness from 5 days (Evans et al., 2002) up to 3 months (Cheesbrough et al., 2000) afterwards.

A more recent study by Thornley et al. (2011) reports an incident on an airplane used for international travel, where an infected individual vomited and the consequential infection of flight attendants the next seven days were followed. Two days after the initial outbreak, the first crew members got reported sick. New outbreaks among crew members emerged every day for the next seven days, even though different crews worked throughout the week. Thornley states that there were no or little direct contact between different crews and concludes that the infections must have occurred inside the airplane. A longer survival time of the *Norovirus* increases its chances of infecting new hosts in addition to increasing the possibility of further proliferation via re-aerosolization or by other transportation mechanisms. The risk of spreading increases further as the virus can remain for extended periods of time in a host after the symptoms of disease have disappeared (Atmar et al., 2008).

Previously mentioned studies (Matthews et al., 2012, Marks et al., 2000, Evans et al., 2002, Cheesbrough et al., 2000, Thornley et al., 2011) on outbreaks of *Norovirus* all involve infection via indirect contact, e.g. viruses deposited on surfaces. Barker et al. (2004) investigated the transmission of viruses onto surfaces from infected individuals and found it to be worryingly effective, as an infected finger had the possibility of transferring viruses to up to seven clean surfaces sequentially. Barkley continued by investigating the effectiveness of different methods of cleaning and concluded that simply wiping with a detergent-soaked cloth was not enough to disinfect the surface, but rather increased the risk of infection transmission via the hands of the cleaner. To properly disinfect the surface, the use of disinfectant in addition to wiping with detergent needed to be performed. This shows the need for extensive and careful cleaning when dealing with *Norovirus*, and that inadequate cleaning may be the cause of many outbreaks.

Norovirus have been shown to survive outside a host for extended periods of time and can stay infectious in water for at least 61 days, with intact capsids detected after as long as 3 years (Seitz et al., 2011). The quality of surviving outside a host is believed to be of significant importance for the prevalence of virus. Statistical analyses of the prevalence of *Norovirus* have shown seasonal increases in outbreaks during cold, dry periods such as the winter months from January to March (Lopman et al., 2009). However, studies like this contains important limitations that may impose critical concerns regarding the conclusions of the findings. Infectivity is affected by a number of different parameters and not only temperature and relative humidity, which are harder to represent and account for. Such parameters include solar radiation, chemical composition of air and immunity of population (Verreault et al., 2008). Hence, proper in vitro studies under controlled conditions are necessary to deduct reliable information about the effect of environmental parameters on airborne *Norovirus*.

In all the studies mentioned previously in this section (Matthews et al., 2012, Marks et al., 2000, Evans et al., 2002, Cheesbrough et al., 2000, Thornley et al., 2011, Barker et al., 2004) viability of the virion have not explicitly been investigated. Instead analysis have been primarily focused on proving presence of *Norovirus*, *Norovirus* genome and epidemiological investigations of infectivity. Traditional infectivity studies (Plaque assays) can yield information about if, and to what degree, the virus may infect, but it is a rough and protracted process comprised of tedious manual labor due to the cultivation and infection of cells (Gonzalez-Hernandez et al., 2012). Current microbiological techniques such as Reverse transcription real-time quantitative Polymerase Chain Reaction (RT-qPCR) may yield information about presence of *Norovirus*, but in its basic form is not able to determine whether or not the virions are viable. Recent development on PCR techniques (Parshionikar et al., 2010, Vashist et al., 2012) presents the possibility of quantitative analysis of infectious virus using RT-qPCR methods, which will be further discussed in the section Viral analysis.

2.2 *Pseudomonas syringae*

Pseudomonas syringae is rod-shaped, gram-negative bacteria roughly 5 μm long and 0.8 μm wide. The *P. syringae* species consist of approximately 40 pathovars (strains of bacteria with similar characteristics but different on a infrasubspecific level, e.g. different host variability), but is considered to be one species (Hirano and Upper, 1990). It is a plant pathogen, but also known for its ice-nucleation capabilities (Šantl-Temkiv et al., 2015). The bacteria release a protein that facilitates ice formation at higher temperatures in air than otherwise possible. *P. syringae* have been found on various locations in the environment, not limited to biofilms on plants. For instance, the bacteria have been sampled from rain, snow and lakes (Morris et al., 2008). The bacteria have even been isolated from clouds several kilometers up into the atmosphere. The spreading of bacteria between plants and locations are referred to as immigration and emigration. Immigration being transfer of bacteria from the environment onto the

plant, and emigration vice versa. The bacteria are transferred by wind and rain, where wind can both cause emigration and immigration, while rain primarily causes emigration. The rain is believed to sweep the bacteria off the leaves of the plant, a theory supported by the high quantity of bacteria found in rainwater beneath plants after rain (Hirano and Upper, 2000). As the bacteria migrate between host due to weather effects, it is abundant in the atmosphere at all times, which makes it an excellent model organism for aerosolization studies.

2.3 Generation of bioaerosol

Generation of airborne microorganisms is a delicate process regarding their survival during the rather fierce conditions of aerosolization. Most research has been made for bacteria as organism and not so much work on viruses have been conducted (Bonifait et al., 2015, Hermann et al., 2007). As a result of this, the findings on bacterial aerosolization and to what extent it may be translated into implications on virus aerosolization will be reviewed.

The majority of aerosolization methods include processes with high airflows, high shear forces, forceful impaction and possible re-aerosolization. In addition to this, the process introduces the microorganisms into the air, which in itself can be very stressful. Factors such as relative humidity and oxygen toxicity greatly affect the ability of the organism to survive, as the atmosphere usually is drier than the normal environment of the bacteria, which leads to desiccation (drying) of the bacteria(Cox, 1987, Cox, 1989). In addition, radiation have been proven to play a significant role in the survival of airborne bacteria, as longer exposure to certain wavelengths will injure the cell (Webb, 1961).

Even if the process of aerosolization is fierce, naturally occurring bioaerosol generation is all around us. Such processes includes, but are not limited to, our own coughs and sneezes but also wave breaking of the sea and rain droplets bursting (Aller et al., 2005, Joung and Buie, 2015). Bacteria, bacterial fragments, viruses and spores are constantly released contributing to the prevalence of airborne microorganisms in the atmosphere. These natural processes consist of mostly uncontrolled generation, resulting in vastly different size ranges and concentrations. In laboratory experiments it is desired to accurately and reliably generate an aerosol of high concentration with minimal damage to the organisms. The process is sought to be as controlled in every aspect as possible, from size distributions and concentration to the applied stress level to the organisms. In vitro testing with bioaerosols may result in valuable information regarding exposure and health effects, transportation, deposition and control of contamination with microorganisms. Typically, the generation of bioaerosols occur through creation of droplets of a cell suspension and then sequential drying leading to airborne microorganisms. However, in the process a lot of droplets burst and fragmentizes the organisms, rendering them non-viable. The challenge lies in developing a gentle generation of droplets while maintaining a narrow size distribution and high

concentration. In this section a few different techniques will be explained and compared, with a more in-detail description of the Sparging Liquid Aerosol Generator (SLAG) which was the method of choice in this thesis.

Microorganisms is a broad and immensely diverse category of organisms with different properties. They vary in size, structure and function and include anything from viruses and spores to bacteria. Thus different microorganisms have different demands regarding the aerosolization process, and finding a universally suited method will prove to be almost impossible. Studies on aerosolization of microorganisms usually contain either *Escherichia coli* or atmospheric bacteria, e.g. *Pseudomonas syringae,* as model organisms. *E. coli* is the most studied bacteria to date and its properties are well-known. However, it is not an atmospheric bacteria and thus has poor survival during such conditions. *P. syringae* on the other hand is a naturally occurring bacteria in the atmosphere (Constantinidou et al., 1990, Šantl-Temkiv et al., 2015) better suited for the environment and surviving harsher conditions than *E. coli*. It is a requirement to understand the properties of the microorganism of interest to tailor the process according to its needs. Many common bacteria and viruses are cultivated in liquid growth media, e.g. *P. syringae* (Elvira-Recuenco and van Vuurde, 2003)*, E. coli* (Reiling et al., 1985) and *Norovirus* (Wobus et al., 2004), and a common principle of generation, as mentioned before, thus revolve around the creation of droplets as a way of aerosolizing the organisms.

A traditional and widespread method of aerosolizing particles in suspension is the pneumatic nebulization method (also known as the Collison nebulizer) (May, 1973). The Collison nebulizer uses high flows to disperse a suspension into droplets and in the process aerosolizing those. Air flow with high velocity enters the channel section in the middle, and exits through a horizontal orifice. The air creates a jet stream and a region of under pressure in the channel, sucking the liquid suspension up into the jet stream. Once in the jet stream, the liquid suspension experience high shear forces tearing it into droplets which are accelerated out of the orifice. Droplets with large inertia (larger droplets) will impact on the side of the flask and recirculate into the liquid suspension. Smaller droplets will follow the airflow out of the flask and the droplet will evaporate leaving a dry airborne particle.

The Collison nebulizer is an old method and proven to not be very suited for aerosolization of microorganisms, as will be discussed further on in this section. Ulevicius et al. (Ulevicius et al., 1997) presented an improved version of the traditional Collison nebulizer for generation of bioaerosols. The novel instrument, which was named the bubbling aerosol disperser (BAD) after its principle of action, would subject the microorganisms to less shear forces in the aerosolization process. As before, the idea was to create droplets from a liquid suspension of microorganisms with subsequent drying to obtain airborne microorganisms.

While the bubbling aerosol disperser did improve the Collison nebulizer in some areas, there was still further improvements to be done. The next iteration of the nebulizer was presented by Mainelis et al. (2005) and named the Liquid Sparging Aerosolizer (LSA, although commercially available as Sparging Liquid Aerosol Generator, SLAG,

the denotation used in this thesis). Maintaining the principle of creating aerosols from droplets, the SLAG introduced a novel way of creating the droplets. Instead of bubbling a liquid suspension, the liquid was dripped down on a membrane, as seen in Figure 2, and aerosolized by an air flow through the membrane. The air break apart the liquid into droplets and carries the droplets out of the flask. Any droplet too large to follow the airflow will fall to the bottom of the flask, not affecting the process anymore. The large droplet on the membrane will eventually burst, releasing the smaller droplets, as a result of an increasing pressure gradient between the inside and the outside of the droplet. The idea behind this method of droplet generation was to eliminate the recirculation of droplets in the process and make use of a gentler dispersion technique. The aerosolization process subjects the organisms to a lot of stress and repetitive aerosolization will decrease the viability of the organisms.

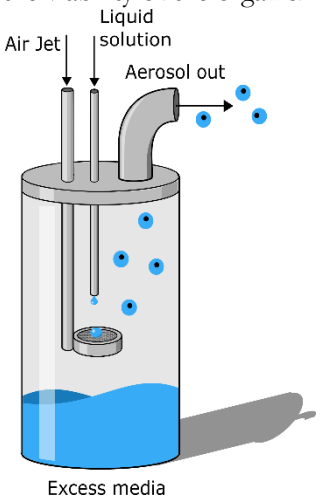

Figure 2. Illustration of the SLAG. The liquid is dripped down on the membrane, where it bursts and releases small droplets into the air. The small droplets contain the material of interest to aerosolize; in this thesis Norovirus.

Several studies have compared the performance of the different methods of bioaerosol generation in regard to various microorganisms and physical parameters. Reponen et al. (1997) investigated the performance of the original Collison nebulizer compared to the then newly developed BAD generator by generating airborne *Pseudomonas fluorescence*, which is a common bacteria found in the ambient air. Their study included a comparison of size distribution and metabolic injury. The Collison nebulizer was shown to produce a broader size distribution than the BAD, but with the same mean size. The concentration of smaller sized particles in the Collison nebulizer increased with longer duration of the experiment suggesting that the process created injury-related fragments from the bacteria. Reponen attributed the high shear forces of the Collison nebulizer as the cause of the fragments. When it came to metabolic injury (measured as culturable cells on complete media versus minimal media, presented in percent values), the difference between the two instruments were greater. A low value of metabolic injury indicates low stress and damage on the bacteria. While the BAD

instrument kept below 1 % metabolic injury for the entire duration of the test, the Collison nebulizer gradually increased up to almost 70 %. Rule et al. (2009) did a similar comparison between BAD and Collison nebulizer, with the bacteria *Pantoea agglomerans,* and found results supporting Reponens findings (15 % survivability loss after 10 minutes with the Collison). However, Reponen also found that the recovery of cells was not affected to the same degree, suggesting that injured cells may recover once on agar. Even so, the stress applied by the Collison nebulizer may be lethal to the cells if any other minor stress element is introduced.

Zhen et al. (2014) did a comparison between the SLAG and the Collison nebulizer, determining their respective characteristics at different working pressures when generating airborne *E. coli*. They found that the Collison nebulizer produced higher concentrations of particles and could function at higher pressures (40 psi for Collison compared to 25 psi for SLAG). However, it was also shown that an increase in pressure increased the fraction of smaller particles produced by the Collison nebulizer, while the SLAG showed no difference. Particles smaller than the known size of the bacteria was considered to be cell fragments, which is the same conclusion as Reponen et al. made. It was concluded that an increase in pressure for the Collison nebulizer also increased the shear forces to such an extent that a majority of the cells were fractionated. Furthermore, Zhen et al. produced evidence that the Collison nebulizer indeed reduced the cultivability of the cells and increased the cell membrane damage compared to the SLAG. This led to the conclusion that the SLAG is a better suited and gentler technique for dispersion of microorganisms into air and the reason for using it in this thesis.

2.4 Collection of Bioaerosol

Microorganisms can be difficult to handle when airborne. Yet they affect us to a great extent (e.g. spreading of diseases) and therefore it is in our interest to study them more. Bioaerosols come in many shapes and varieties, complicating research (Cox and Wathes, 1995). Some microorganisms are naturally airborne and thus suited for the air environment, while others became airborne by chance. No matter the cause of their aerosolization, the need for reliable monitoring is great whether it be to assess workplace hazard or for laboratory studies. As bioaerosols often are living organisms they impose high demands on the detection method. The monitoring should not inflict any harm to the microorganisms to disrupt their viability but still be able to sample high volumes for representative measurements. This calls for thoroughly investigated methods of sampling and sample storage. *Norovirus* are not a common airborne microorganism and little is known about its persistence in the environment.

Aerosol sampling of non-biological substances is often characterized by which sizes it has the possibility to measure, highest particle concentration and sampling efficiency. Sampling efficiency may refer to a variety of different things, but in the general case it is how much that is detected compared to the true sample. The lower amount detected is due to losses by for instance impaction or diffusion in the detection apparatus or

associated equipment. When collecting bioaerosols, an additional even more important factor is introduced: the bioefficiency. The bioefficiency refers to how much of the sample organism that survived the sampling (Griffiths and DeCosemo, 1994). In the case of collecting airborne bacteria, bioefficiency refers to how much of the bacterial population that is viable after sampling versus in the sample (i.e. air). A decrease in viable organisms is a decrease in bioefficiency.

Sampling techniques for inorganic dust aerosol is often unsuitable for bioaerosol as it includes high shear forces, static forces or desiccation which decreases the viability of the microorganisms (Griffiths and DeCosemo, 1994). Sampling of airborne microorganisms vary depending on target organisms and post-sampling analysis method. The largest difference lies in the shape of the collection media, where prominent choices are solid-support sampling, liquid sampling or filtration sampling (Figure 3). Solid-support sampling commonly involves different impactors, with an agar plate as capturing surface (Li, 1999) (Figure 3b). Liquid sampling on the other hand are often varieties of impingers, i.e. bubbling of sample air into a liquid (Grinshpun et al., 1997) (Figure 3c). The third category filtration sampling captures the bioaerosol, as the name suggests, in an air filter (Wang et al., 2001) (Figure 3a).

Solid-support in an impactor is comprised of one or several nozzles, e.g. the 6-stage cascade impactor with a nozzle and solid support for every stage (Mercer et al., 1970), and solid support for collection of aerosol. The radius of the nozzle and air flow rate determine which sizes of aerosols are collected or passing through (i.e. a cut-off diameter). Any particle of a larger diameter than the cut-off diameter is collected through impaction on the solid support as the particle is unable, due to inertia, to follow the airflow as it flows around the solid support. Particles smaller than the cut-off diameter will be able to follow the air stream and pass by the collection plate. An

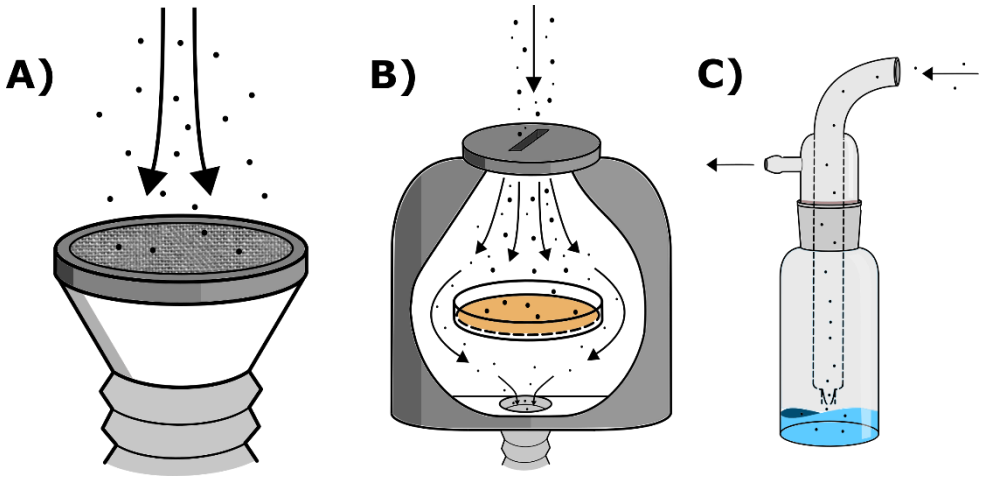

Figure 3. A) Filtration sampling where particles are sucked into and captured by a filter. B) Solid support sampling, where the particles are sucked through a narrow orifice and particles too large to follow the airflow impact on the agar plate. C) Liquid impinger, particles are sucked into the impinger by vacuum and impacted into the liquid.

advantage of the impactors is that the collection of microorganisms may occur directly onto cultivation media, allowing for enumeration without transfer. The cultivation media also increases the organism's chance of surviving the storage. However, the impactors only captures particles larger than the cut-off size and a 6-stage cascade impactor for instance, may only capture particles as small as 0.57 μm (Tseng and Li, 2005). As viruses usually are much smaller than this, it is limited to the capture of virus-laden particles (Verreault et al., 2008).

Filtration sampling have no direct collection media, but instead uses filters with various pore sizes to capture aerosols. The method of capture is attributed to impaction and gravitational settling for large particles, and diffusion and interception for smaller particles. Due to the physical collection properties the filter is the least effective for particles of intermediate sizes (300-500 nm) as neither capturing mechanism works well for these sizes (Kulkarni et al., 2011). Filters are usually characterized by their collection efficiency at a given size. By using filters with different pore sizes various particle sizes can be captured, or by using HEPA (High Efficiency Particulate Air) filters theoretically almost all particle at all sizes can be captured (classified HEPA when filter efficiency above 99.97% at 300 nm). Since the filters does not contain any culture media to keep the microorganisms alive, the sampling duration is limited before a large loss in viability is observed. The captured organisms are subjected to high air flows during the entire sampling process which greatly increases the risk of desiccation (Hinds, 1999). Some filters can be transferred to an agar plate, to allow for cultivation and enumeration of the microorganism. A solution to the desiccation problem may be to use gelatin filters, which decreases the number of cells rendered non-viable by the sampling (Parks et al., 1996). The gelatin filters reduced the desiccation of the cells and thereby improves viability. However, using gelatin filters only prevent desiccation up to 10 minutes where after the cells gradually are rendered non-viable (Parks et al., 1996).

Sampling into liquid, like solid-support sampling, also uses inertial forces (i.e. impaction) as capture mechanism with the addition of diffusion for smaller particles. The most common liquid sampler is the traditional liquid impinger (May and Harper, 1957), which also may be called the AGI impinger (Ace glass, incorporated). The AGI impinger uses inertial forces to collect aerosols and have a horizontal inlet which is curved vertically down into a liquid to imitate the airways of the human nose. Large particles will impact in the curvature, but smaller particles will continue down vertically and exit through a critical orifice. The orifice is located at a fix distance from the bottom of the glass flask. The smallest possible cut-off diameter have been calculated to 0.31 um (Nevalainen et al., 1992). Underpressure inside the flask draws the air through the orifice and in the process accelerating the air. Large aerosols will impact into the liquid, while smaller particles may be captured through diffusion from air bubbles created. However, the air bubbles also contribute to the re-aerosolization of particles as they rise from the liquid and burst.

A notable drawback of the liquid collectors is the evaporation of collection media, limiting the duration of sampling. Even so, studies have shown sample efficiencies with polystyrene latex particles of up to 95 % for particles larger than 0.5 um (Henningson et al., 1988) and with *Bacillus cereus* >98 % for sub-surfaces impingement (Grinshpun et

al., 1997). To achieve such good efficiency, the flow rate is required to neither be too low (inefficient sampling) or too high (bounce off or re-aerosolization). More recent advancement in sampling have tried to minimize the evaporation of the liquid impinger by having several nozzles and introducing a swirling motion of the collection media (Willeke et al., 1998). This was shown to decrease the bubbling which in turn reduces the number of re-aerosolization instances and subsequent stress on the microorganisms (Lin et al., 2000). The research resulted in the commercially available sampler BioSampler (Skc Inc., Eighty Four, Pennsylvania, USA) which was the sampling device of choice in this thesis.

2.5 Viral Analysis

Analysis of viruses can be performed by executing either of several methods, including plaque assays, immunoassays and polymerase chain reaction (PCR) assays. The analysis should provide information about presence of virus, quantity of virus and infectivity. Plaque assay utilizes the infection and subsequent death of cells to determine infectivity. Immunoassays utilizes monoclonal antibodies to mark virus for quantification. PCR depends on amplification of the viral genetic material to quantify viruses.

The plaque assay is the golden standard in viral analysis for measuring the infectivity of the virus. The assay makes use of the viral particles' ability to lyse the infected cells. This is the main advantage as the infectivity can be directly quantified as a result of killed cells. The plaque assay is performed by cultivation of a monolayer of cells on agar plates and sequential infection with the viruses. In the plaque assay for MNV (Gonzalez-Hernandez et al., 2012) the virus-containing sample is prepared in a dilution series where it is diluted 10 times in steps to reach appropriate concentration for quantification (illustration in Figure 4). After incubation, the cells and viruses are covered with gel agarose, which enables proliferation of viral progeny to cells close by, and limits it to cells further away. Infected cells are then lysed by the viruses, leaving empty spots on the agar. The cells are stained with neutral red (a dye that is only incorporated into the cell if it is alive and thus works as a stain for cell viability) to allow for visible quantification. A region where a lot of cells have died is an infected region and called a plaque. A plaque is considered to be the result of one infectious virus and its progeny. The number of plaques per plate is counted and the infectivity of the virus can be denoted in plaque forming units per ml sample (pfu/ml). The dilution series was made to improve certainty of the result, as the number of plaques should be relative to the volume of sample added and level of dilution. This method is very tedious due to the cultivation of cells, and consecutive infection, and takes several days to perform. However, it is a reliable and acknowledged method for quantification of viable and infectious viruses, which otherwise can be a difficult thing to measure. The limit of detection relies on the dilution of the sample, but is in the order of 10 pfu/ml.

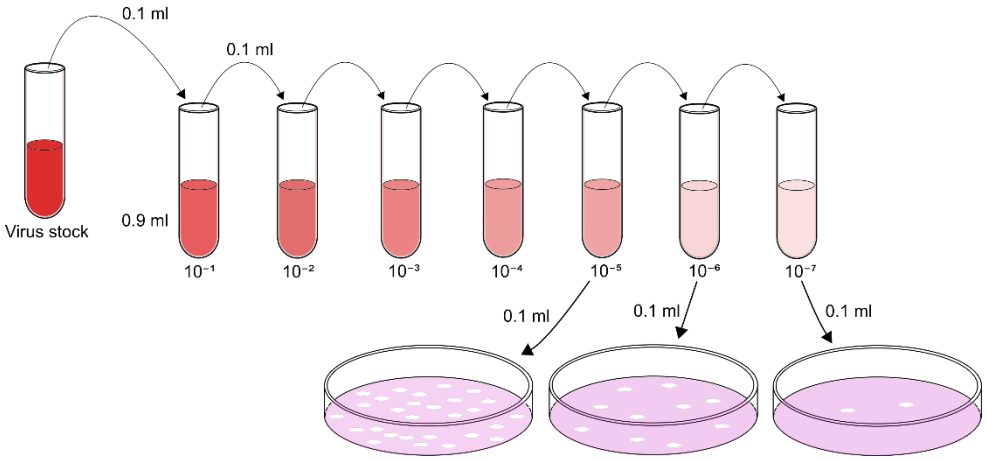

Figure 4. Schematic illustration of the workflow and outcome of a plaque assay. An appropriate dilution has been reached when the visible plaques are in the range of 10-100 per plate.

Immunoassays apply the use of antibodies for detection of viral particles. The antibodies detect proteins (antigen) in the capsid of the virus. The antibodies are in turn marked with enzymes. The enzymes convert a substrate into a detectable product, which allows for detection of viral presence. This principle of immunoassay is called Enzyme Linked ImmunoSorbent Assay, or ELISA, and is a widespread method. Immunoassays are a rather fast and simple technique compared to traditional plaque assays. A major drawback is the need for effective monoclonal antibodies. The viruses evolve fast, and therefore continuous research is required to develop new antibodies (Kele et al., 2011). Several studies (Kele et al., 2011, de Bruin et al., 2006, Gray et al., 2007) have been performed comparing the performance of immunoassays and RT-PCR and found immunoassays to be less sensitive. The assays for detecting *Norovirus* are continuously developing and are gradually catching up to PCR in terms of sensitivity.

Comparison to *Norovirus* PCR-analysis have proven immunoassays methods to be less sensitive, where even de Bruin et. al (2006) found that nearly 40 % of *Norovirus* outbreaks would not be detected using at that time commercially available assays. The immunoassay works well as an initial screening of many samples, but would not suffice as primary diagnosis method of infected patients or for genotype determination (which is done by PCR). A compilation study (Gray et al., 2007) from various countries in Europe have concluded that the result vary greatly, but that the immunoassay method is less sensitive than PCR in all cases. However, a newer study (Kele et al., 2011) done to compare the next generation of immunoassays to PCR shows an improvement in immunoassay performance, although still concluding that the method is not sensitive and reliable enough to compete with PCR for patient diagnosis. Novel techniques arise continuously and improve the performance of the immunoassays. A newly presented bioluminescent enzyme immunoassay (Sakamaki et al., 2012) shows high sensitivity and broad reactivity against various *Norovirus* genotypes and the authors suggest that it may be useful in screening of epidemic and sporadic outbreaks of gastroenteritis. The

method is still very new and need further testing, but it works as an example of a field in continuous progress.

The PCR is a molecular biology method for amplification of genetic material (i.e. Ribonucleic acid, RNA or Deoxyribonucleic acid, DNA). It makes use of DNA polymerase enzymes (enzymes that build up DNA from nucleotides) and the denaturation property of DNA at higher temperatures. The method is used to study a specific sequence of nucleotides, for instance a particular gene. The gene is amplified with PCR until the total amount of the gene has reached satisfactory levels for analysis. Common PCR works in three steps, defined by their different temperatures (Figure 5). Before the initiation of the method, the sample is mixed with DNA polymerase from the bacteria *Thermus aquaticus (Taq)*, free nucleotides as building blocks and primers. The *Taq* DNA polymerase was chosen due to its thermostability. Primers are short complementary sequences of nucleotides that are specific to certain parts of the genome, typically the ends of the target gene. The primers hybridize to the target gene and marks the start of the DNA polymerase synthesis. The heat cycle then begins with increasing the temperature to 94 C, which makes the two strands of DNA denature and the double helix structure is broken. The DNA is then present as single strands. The next step in the heat cycle is to reduce the temperature to 50°-60° C and allow the primers to get attached to the single strands of DNA. This step is referred to as the annealing. The last step in the heat cycle begins by increasing the temperature to 74° C, just below the optimum temperature for the *Taq* polymerase. At this temperature, the *Taq* polymerase starts to synthesize the new strands of DNA from the ambient nucleotides present. The completion of synthetization marks the end of the first cycle and the product will be a nucleotide sequence starting from the primer and ending by chance anywhere after. However, as the heat cycles are repeated and the DNA is amplified, the gene of interest will be multiplied more frequently than the longer sequences. This is due to the fact that the *Taq* polymerase always works from the 5' end of the DNA strand to 3' end (5' and 3' are denotations of carbon groups in DNA

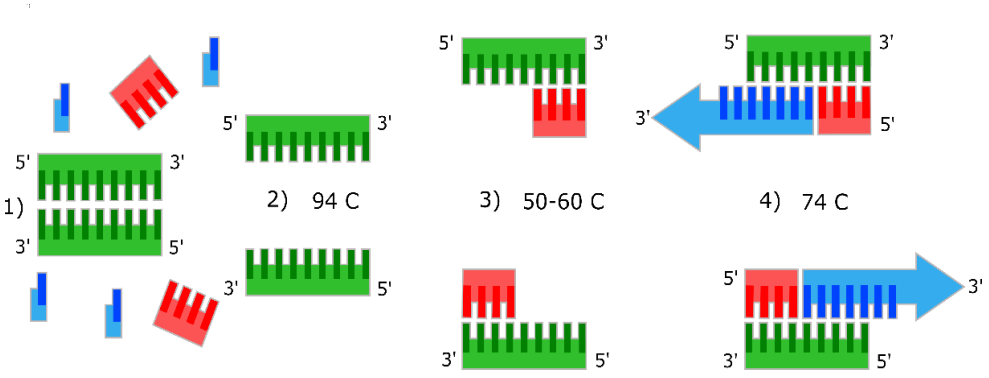

Figure 5. 1) Double stranded DNA (green), primers (red) and free nucleotides (blue). 2) At 94° C the double stranded DNA is denatured, resulting in two single stranded DNA. 3) At 50°-60° C the primers are annealed to the DNA, meaning they latch on to each other. 4) At 74° C the *Taq* DNA polymerase make use of the nucleotides to build up the complementary DNA strand. After step 4), the process is repeated from step 2.

and a strand always have one 5' end and one 3' end. They are used to denote direction on the DNA strand), leading to a product which is defined by the positions on the strand marked by the primer (Brown, 2010).

As *Norovirus* contain RNA as its genetic material, it needs to be converted to complementary DNA (cDNA) before PCR can be used for analysis. This is done by the enzyme reverse transcriptase, and the assay is called reverse transcription - polymerase chain reaction (RT-PCR). The following procedures are the same as for traditional PCR. Next generation of PCR assays implemented real time quantitative PCR (qPCR) with the RT-PCR, to allow for quantification of species containing RNA as well. To monitor the amplification of DNA copies in real time, various fluorescent probes are used. The simplest and cheapest is the molecule SYBR green, which binds to any double stranded DNA (dsDNA) and emits light when excited. However, since SYBR green binds to any dsDNA it has low specificity as it can bind to the primers as well, leading to possible overestimation of the product amplification.

Another, more accurate way to mark the product is to use the Taqman probe. The Taqman probe uses a technique called Fluorescent Resonance Energy Transfer (FRET), where energy is being transferred between two molecules in close proximity (Clegg, 1995). The probe is made to bind specifically to the target sequence of the genome. Figure 6 illustrates the mechanisms behind the Taqman probe. In addition to the binding sequence, the probe contains one reporter and one quencher molecule. The reporter molecule can be excited by a certain wavelength and will fluoresce as a result. The quencher molecule is chosen to absorb light with the wavelength the reporter molecule emits. As long as the quencher molecule is in close proximity (<10 nm) to the reporter molecule, it will absorb all light and no signal will be detected. However, as the *Taq* DNA polymerase starts to build up the DNA it removes the Taqman probe, separating the reporter and quencher from each other allowing the signal from the reporter molecule to be detectable. As the total signal (fluorescent intensity) increases, information on how much product is being formed can be gathered (Trujillo et al., 2006).

A novel PCR technique presented by Parshionikar et al. (2010) displays the use of Propidium Monoazide (PMA) to distinguish between infectious and non-infectious enteric viruses. The PMA have the ability to penetrate damaged capsids of viruses to, after exposure to light, covalently bind to the viral RNA. The covalent bond between the PMA and the viral RNA effectively renders the RNA unavailable for amplification in RT-PCR. Therefore, when proceeding with RT-PCR, it can be assumed that only RNA from viruses with intact capsids is amplified and a positive signal thus implies presence of viruses with intact capsid. However, an intact capsid does not necessarily mean a viable and infectious virus as the virus may be inactivated by other means. An evaluation of the PMA-PCR technique (Karim et al., 2015) has reported that the method only may differentiate *Norovirus* inactivated by capsid damage and not by other means (e.g. receptor damage by chlorination), leaving a possibility of overestimating the number of infectious viruses.

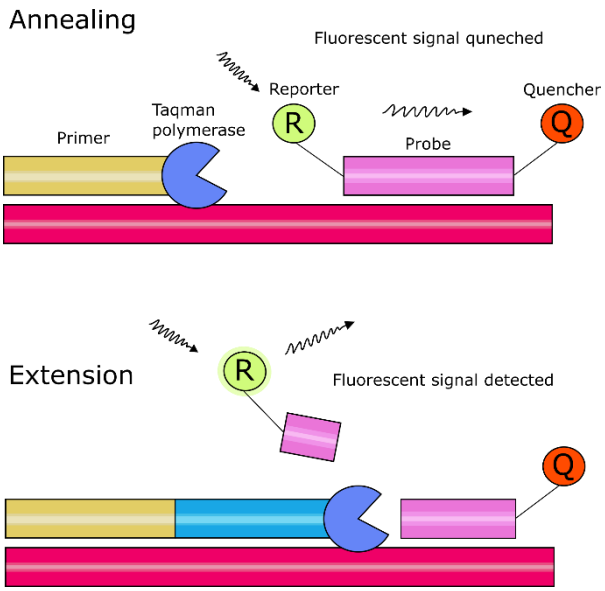

Figure 6. During the annealing phase, the primer and the Taqman probe are attached to the genetic material. Once the extension begins, the Taq polymerase will break apart the Taqman probe, dislocating the reporter and the quencher from one another. Now the fluorescent signal from the reporter is not quenched anymore and can be detected.

Another, more acknowledged method of distinguishing infectious from non-infectious virus have been published using reverse transcription primers specific for (-)RNA (Vashist et al., 2012). By only converting (-)RNA to cDNA, exclusively the (-)RNA will be amplified. (-)RNA from *Norovirus* is, as more thoroughly explained in the section Virus and in particular *Norovirus,* only present when the virus is replicating. Therefore, a presence of (-)RNA indicates *Noroviruses* in the process of replication, which in turn implicates viable and infectious virus. This PCR technique relies on the high specificity of the primer to the (-)RNA strand, as a primer with low specificity may bind to more than (-)RNA and amplify a false response.

2.6 Bacterial analysis – Flow cytometry

Flow cytometry is a biological analysis method where single cells or particles in a directed fluid stream pass through a laser beam. The cells will interact with the laser and scattering, absorbance and possible fluorescence will be detected. The cells are hydrodynamically focused into a small stream by a surrounding laminar sheath flow. The principle of flow cytometry is illustrated in Figure 7. The focusing make sure that one cell at the time crosses the laser (if the concentration of the sample is not too high). The detected signal from the laser can give information of a range of properties of the cells. Forward-scattering will mostly depend on the size of the cell, while side-scattering will depend on intracellular properties as granularity but also morphology. Flow

cytometry provides the user with the option to achieve singe cell information for an entire population of cells. As a result, sub-populations can be detected and visualized. Flow cytometry is used in the majority of the cases for medical and clinical applications, e.g. oncology (diagnosis of cancer) and hematology (blood analysis) (Rieseberg et al., 2001).

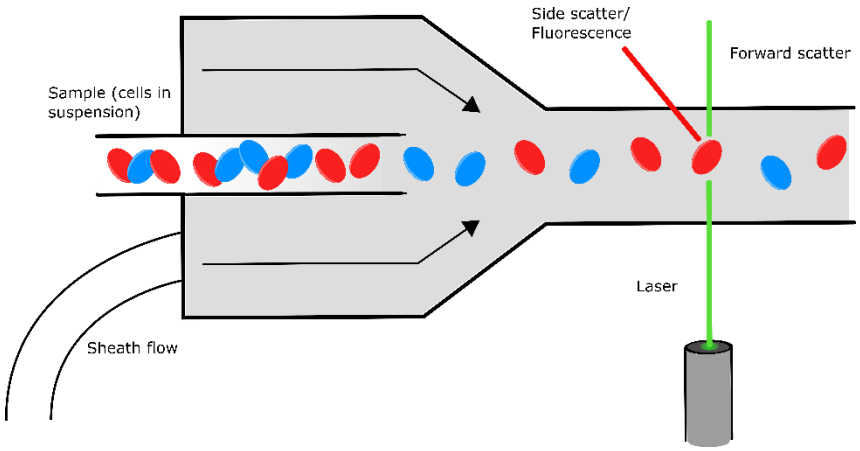

Figure 7. Illustration of the working principle of flow cytometry. The cells enter from the left and are then hydrodynamically focused by the sheath flow into a single stream line. One cell at the time crosses the laser and the scattering/fluorescence is detected.

To yield even more information about the cells, staining with fluorescent dyes can be performed. A fluorescent dye can for example stain the genetic material of the cell. A pair of fluorescent dyes commonly used are Syto9 and propidium monoiodide. They both bind to nucleic acid (DNA/RNA) to yield a fluorescent signal, but only Syto9 can enter a cell with intact membrane. Propidium monoiodide on the other hand can only enter and stain a cell if the membrane is destroyed or incomplete. Therefore, this combination of dyes can be used in conjunction to gain information of the live and dead fraction in a cellular population.

Flow cytometry is a technique mainly applied to mammalian cells. As bacteria are smaller in size than mammalian cells and flow cytometry rely on scattering of light, it might impose some critical limitations. The limitation specifically could be in distinguishing between small cells and cellular debris. Several things to overcome this limitation can be done. By always fluorescently stain the sample, both the fluorescent signal as well as the forward scattering can be used as a dual trigger for detecting a bacterium, giving more reliable results. Staining of the nucleic acid may also give distinct difference between cells and abiotic particles. Lastly, to reduce aggrupation (formation of bacterial clusters) the sample needs to be homogenized before analysis. This can be done by mild sonication or vortexing of the samples (Ambriz-Aviña et al., 2014).

2.7 Aerosol instrument

Aerodynamical particle sizer (APS) is an instrument that relies on light scattering to measure size and concentration of airborne particles. The airborne particles are introduced into the instrument in a sample flow of 1 l/min with a controlled sheath flow of 4 l/min, perpendicular to twin laser beams (Figure 8). A detector registers if a laser beam is scattered and measures the time between the obstruction of the first laser and the second. This time is referred to as the time of flight of the particle. As the flow rate is known and the distance between the lasers are set, the aerodynamic size of the particle can be calculated. Aerodynamic diameter means what spherical diameter with the density of water a particle of unknown shape has the same aerodynamical properties as a particle of unknown shape and density. In this case, the travel speed between the lasers is the aerodynamical property. For example, a rod-shaped particle may move as fast as a spherical particle with the same diameter as the short side of the rod-particle, if it is aligned with the flow. Then, the rod-shaped particle will have an aerodynamic diameter that will be smaller than its actual spatial size. As the APS relies on light scattering, it is limited in the smallest size that can be detected. Thus, the APS is suitable to measure particles between 0.5 – 20 μm in size (Kulkarni et al., 2011).

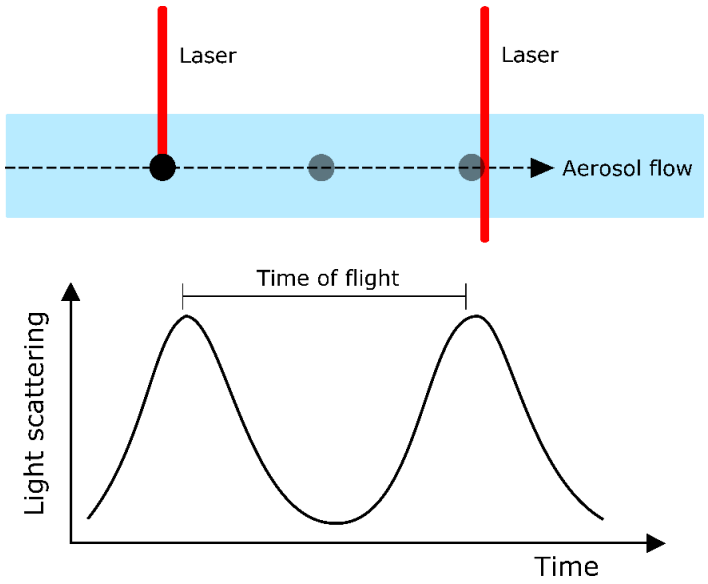

Figure 8. The function principle of the APS. Top: A particle enters from left and crosses the first laser. After a certain time, it crosses the next laser and the time in-between is recorded. Bottom: The registered signal by the detector, with the time of flight marked.

The scanning mobility particle sizer (SMPS) consists of two subparts: a differential mobility analyzer (DMA) and a condensation particle counter (CPC). The DMA electrically charges the particles before introducing them, in a flow, to an electrical field perpendicular to the flow. Depending on their charge, the particles will deviate from

their streamline differently. At a certain distance from the particle inlet, a small slit is placed and serves as outlet. Only particles with a certain charge exits through this outlet. By calculation, the electrical mobility diameter of the particles that exits can be determined. The voltage of the electrical field is then scanned, allowing for a wide range of particle sizes to be chosen for extraction. The particles that exits the DMA are all of the same electrical mobility diameter and are led into the CPC. The CPC counts the particles with a laser. To detect as small particles as sub 10 nm, the CPC condenses butanol onto the particles, effectively growing them up to sufficient size for detection. As particles of one size at the time enters the CPC, a size distribution can be determined. The SMPS system is useful for counting particles between 10 and 1000 nm (Kulkarni et al., 2011).

2.8 Scientific contribution – the context of this thesis

Prominent research on the topic of bioaerosol, and specifically airborne viruses, have been conducted and published by the research group headed by C. Duchaine (Laval University, Quebec, Canada). Especially a few publications are of interest (Bonifait et al., 2015, Turgeon et al., 2014, Verreault et al., 2008) as comparison for the work in this thesis. In these publications they have recognized a similar issue in aerosol and infectivity science that was the foundation to this thesis: the lack of, and need for, controlled laboratory studies on virus survival in air. Verreault et al. (2008) reviewed the research on airborne viruses and compiled it into an knowledge bank for work in this field. However, the novel research of Bonifait and Turgeon is of more interest since their publications are of the first to use molecular biology analysis techniques for airborne viruses. Turgeon et al. (2014) used five different bacteriophages as model organisms to compare three generators, two sampling devices and the effect of the aerosolization media. Their findings determined which of the evaluated generators and sampling devices that were best suited for the aerosolization and collection of bacteriophages. Although their study did not include viability analysis with PCR, it was the first to investigate the damage done to viruses by the aerosolization and sampling.

The study by Bonifait et al. (2015) can be seen as a continuation on the work done by Turgeon, as they realize a laboratory setup for aerosolization of viruses (specifically *Norovirus*, the same virus as in this thesis). The aim of their study and the aim of this thesis very much coincide, but the approach differs on most points. Bonifait relied on Turgeons findings when deciding on generation and sampling device, using a single-jet atomizer and the NIOSH sampler (The National Institute for Occupational Safety and Health, USA). This thesis chose a different approach than Bonifait by using the SLAG and the BioSampler, resulting in different setups even though similar problem formulations. The viability analysis of collected virus samples differs as well. Bonifait used the qPCR technique published by Parshionikar et al. (2010), whereas the analysis in this thesis was done with the qPCR technique published by Vashist et al. (2012). The methods are described in the section *viral analysis* and as presented there, they can

produce different results. The method developed by Parshionikar separates viruses with damaged capsids from viruses with complete capsids. However, not all non-infectious viruses have damaged capsids, but can instead have been rendered non-infectious by other means. The methods described by Vashist on the other hand amplifies negative sense RNA, that only is present during the replication of *Norovirus*. The work in this thesis and the work done by Bonifait complement each other well, as they can act as direct comparison to determine the best setup for laboratory studies of *Norovirus*.

3 Material and Methods

3.1 Safety precautions

The work in this thesis included biological material and to ensure the safety of all personnel involved, a risk assessment was performed prior to all practical work. Valid risk assessment and documentation for work with biological material in risk class 1 (non-infectious/non-pathogenic biological agents (Arbetsmiljöverket, 2005)) existed before this thesis commenced, but needed to be complemented and updated to also cover work with material in risk class 2 (infectious organisms who causes illness that can be prevented, cured or self–healed. All viruses isolated from humans, which do not belong to a risk class of higher degree, belong here (Arbetsmiljöverket, 2005)). The risk assessment for work with *Norovirus* submitted to Arbetsmiljöverket (Eng. Swedish Work Environment Authority) is included, in Swedish, in Appendix A. As Arbetsmiljöverket demands specific safety measures to ensure proper work place safety, laboratory routines for work with agents in risk class 2 were prepared. Regulations listed in AFS 2005:1 (Arbetsmiljöverket, 2005) were followed and the laboratory setup was designed in line with these demands. Examples of precautionary actions taken are the purchase of a table top autoclave (CertoClave, Traun, Austria), appropriate virus disinfectant (DuPont, Wilmington, Delaware, USA) and the manufacturing of a sampling port to enable the construction of a closed system (Figure 9).

3.2 Study design

This thesis involved the construction and evaluation of a laboratory setup for aerosolization of *Norovirus*, the virus causing winter vomiting disease. The laboratory setup included: 1) generation of airborne bioaerosols (primarily *Norovirus*, but the bacteria *Pseudomonas syringae* as well), 2) exposure to controlled environmental settings, 3) representative collection of bioaerosol and 4) biological analysis. An illustration of the complete laboratory setup can be seen in Figure 9. Biological analysis was performed on the collected bioaerosol to prove presence, and determine quantity and viability in the sample. The setup was constructed with the purpose of examining the survival properties of airborne *Norovirus* to enable conclusions on transmission of the virus. The knowledge, routines and hardware gained were intended to act as a foundation for future research on other viruses and bioaerosol of interest.

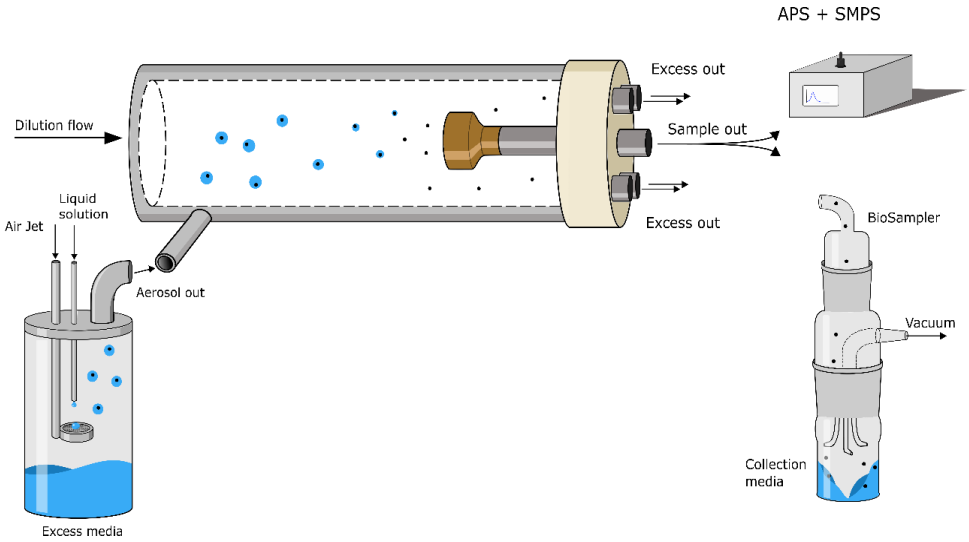

Figure 9. The total laboratory system constructed and used in this thesis. From left: The SLAG creating aerosol which is lead into the exposure tube. From the exposure tube, via the sampling port, continuous sampling occurs to both the BioSampler and aerosol measurement instrument.

The laboratory setup was evaluated with inorganic aerosols (salt and polystyrene latex particles) to study the generation characteristics and concentration homogeneity. In addition, experiments with fetal calf serum (FCS), which is a major component in the cell medium viruses are harvested from, were performed to investigate if FCS could be aerosolized and how much background the viral medium caused. The bioefficiency was determined by performing complete experiments with *P. syringae,* from generation to collection and viability analysis with flow cytometry. Finally, *Norovirus* aerosolization was performed and the samples were analyzed with qPCR.

3.3 Experimental setup

3.3.1 **Aerosol generation**

The generation of aerosol was conducted with a SLAG, Sparging Liquid Aerosol Generator (CH Technologies, Westwood, New Jersey, USA), and with a constant output atomizer (TSI, Minneapolis, Minnesota, USA). The majority of the work was performed with the SLAG, while the constant output atomizer functioned as a comparison, and as a prospect for virus aerosolization.

To generate bioaerosol from the SLAG, a biological sample solution in a syringe with maximum sample volume of 40 ml, was pushed with a speed of 1 ml/min into the SLAG and down on the porous membrane. A constant flow rate of 16 l/min was

supplied to the SLAG as the air jet in (Figure 2, section 2.2 Generation of bioaerosol). The sample aerosol exited the generator with a flow rate of 14 l/min.

The process to generate bioaerosol in the constant output atomizer was similar to the process for the SLAG. A 40 ml syringe containing the biological sample solution was connected to the atomizer to remove the recirculation of sample which is otherwise standard for this type of generator. The sample solution was pushed with 1 ml/min into the atomizer. The input flow of air to the atomizer was set constant to 0.85 l/min (830 mBar overpressure) and the output sample flow to 0.14 l/min.

3.3.2 **Container**

The aerosol from the generator was led via a short connection (5 cm) into a 1 m long metal cylinder with a diameter of 6 cm. The cylinder, from now on referred to as the exposure tube, had two inlet ports on the same end of the tube. The first inlet, axial to the tube direction, was used for the dilution flow and the second inlet, perpendicular to the tube direction, was used as sample inlet (Figure 9). The dilution flow was in all cases HEPA-filtered air and in addition had the possibility to be connected to a simple impinger bubbling device. The impinger had the purpose of generating air with higher relative humidity through bubbling of the air down into distilled water.

The SLAG used a dilution flow of 30 l/min as standard and the constant output atomizer used a dilution flow of 45 l/min. The residence time of the sample in the exposure tube was close to 4 seconds with a total flow of 45 l/min in the exposure tube.

3.3.3 **Sampling**

The outlet of the tube was initially open and, in purpose of creating a closed system, a sampling port was created. The sampling port was specifically designed for this setup with an end cap 3D-printed in polyvinyl chloride (Figure 10). To the PVC end cap, five metal pipes were glued to act as outlet connections. The sampling outlet in the center consisted of a longer metal pipe with a sampling probe attached, reaching 15 cm into the exposure tube to minimize the disturbance from the turbulence from the four surrounding excess outlets.

The sampling from the exposure tube were done in two ways, depending on if the sampling port was used or not. Without the sampling port, a cone-shaped probe connected to a branching of tubes was placed in the end of the exposure tube. The tube-branches led to the different sampling instruments. With the sampling port, the tube-branching was connected directly to the sample outlet in the center of the sampling port. The four excess outlets on the sampling port was connected to a HEPA-filter and led to the waste ventilation.

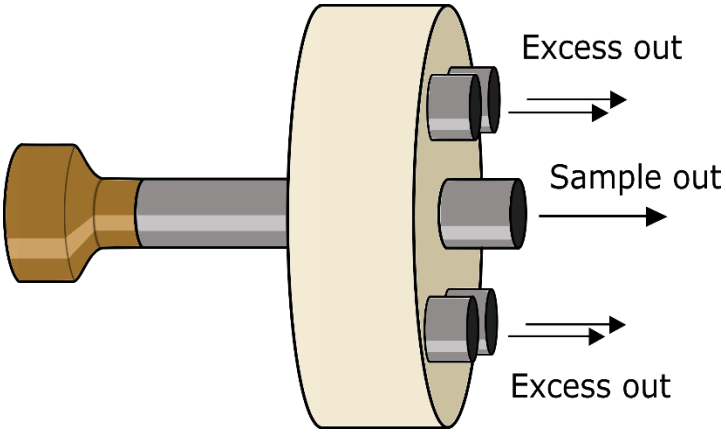

Figure 10. Illustration of the designed and constructed sampling port. The light part was printed in PVC with a 3D-printer and the metal pipes were glued in place. The center pipe reached further into the exposure tube, to decrease the effect of turbulence on the sampling from the excess outlet.

3.4 Analysis techniques and instrumentation

To assess the size distribution of the aerosol generated, two different instruments were used. A scanning mobility particle sizer, SMPS (LTH, Lund, Sweden), was used for particles between 10-650 nm and an aerodynamic particle sizer, APS (TSI, Minneapolis, USA), for particles between 0.5-20 μm. The SMPS had an aerosol flow of 0.95 l/min and a sheath flow of 7 l/min while the APS had an aerosol flow of 1 l/min and a total flow of 5 l/min. The instruments were used for estimation of the number size distribution of the aerosol and to determine the total concentration of aerosolized material.

During the bacterial experiment with *P. syringae*, an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA) was used for quantification and viability assessment of the original bacterial solution as well as the collected bacteria from the BioSampler. The bacterial population counted in the flow cytometry were stained with fluorescent markers for intact cell membrane with Syto9 (ThermoFisher, Waltham, USA), non-intact cell membrane with propidium monoiodide (ThermoFisher, Waltham, USA) or both at the same time. As a reference to the flow cytometry, traditional cultivation on agar plates was performed to determine the number of colony forming units (cfu). Dilution series, with tenfold dilution down to a sample concentration of 10^{-8} of the original concentration, of the bacterial samples were prepared and the most diluted samples were cultivated on the agar plates and enumerated.

Analysis of collected *Norovirus* were performed according to a RT-PCR protocol in a qPCR instrument (ThermoFisher, Waltham, USA). All analysis with PCR were performed at the division of clinical virology (Faculty of medicine, Lund university) by a lab technician, according to the procedure described by Vashist et al (2012). The RNA was extracted from the sample, cDNA was synthesized and the product amplified and quantified in the qPCR. qPCR for positive sense RNA was the only PCR analysis performed, the negative sense did not yet yield reliable results. The positive sense RNA yielded information about the total quantity of virus in the collected sample.

3.5 Experiments and calculations

3.5.1 **Validation of setup with non-biological particles**

Phosphate Buffered Saline (PBS) was used as aerosolization agent to investigate the size distribution and initial droplet size generated by both aerosol generators. From the size distributions, support for a theory about the mechanisms of aerosolization in the generators was created (presented in the discussion). The sizes of dry particles in the generated distribution were used for calculations of initial droplet sizes. By knowing the volume of the dry particle ($V_{dry,}$ assuming all water has evaporated) and the concentration of salt in the PBS solution (D_f, dilution factor, in this case the salt was diluted 125 times), the initial droplet size (d_{wet}) can be derived from the droplet volume by calculations of the particle mass according to equation 1a and 1b (M_{dry} is the dry particle mass, ρ_{dry} is the dry particle density, ρ_{wet} is the wet particle density, V_{wet} is the initial droplet volume).

(Eq. 1a)

$$M_{dry} = V_{dry} \times \rho_{dry}$$

(Eq. 1b)

$$d_{wet} = \sqrt[3]{\frac{6\,V_{wet}}{\pi}} = \left[\, V_{wet} = \frac{M_{dry}\,D_f}{\rho_{wet}} \right] = \sqrt[3]{\frac{6\,M_{dry}\,D_f}{\pi\,\rho_{wet}}}$$

Theoretical evaluation of the mixing of sample flow and dilution flow was done by calculating Reynold´s number (Eq. 2). Reynold´s number indicates if the flow in a vessel is laminar (only diffusive mixing) or turbulent (convective mixing). To ensure homogenous sample concentration throughout the exposure tube, turbulent flow was desired.

(Eq. 2)

$$Re = \frac{\rho\,v\,D}{\eta}$$

The Reynold´s number (where ρ density, v velocity, D diameter of the tube and η viscosity) should exceed 2000 for turbulent flow, otherwise it is considered laminar. Displacement by diffusion (mean square displacement) in a laminar flow was calculated by using equation 3

(Eq. 3)

$$x_{rms} = \sqrt{2Dt}$$

where *D* is the diffusion coefficient for the particle and *t* is the time. This yields the mean distance a particle of a specific size has moved by diffusion in the time given.

To ensure that the sampling port did not affect the collection efficiency of the system, a throughput test was conducted. A solution with the food dye Brilliant blue (Dr. Oetker, Bielefeld, Germany) in MilliQ water was aerosolized and the concentration of the solution was estimated by measuring absorbance at 400 nm. The absorbance of the solution was measured before and after aerosolization on the original solution and the solution in the BioSampler respectively. Collection efficiency (CE), in %, was calculated as

(Eq. 4)

$$CE = \frac{abs_{after}}{abs_{before}} \times 100$$

In addition, a test was performed to determine whether the sampling efficiency would be different when sampling only small particles (<100 nm, as the color pigment in brilliant blue) or a mix of small and large particles (>1 μm). The color solution with Brilliant blue in MilliQ water was mixed in 0.1 % w/v NaCl in MilliQ solution, and the throughput was calculated according to Eq. 4.

Losses of small particles (<100 nm) in the tube was theoretically estimated by calculating the deposition due to diffusion. Only losses due to diffusion were considered as the particles of interest were smaller than 100 nm. The diffusion losses were calculated from the penetration efficiency, P,

(Eq. 5)

$$Diffusion\ losses = 1 - P = 1 - \frac{n_{out}}{n_{in}} = 5.5\,\mu^{2/3} - 3.77\,\mu$$

where n denotes total number of particles and μ is a dimensionless deposition parameter (Hinds, 1999). The deposition parameter (μ, eq. 6) is a function of the diffusion coefficient (D) for the particles of interest, the length of the tube (L) and the volumetric flow rate through the tube (Q).

(Eq. 6)

$$\mu = \frac{D\,L}{Q}$$

Experimental examination of concentration homogeneity in the exposure tube was conducted by using 50 nm PSL particles. These particles were chosen because they are

in the same size range as *Norovirus*. Spatial repositioning of the sample probe to five different positions in the outlet of the exposure tube (open-ended exposure tube) indicated if the concentration was not homogenous throughout the tube. This was performed to evaluate if the concentration was homogenous in radial direction of the tube (e.g. if proper mixing occurred in the tube or the flow was laminar).

Furthermore, the impact of the dilution flow on total aerosol concentration of PBS was evaluated, as well as if the location of the sample flow inlet affected the mixing. The sample flow inlet was tested at two different locations: radially into the dilution flow in the beginning of the exposure tube (as in Figure 9), and axially into the dilution flow before the exposure tube. The effect of the dilution flow was studied at four levels: 0, 15, 30 and 45 l/min.

3.5.2 Aerosolization of bacteria – *Pseudomonas syringae*

The atmospheric bacteria *Pseudomonas syringae* was aerosolized with the SLAG to evaluate the bioefficiency of the experimental setup. As bacteria generally are more sensitive than viruses, it was reasoned that the survival of *P. syringae* would be lower than for *Norovirus*. A cultivation of *P. syringae* was concentrated 10 times by centrifugation and subsequent resuspension in a sodium chloride solution (0.1 % w/v NaCl in MilliQ water) before aerosolization to a final OD of 1.3.

The collection of bacteria was performed with the BioSampler (Skc Inc., Eighty Four, Pennsylvania, USA) liquid impinger sampling device. The flow into the BioSampler was constant at 12.5 l/min for all experiments. The collection media consisted of 20 ml 0.1 % w/v NaCl in MilliQ water. Every sample was aerosolized and collected for 30 minutes to allow for high enough concentration in the collected sample to measure. This corresponded to 30 ml of sample solution introduced into the SLAG generator. *P. syringae* was aerosolized in biological duplicates at two different levels of relative humidity (RH), 25 % and 60 %. Throughput of the system, in %, was measured as

(Eq. 7)

$$Throughput = \frac{C_{col}}{C_{ini}} \times 100$$

where C_{col} is the concentration of bacteria in the collected sample and C_{ini} is the initial concentration of bacteria before aerosolization.

3.5.3 Aerosolization of virus – *Norovirus*

Norovirus was received in batches of 5 ml solution with DMEM (Dulbecco's modified eagle medium) cellular growth medium (Biochrom, Cambourne, United Kingdoms) and 10 % FCS. The viral solution was diluted to 40 ml with PBS to achieve a larger amount of liquid to aerosolize with the SLAG. To adjust for the background,

aerosolization of growth medium diluted in PBS were performed. In addition, PBS were aerosolized with and without FCS as a component to determine the effect of having proteins in the aerosolized solution.

The aerosolized viruses were reasoned to be coated in salts and proteins from the growth medium, effectively changing the properties of the virus particle. To estimate the thickness of such a potential coating layer, a rough mathematical model was formulated. The model builds upon, and includes, the assumptions: completely spherical particles; 30 nm in diameter viruses; only one virus per coated particle; the coating layer has the same volume as a dry particle without a virus. In the following mathematical derivation, the volume of a particle consisting of a virus with coating will be denoted V_{vp}, a particle without virus V_{dry} and the volume of a virus V_{virus}.

(Eq. 8)

$$V_{vp} = V_{virus} + V_{dry} \rightarrow d_{vp}^3 = d_{virus}^3 + d_{dry}^3$$

Equation 8 is the central assumption in this model and describes that a coated virus has the same volume as a virus and a dry non-virus containing particle together (Figure 11). The thickness of the coating layer can then be stated as Eq. 9, where *d* denotes diameter.

(Eq. 9)

$$d_{coating} = d_{vp} - d_{virus} = \sqrt[3]{d_{virus}^3 + d_{dry}^3} - d_{virus}$$

The diameter of the dry particle was measured with the aerosol sampling instruments. A particle present when only aerosolizing salt solution was assumed to be a non-virus containing particle.

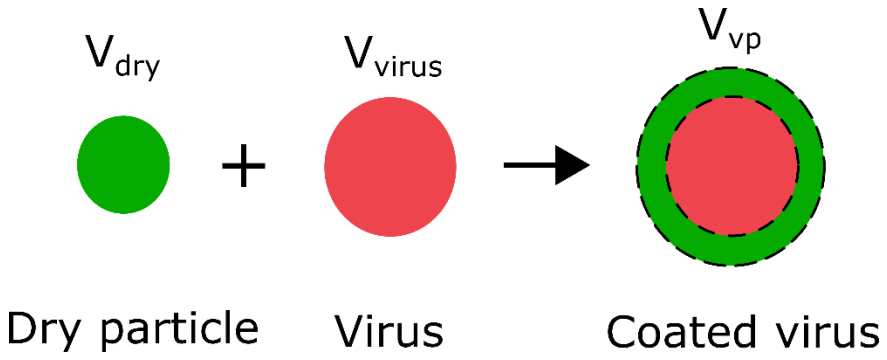

Figure 11. Illustration of the assumption behind the coating thickness equation.

Throughput of the system, in %, was measured as

(Eq. 10)

$$Throughput = \frac{C_{col}}{C_{ini}} \times 100$$

where C_{col} is the concentration of virus (copies of RNA) in the collected sample and C_{ini} is the initial concentration of virus (copies of RNA) before aerosolization.

3.6 Model organisms

Pseudomonas syringae was acquired from the microbiology section at the Department of biosciences, Aarhus University, Denmark. The bacteria were originally isolated from rain and snow samples. Bacterial solutions used in this thesis were cultivated and prepared at the division of biotechnology, Lund University. The cultivations were inoculated in the late afternoon and left in room temperature, with no external oxygen supply, on a shaking plate at 120 rpm overnight to allow the bacteria to reach the stationary phase. The cultivations were done in R2A (Reasoner's 2A) broth, a complex growth medium for slow growing bacteria. The cultivations had a final optical density, OD, of 1.3-1.4.

The murine *Norovirus* was acquired from the Division of clinical virology at the Faculty of medicine, Lund University. The murine *Norovirus* was used as a model organism for the human strain of the same virus. The virus solution was prepared by the laboratory technician of the virus lab according to their standard procedures. The *Norovirus* was harvested from murine macrophages in cellular growth medium DMEM 1X 3.7 g/l $NaHCO_3$ with 10 % FCS (VWR, Radnor, Pennsylvania, USA) and received in 5 ml frozen batches.

3.7 Statistics

To determine if significant difference was present between measurement series, ANOVA (analysis of variance) tests were performed with a follow up Tukey-Kramer test if more series than two existed. The ANOVA determines if there is significant difference between a number of measurement series, but not which it is that differ. The Tukey-Kramer test allows to test the samples against each other to specifically determine where the difference arises from. The p-value was in all cases set to 0.05.

4 Results & Discussion

4.1 Validation of setup with non-biological particles

4.1.1 **Generator characterization**

Initial droplet size from the aerosol generators calculated (according to eq. 1a and 1b) from the highest concentration dry particle sizes (Figure 12 and Figure 13) of aerosolized PBS (Phosphate buffered saline) showed that the particles shrink to approximately one sixth of the particle size after the water have evaporated (Table 1).

Table 1. Droplet size of two different aerosol generators calculated from aerosolization of PBS buffer.

SLAG		**TSI Atomizer**	
Dry size (nm)	Droplet Size (nm)	Dry Size (nm)	Droplet Size (nm)
25	161	17-32	109-206
130	837		

The size distribution of PBS from the SLAG yielded two distinct peaks at 25 nm and 130 nm (Figure 13). 1 % FCS (fetal calf serum) in PBS proved to yield roughly the same size distribution as the PBS without FCS. 10 % FCS in PBS did however yield another size distribution with three peaks at 15 nm, 40 nm and 175 nm instead. The same solutions aerosolized by the TSI atomizer generated very different size distributions (Figure 13). For PBS and 1 % FCS in PBS, a single broad peak at 20 nm was observed. 10 % FCS in PBS did not give a distinct peak, but rather a decrease in concentration as the size increased. 10 % FCS was concluded to affect the aerosolization to a great extent.

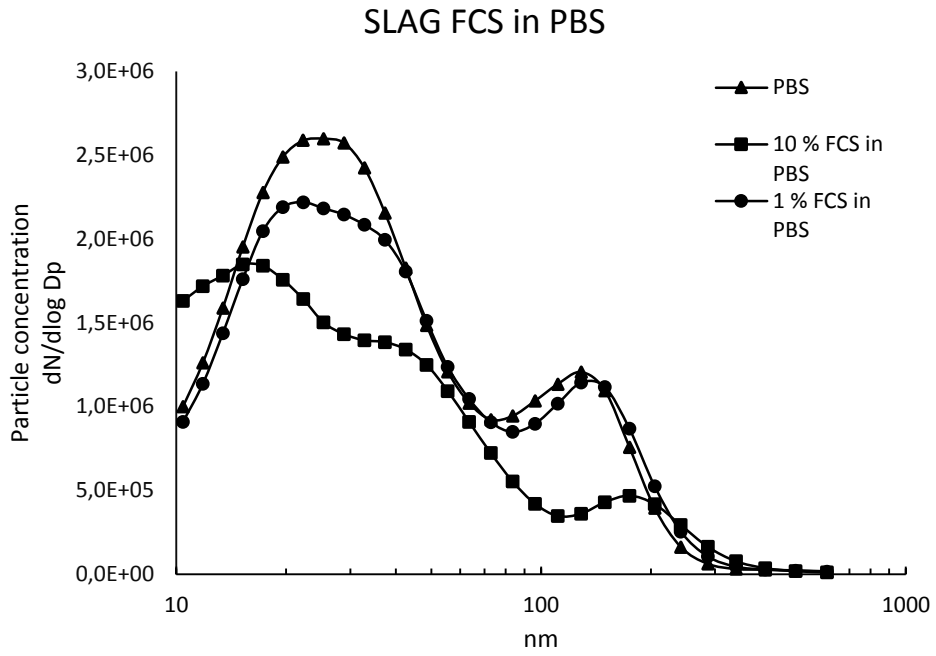

Figure 12. Size distribution of 10 % FCS in PBS, 1 % FCS in PBS and solely PBS particles smaller than 600 nm generated by the SLAG.

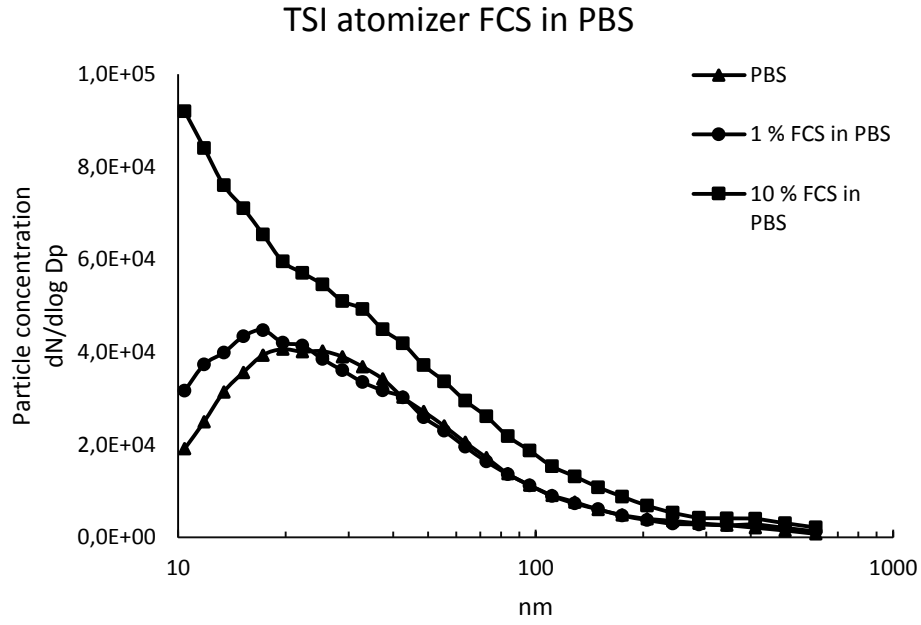

Figure 13. Size distribution of 10 % FCS in PBS, 1 % FCS in PBS and solely PBS particles smaller than 600 nm generated by the TSI atomizer.

The two tested aerosol generators have different formation mechanisms which results in different size distributions of the same aerosolized solution. The SLAG relies on the bursting of bubbles to form aerosols and the process can be compared to the formation of marine aerosols by sea spray (Agranovski, 2011) (the mechanisms are illustrated in Figure 14). Bubbles of the solution in which the prospective aerosol is present are formed and when the bubbles burst, small droplets are ejected. These droplets originate mainly from the surface of the bubble and are denoted film drops (FD). After the bubble burst, a void is left which causes the bubble to implode on itself. During the implosion larger droplets are ejected into the air in a jet stream. These secondary droplets are denoted jet drops (JD). FD are most often smaller and more numerous than the JD. These two processes are believed to be the underlying mechanism for the double peak observed in the size distribution of PBS aerosolized in the SLAG.

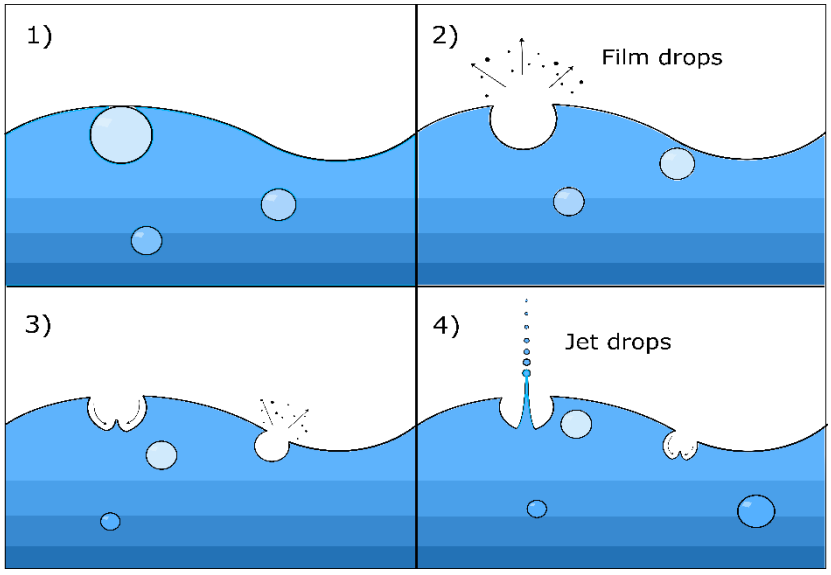

Figure 14. Bubble burst mechanisms of forming aerosol. Both film drops and jet drops included in the image. 1) A bubble rises to the surface. 2) When reaching the surface, it bursts, emitting small droplets called film drops from the thin film of compound on top of the liquid. 3) After the burst, a void is left behind and the bubble will implode on itself. 4) The liquid falling back into the bubble will create a jet stream emitting larger droplets, jet drops.

Recent studies have shown the formation of aerosols from raindrops hitting a solid surface by bubble bursting (Joung and Buie, 2015). The scenario of raindrops hitting a surface is comparable to the process in the SLAG where a solution is dripped onto a porous filter. Joung's study proved that the raindrops captures air bubbles at the water/solid interface when impacting on the surface, which then rises through the droplet and release aerosols upon bursting. The mechanism in the SLAG can be assumed to be more violent as an air flow is supplied from beneath the porous filter, reasonably increasing the number of bubbles while also causing a never ending supply of bubbles.

The mechanism of the constant output atomizer is different and can be compared to a third sea spray mechanism, so called spume drops. Spume drops are generated when sufficiently high wind speeds tear the crest of the waves directly (Lewis and Schwartz, 2004). This mechanism results in a more polydisperse aerosol (Figure 13) compared to the two mechanisms forming aerosols in the SLAG. The formation of spume drops requires high wind speeds, which is created in the TSI atomizer as the air jet enter with high speed. Spume drops from the sea are prominently large droplets, but as the TSI atomizer has a small channel where the aerosol is ejected into directly after aerosolization, large droplets will impact on the wall. The impacted droplets are effectively removed from the aerosol, resulting in an aerosol consisting of only the smaller droplets that are able to exit the atomizer without impaction.

Comparing the three solutions (PBS, 1 % FCS and 10 % FCS) to each other, the SLAG proved to yield less aerosols for the 10 % FCS solution while the TSI atomizer yielded more aerosols for the same solution. However, both generation methods have in common the increase of small (10-20 nm) aerosols. This increase may be explained by the formation of small protein agglomerates or even single protein aerosols. The geometrical diameter of BSA have been measured to be 3 nm (Su et al., 1998). Thus particles in the sizes of 10 - 20 nm may be small agglomerates of proteins or salt coated in protein. As the SLAG have similar mechanism of generating droplets as sea spray it may give a hint that small clusters of, or even single, proteins may be aerosolized even during natural circumstances. The result proved that a solution with 1 % FCS did not yield different sizes or concentrations of aerosol than a salt solution. However, the aerosol generated from a 10 % FCS solution did differ to a greater extent and as both the concentration and sizes became different, it was decided to not aerosolize virus solution with 10 % FCS.

4.1.2 **Homogeneity in the sampling tube**

The results from the concentration homogeneity tests proved that the position of the sample inlet did matter (Table 2). For radial inlet of sample, the concentration differences between five different positions was not significant (at p-value 0.05). When inlet of sample occurred axially to the drying flow, a significant difference (at p-value 0.05) in concentration was observed. The concentration difference was tested with an ANOVA-test.

Table 2. The dependence of sample inlet position on the concentration difference in the sample outlet position. Radial inlet refers to 90° difference in sample and sheath direction. Axial inlet consequentially has 0° between sample and sheath inlet direction.

Sample inlet	Concentration difference
Radial	Not significant
Axial	Significant

These findings were supported by theoretical calculations of the Reynolds number to ~700, which implies laminar flow in the exposure tube. If the flow is laminar, no turbulent mixing occurs and the air streams will follow alongside each other and only mix by diffusion. When the sample inlet was axially placed, the sample flow and the dilution flow were positioned on different flow paths from the beginning, causing them to only mix by diffusion. The residence time in the exposure tube was 4 seconds and as a result, diffusion did not have enough time to allow for effective mixing. Calculation of the mean square displacement by Brownian motion for a particle with diameter of 50 nm during the present conditions, showed a mean displacement of only 130 μm. Compared to the diameter of the exposure tube, which was 6 cm, this is an insignificant distance. As a result of the inadequate mixing, there will be heterogeneity in the sample concentration throughout the tube. The radial inlet injected the sample flow perpendicularly into the dilution flow and thus the flows mixed from the start. Therefore, as the work continued, the radial sample inlet was utilized to ensure proper sample mixing.

4.1.3 **Throughput of the system**

The experiment without sampling port versus with the sampling port (when using a dye mixed with a salt solution), proved that the difference in throughput efficiency was negligible (Table 3). When using a dye solution mixed with only MilliQ water the difference was larger although not large. The aerosol generated from the blue dye solution was monodisperse with a peak concentration at 50 nm particles, roughly the same size as *Noroviruses*. Overall, the throughput was low at only 2 % and a lot of material was lost in the process.

Table 3. Throughput measured by using a blue dye and measuring absorbance at 400 nm. The dye was either mixed in salt solution or MilliQ water. Calculated according to Eq. 4.

	Open tube sampling (MilliQ)	**Sampling port (NaCl)**	**Sampling port (MilliQ)**
Throughput efficiency	2.32 %	2.27 %	2.10 %

Losses due to deposition by diffusion on walls in the exposure tube were calculated using equation 4, to determine if the diffusion caused the low throughput. Theoretically, only 0.12 % of the particles with a diameter of 50 nm would diffuse and impact on walls in the tube (Table 4), leading to the assumption that the majority of the measured losses occur somewhere else in the system.

Table 4. Theoretical losses due to diffusion in the exposure tube presented as % of total number of particles lost for different particle sizes. The losses are calculated for a 1 m exposure tube and a total flow of 45 l/min.

Particle size (nm)	Losses in %
25	0.28
50	0.12
150	0.03

The total particle concentration measured for different flow rates on the dilution flow in the exposure tube proved an exponential decrease in concentration when the flow increased (Figure 15). Each flow rate is a mean of a minimum of three individual concentration measurements. The red dotted line shows the exponential fit to the data, which allows for interpolation.

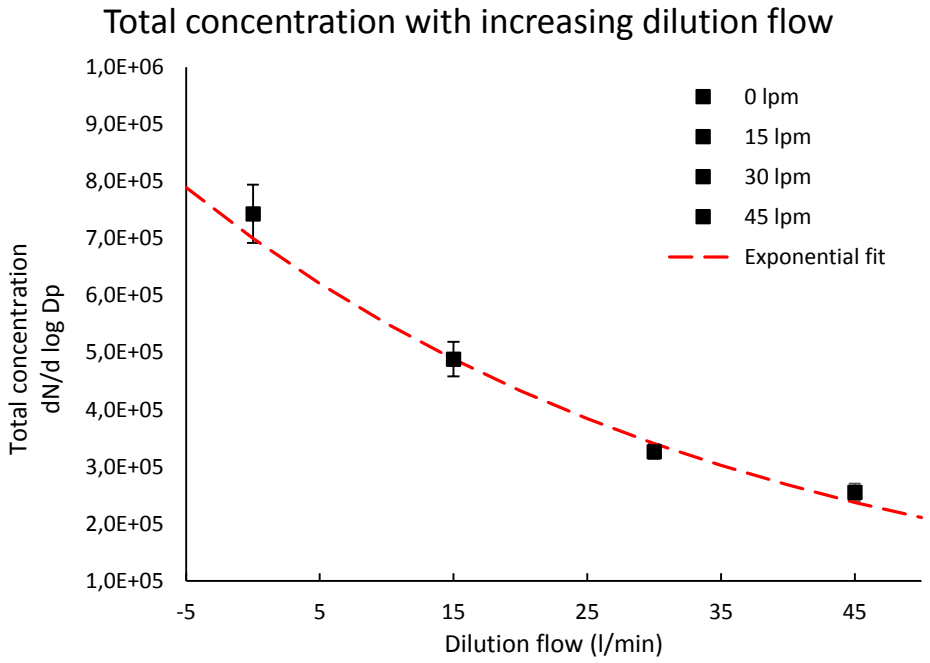

Figure 15. Total particle concentration of aerosolized PBS solution versus dilution flow. The concentration was measured for four flow rates on the dilution flow, and for each flow rate a minimum of 3 individual measurements were collected. The red dotted line shows an exponential fit to the measured data with a R^2-value of 0.98.

Diffusion was not the major cause for the low throughput as it only accounted for less than 1 percentage of the losses. Instead, the most probable place for the losses is in the generation process in the SLAG, as only 17 % of the sample liquid was aerosolized. The SLAG is a novel generator and more work to optimize generation efficiency may improve the performance of it, but complete elimination of losses does not seem

feasible. Therefore, it is of most importance to generate an aerosol of high concentration to be able to have a detectable concentration in the biological sampling.

The purpose of the dilution flow was to ensure that a measureable aerosol concentration for the instrumentation was achieved, as well as making sure dry particles, not droplets, were measured. In addition, it controlled the environment for the sample, and the change of RH in the exposure tube was achieved by changing RH of the dilution flow. The information on how the total particle concentration is affected by the dilution flow, even though not used much in this thesis, may be useful for future work on the setup. For example, knowing the relation between the dilution flow and the total particle concentration allows for comparison between experiments with different flow rates on the dilution flow. A measured concentration at one flow rate may thus be compensated for, and it can be extrapolated what concentration it would correspond to at other flow rates. Changing of environmental parameters such as RH and temperature may involve a change of flow rate. Therefore, it is useful to have a relationship between concentration and flow rate to compare measurements.

4.2 Aerosolization of bacteria - *Pseudomonas syringae*

4.2.1 **Size distribution**

The size distribution of the aerosolized *P. syringae* measured with the APS for both a lower and a higher level of relative humidity showed a shift to larger sizes for the bacteria in humid air (Figure 16). The distribution for the lower humidity has a distinct peak at 0.8 μm, which was considered to be close to the actual size of the bacteria. The size distribution for the higher RH instead has a peak at 0.9 μm, a result of less evaporation of water from the droplet particles.

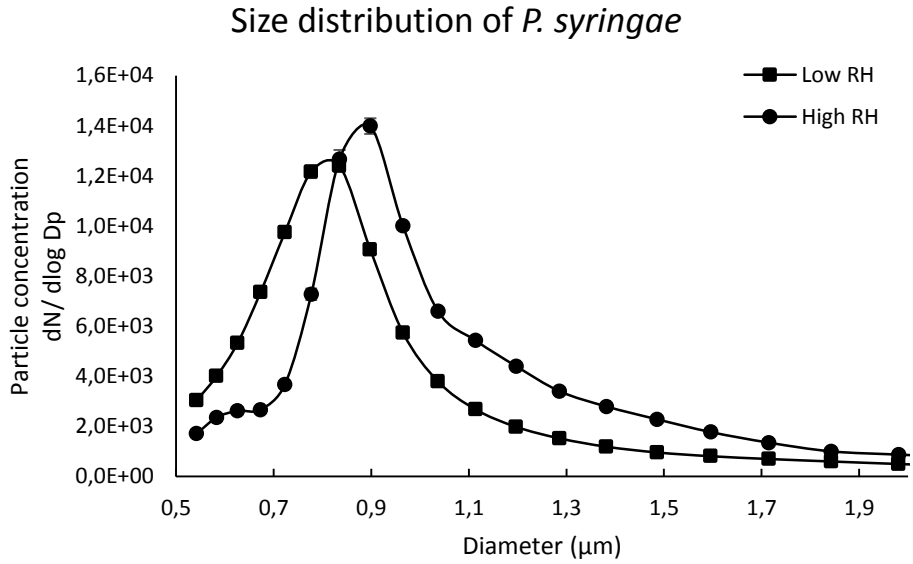

Figure 16. Size distribution mean from 30 measurement series of 30 seconds for P. syringae. Both distribution for aerosolization at high and low RH are displayed. Error bars are presented, although too small to be visible for most points.

It was assumed that the droplets containing bacteria generated from the SLAG had the same initial size for both low and higher RH. What is interesting in this distribution however, is that the peak occurs at roughly 0.8 μm. *P. syringae* are rod-shaped bacteria with a width of 0.8 – 1 μm and a length of ~5 μm (Choi et al., 2014). That means that the airborne bacteria align with the flow in the APS instrument and the aerodynamic size corresponds to the smallest dimension of the bacteria (Figure 17).

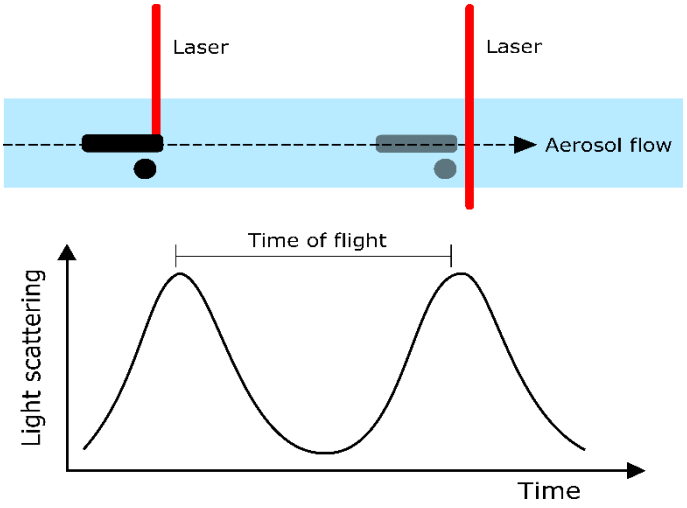

Figure 17. The principle of work for the APS with both a spherically shaped and a rod shaped particle.

4.2.2 Traditional cultivation

The result from the cultivation on agar plates proved a throughput of culturable bacteria of 0.2 % - 0.6 % (Table 5). There was no significant difference between the collection efficiency at lower respective higher RH (at p-value 0.05). However, the lack of noticed difference between the two levels of RH may be due to cultivation on agar plates being a rough method. The difference between the samples (see flow cytometry analysis results) may have been too small to be detected with cultivation, or at least smaller the variance within samples. Both for lower and higher RH double biological replicates were performed to strengthen the result. The plates were enumerated after 3 days of incubation at room temperature.

Table 5. CFU/ml from enumerated agar plates before and after aerosolization at two different RH. The biological replicate values are an average of three agar plates. Calculated according to Eq. 7.

Sample	Before aerosolization	After aerosolization	Collection efficiency
Lower RH			
Replicate 1	$1x10^{10}$	$2x10^{7}$	0.2 %
Replicate 2	$5x10^{9}$	$3x10^{7}$	0.6 %
Higher RH			
Replicate 1	$1x10^{10}$	$2x10^{7}$	0.2 %
Replicate 2	$3x10^{9}$	$1x10^{7}$	0.33 %

Cultivation is a traditional, but effective, method of determining colony forming units (cfu). However, the method is not very precise and more or less gives an answer in

what order of magnitude the concentration of culturable bacteria is. Therefore, if a difference exists in the same order of magnitude, it can be hard to detect. A collection efficiency of 0.2 % may seem low, but by measuring the waste in the SLAG it could be determined that only one sixth of the sample actually was aerosolized. This partly contradicts the findings of Mainelis et al. (2005) whom stated that for most settings, the particle generation efficiency exceeded 80 %. However, they also stated that the liquid loss and particle generation efficiency were not necessarily related. The concentration in the waste was not determined due to technical problems, but its visual appearance did not differ from the original solution. It was therefore assumed that the waste liquid had a higher concentration than 20 % of the concentration in the original solution, simply from visible comparison to the original solution.

When taking regard to losses at all stages of the setup, one can realize that the collection efficiency can be reduced in several steps in the setup. In addition to the losses, the cultivation method measures colony forming units which implicates that bacteria that have died or been rendered non-culturable would not be counted. Because of this very reason, the samples were also analyzed with the flow cytometer as it counts both culture forming units as well as non-culturable bacteria.

4.2.3 **Flow cytometry analysis**

The flow cytometry analysis of survival of *P. syringae* indicated that there was a difference between the lower and higher RH experiments. The results were obtained by repeated tests with two biological replicates to strengthen the conclusion. The bacteria had been stained first as dead and then as live to allow for gating of the populations. The gates were then transferred to the live/dead stained sample to determine the survival percentage of the population. In the analysis of the higher RH, 66 % of the population was dead after aerosolization (Figure 19). By compensating for that only 85 % of the population was alive before aerosolization (Figure 18), the survival of aerosolized bacteria was 40 % (Table 6).

Table 6. The percent of living bacteria measured by the flow cytometry. % of total indicates fraction of living bacteria in the entire measured population of bacteria. % of living before aerosolization have compensated for the fraction already dead before aerosolization, to present the fraction of bacteria that died during the aerosolization process.

	Before aerosolization (% of total)	After aerosolization (% of total)	After aerosolization (% of living before aerosolization)
Low RH	80.4	46.3	58
High RH	84.5	33.7	40

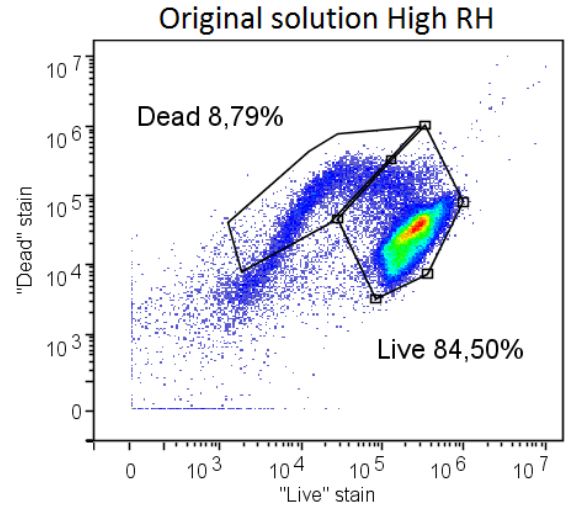

Figure 18. Measurement of the original bacterial solution used for the experiment with higher RH. The x- and y-axis represent staining for live and dead bacteria and the polygons represent gating for the live and dead populations, with corresponding fraction presented. Red indicates a high concentration, while blue is a lower concentration.

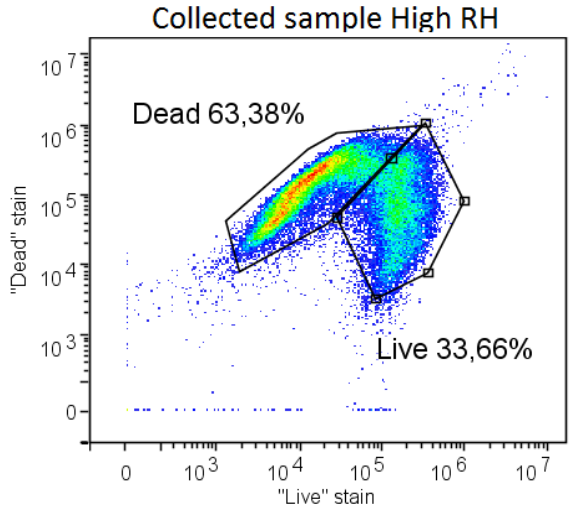

Figure 19. Measurement of the collected bacterial solution used for the experiment with higher RH. The x-and y-axis represent staining for live and dead bacteria and the polygons represent gating for the live and dead populations, with corresponding fraction presented. Red indicates a high concentration, while blue is a lower concentration.

The analysis of the bacterial population from the aerosolized sample at lower RH proved to yield a higher percentage of survival (Figure 20), with 46 % of the population being alive after aerosolization. By compensation for that only 80 % were alive before aerosolization (Figure 21), it was proved that 58 % of the bacteria survived the aerosolization (Table 6).

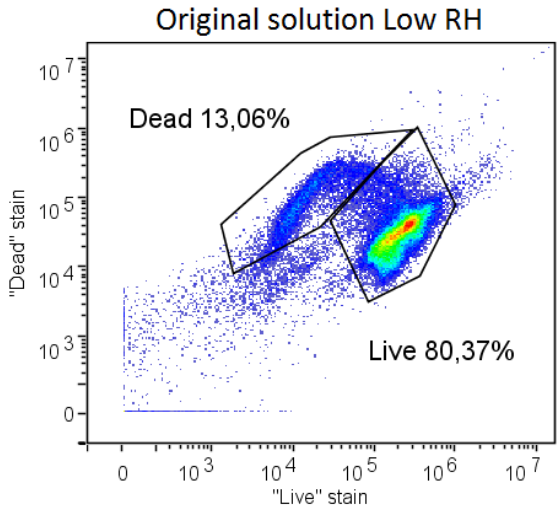

Figure 21. Measurement of the original bacterial solution used for the experiment with lower RH. The x-and y-axis represent staining for live and dead bacteria and the polygons represent gating for the live and dead populations, with corresponding fraction presented. Red indicates a high concentration, while blue is a lower concentration.

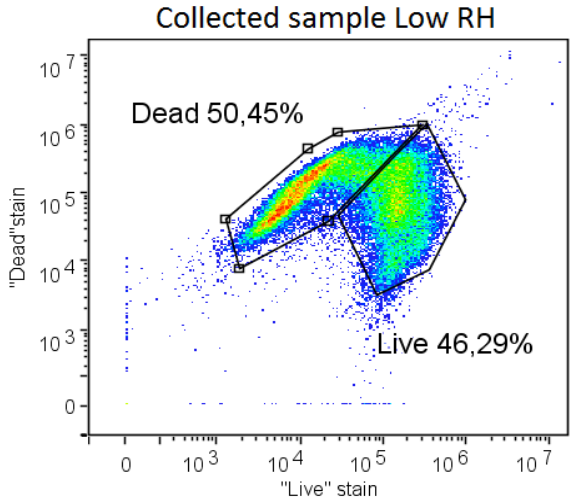

Figure 20. Measurement of the collected bacterial solution used for the experiment with lower RH. The x-and y-axis represent staining for live and dead bacteria and the polygons represent gating for the live and dead populations, with corresponding fraction presented. Red indicates a high concentration, while blue is a lower concentration.

The experiments were done with an original bacterial solution concentrated ten times from its cultivation concentration, meaning the concentration of aerosolized bacteria was drastically higher than atmospheric concentration of *P. syringae*. How well the collection of airborne bacteria is for more realistic concentrations was not investigated, as the main focus of this thesis was to achieve controlled laboratory studies. However, if sampling from ambient air would be desired, an appropriate first step is to evaluate how low concentrations can be collected by the BioSampler and detected by the flow cytometry using the setup of this thesis.

The viability analysis indicates a higher fraction of surviving bacteria for lower RH, findings in line with previous research on airborne bacteria by Theunissen et al. (1993). They report that gram-negative bacteria (such as *P. syringae*) survived better at low

temperatures and low RH. In contrast, gram-positive bacteria survived best at low temperatures and high RH. In this thesis, high and low RH were 60 % and 25 % respectively, which would not be considered extremes in any way. An extremely low RH could be considered to be less than 10 % and an extremely high RH above 90 % RH. 25 % is on the verge of being low, while 60 % could be considered to be a moderate RH. This can be an explanation why the difference in survival at lower RH and higher RH was not greater than 20 percent units.

Recent characterization work on bioefficiency on the SLAG with *E. coli* showed a culturability reduction of up to 60 % by aerosolization (Zhen et al., 2014). Although using the SLAG and a BioSampler for collection (the same as in this thesis), the experiment differed on essential points. Most importantly, the findings were based on survival of *E. coli* which is not a native atmospheric bacterium as *P. syringae*. Therefore, the survival of *P. syringae* could be expected to be higher. The total concentration (all optically measurable sizes) of aerosol were in their case ~250 particles/cm^3 while in this thesis, a total concentration of ~2500 particles/cm^3 (note: not $dN/d\log D_p$) was reached, although in this thesis particles down to 10 nm were measure. The collection flow rates used were comparable (11.9 l/min vs 12.5 l/min) so the concentration difference in the air originated from a concentration difference between the aerosolized solution (10^9 cells/ml vs 10^{10} cells/ml). A parameter that differed was the flow rate of the dilution flow. Zhen et al. used 80 l/min while only 30 l/min were used in this thesis. To allow for an even comparison, the setup in this thesis needs to be tested with *E. coli*.

Decreasing the concentration of bacteria, or increasing the dilution flow, may increase the collection efficiency but would probably decrease the absolute concentrations collected in the BioSampler. Maintaining a high collected concentration was key to allow for flow cytometry analysis, as the analysis is statistically most reliable for concentrations of 10^6 – 10^7 cells/ml.

4.3 Aerosolization of virus – *Norovirus*

4.3.1 **Size distribution and particle coating**

The size distributions of the virus solution diluted in PBS and non-virus containing growth medium diluted in PBS solution proved to be very similar (Figure 22). The virus solution consists of the virus population in growth medium with 10 % FCS. The growth medium has the same composition as the virus solution, except it contains no viruses. Both solutions are diluted eight times in PBS. When adjusting the size distribution of virus in growth media according to background of growth medium in PBS, theoretically the concentration of virus should be what is left. This was not the case, as the non-virus solution even had higher concentrations for some sizes.

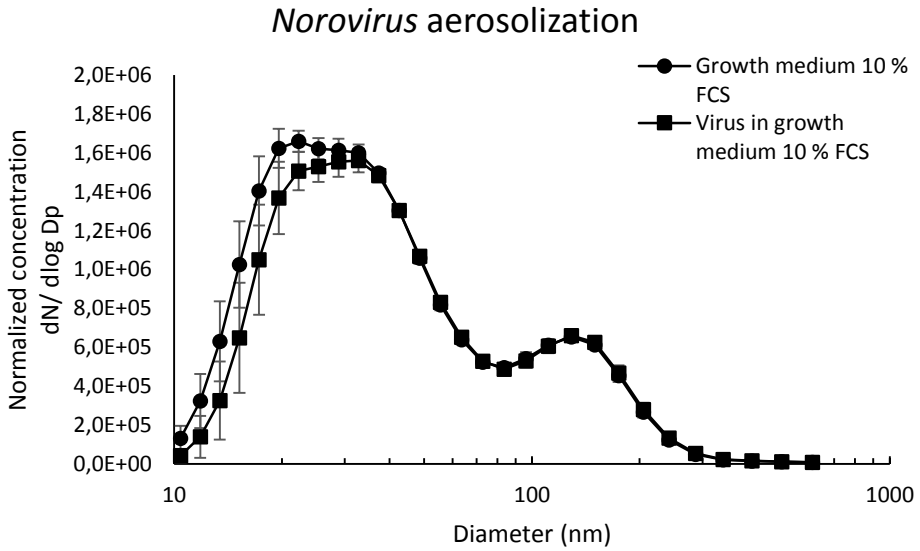

Figure 22. Size distribution of virus solution and growth medium solution. All values are means of eight or more measurements. The error bars represent one positive and one negative standard deviation from the mean value.

From the sizes with the highest concentration when aerosolizing growth medium with 10 % FCS (Figure 22), a dry particle for calculations of coating thickness on airborne viruses were chosen. The sizes of particles chosen for calculations were 22 nm and 130 nm. The result of the calculations are presented in Table 7. The result show how large a particle containing a single virus will become depending on how much material that coat it (which otherwise would have been a non-virus containing particle). A single virus (assumed 30 nm in diameter) coated with the volume of a particle with a diameter of 22 nm will have a diameter of 34 nm.

Table 7. Possible coating thickness of aerosolized viruses. Dry particles were assumed to not contain a virus and a virus was assumed to have a diameter of 30 nm.

Dry particle diameter (nm)	Coating thickness (nm)	Virus with coating diameter (nm)
22	4	34
130	100	130

The size distribution of virus in growth medium does resemble the PBS size distribution, which was expected as the virus solution was diluted eight times in PBS. The dilution was performed to increase the volume of the sample to allow for aerosolization during extended periods of time because, as stated in the methods, a full experiment needed the sample to be collected for 30 minutes. The longer sample duration was done to increase the concentration of virus in the collected media to have a higher probability of detecting the viruses.

Regarding the attempt to size determine airborne viruses, a conclusion was drawn that the viruses could not be detected in the size distribution of the aerosolized material. This is most likely a result of a great concentration difference between salts and viruses in the solution. Another aspect is in what size and composition a virus particle would have been detected. There is a high probability that a virus would be agglomerated or coated with other viruses or salts/proteins from the medium instead of existing as a single virus aerosol. This makes size determination of viruses even more difficult as the virus may be present in agglomerates consisting of different amounts of viruses and coated material, leading to a large span of different sizes of particles containing viruses. To further complicate the task, a *Norovirus* may differ a lot in size with a diameter ranging from 26 to 35 nm (Prevention, 2009).

Airborne *Norovirus* have never before been size determined and it was one of the ambitions in this thesis to do so. However, the large concentration difference between the various salts and viruses in the solutions proved to be a too difficult task to accomplish. One way to determine the actual size of the *Norovirus* is to image the viruses using an electron microscope, either a transmission electron microscope or a scanning electron microscope. Knowing the actual size of the virus eliminates one source of uncertainty in trying to size determine the virus aerosol (with coating or in agglomerates). When using an imaging technology there is also a possibility to estimate the size span of virus particles. Imaging the viruses from the collected aerosol is no easy task either, since only determining which particles contain a virus may be too difficult to establish.

It is probable that salts and proteins from the growth medium coated the aerosolized viruses, which implies that measured sizes of airborne viruses would not be the same as their actual size. It also may affect properties of the virus. For instance, an airborne virus may be less susceptible to environmental parameters, such as relative humidity and chemical composition of the atmosphere, when coated in a protective layer.

4.3.2 **Quantity of *Norovirus***

The concentration of RNA copies per ml solution before and after aerosolization were analyzed and showed a throughput of 0.23 % (Figure 23, calculated according to Eq. 10). The number of amplification cycles to reach the threshold value of fluorescence was compared to a standard curve (Appendix B) to determine the quantity of RNA copies present. The standard curve had a viral solution with an estimation of 10^6 RNA copies/ml and was diluted in steps of 10, 5 times down to 10^1 RNA copies/ml. As the original concentration only was an estimation, all presented values also are rough estimates. The relation between values however, is independent of actual concentration. It was assumed that one RNA copy originated from one virus.

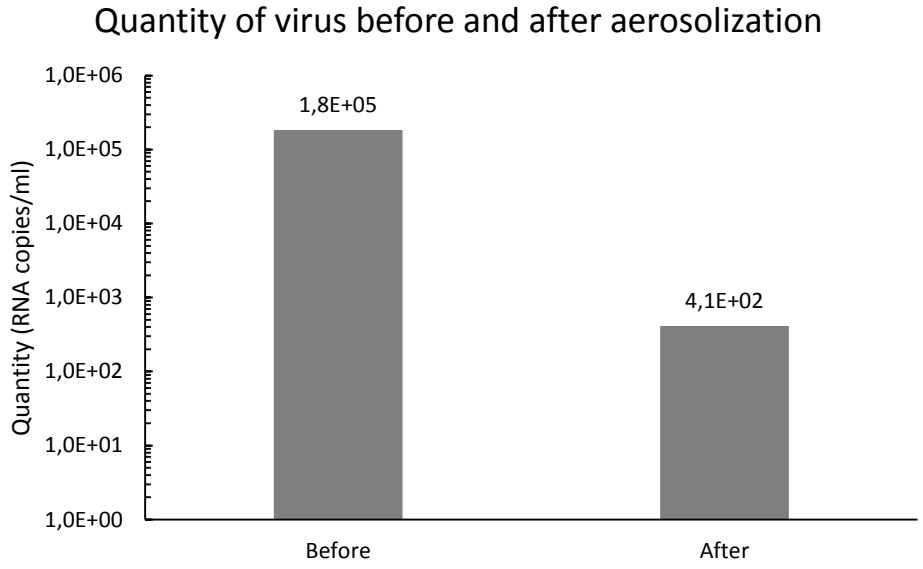

Figure 23. Concentration of *Norovirus* RNA copies per ml before and after aerosolization. The actual values are rough estimates. Please note the logarithmic y-axis.

The qPCR analysis done here does not differentiate between viable and non-viable viruses. Unfortunately, the PCR assay differentiating between viable and non-vible virus did not at the time of the thesis work allow for reliable results, and the work to optimize it extended beyond the duration of this thesis. Because of this, the environmental parameters were not changed for virus aerosolization. As the viability could not be measured, these experiments would not have yielded any useful information. Unfortunately, that limits the analysis of virus experiments. As a direct result, this thesis cannot answer the question about seasonal abundance of winter vomiting disease, as had been stated as an objective in the introduction.

The throughput of 0.23 % for *Norovirus* aerosolization can be compared to the throughput for *P. syringae* that also were roughly 0.2 %. This indicates two things (or either one of them): The throughput of the system does not depend on the size of the

target organism; the throughput does not depend on the original concentration of the target organism.

The fact that the system could be independent of the size of the organisms collected seem unlikely, mainly as the manufacturers of the BioSampler present a worse sampling efficiency for smaller particles (SKCinc, 2016). The sampling efficiency was only measured down to 300 nm particles but already at that, in this context, rather large particle size the sampling efficiency had dropped to 80 %. This can be compared to the sampling efficiency of 1 μm particles which was practically 100 %. The concentration difference between the original bacterial and viral solutions used were almost a factor of 10^4 (10^{10} vs $2x10^6$). Even so, the throughput percentage stayed the same for both. This is highly interesting in the sense that the setup may not need extremely high concentrations to function. In the end, this indicates that the setup might be used for measurements on concentration levels closer to what can be found in ambient air. However, before this can be reality the losses in the setup need to be reduced. Generating ambient concentration in the setup and only collecting 0.2 % will most certainly be below the limit of detection for any analysis method present today. Bonifait et al.(2015) measured ambient *Norovirus* concentration at health care wards and found it to be in the range 10^1-10^3 RNA copies/m^3 air. 0.2 % throughput efficiency would then in almost all cases result in zero viruses collected. Nevertheless, if the throughput of the setup could be increased, the possibility of generation and collection of ambient concentrations of *Norovirus* exist, as the limit of detection for PCR is low.

Continuing the comparison to the study of Bonifait et al., the system in this thesis proved a significantly worse recovery percentage of virus. They state that their setup recovered 89 % of the aerosolized viruses, in comparison to the 0.2 % in this thesis. They did however use substantially higher concentrations of virus, with as many RNA copies per ml as $5x10^{10}$. Again, in comparison to this thesis that is much higher (this thesis had $2x10^5$, an increase in concentration 10^5 times).

As the result produced work as a proof-of-concept that the setup actually can aerosolize and collect virus, there are improvements to be done with all sections of the setup. The generation of aerosol, the sampling from the exposure tube and the sampling device all can be improved upon. By tuning the parameters of the SLAG a higher generation efficiency could be reached, as of now one sixth of the sample is lost before even being aerosolized. Sampling from the exposure tube was done through the sampling port. However, not all air with aerosol passed through the sampling section. About two thirds of the aerosol went directly to waste. Even if it is concentration that is the measurement of quantity, it can be established that the air in the exposure tube is diluting the viruses, and then a majority is led directly to waste. Finally, the BioSampler has a sampling efficiency of less than 80 % for particles smaller than 300 nm. As the majority of the generated aerosol in the virus experiments was below that size, the sampling efficiency most likely was below 80 %.

5 Reflections on the work

5.1 Outlook and improvements

The work of constructing and testing a setup for aerosolization of biological material in this thesis works both as a proof-of-concept and as a platform for further research. Concept that needed verification was that the setup indeed managed to aerosolize and collect biological material, without rendering it non-viable. Although verification was provided, the setup can be improved a lot. Firstly, the generation method needs to be optimized to minimize waste created. Only aerosolizing one sixth of the sample is not good enough when, for example, virus samples exist in small volumes. Optimization of the SLAG mainly involves configuration of air flow rates, membrane pore size and liquid delivery rate. Research have shown that the SLAG, with the correct settings and circumstances, can aerosolize 95 % of all particles in the sample (Mainelis et al., 2005). However, the setup is not limited to the use of the SLAG. If any other generator is found to be more suited, it can easily be incorporated instead. The SLAG was chosen due to the, in comparison to other aerosol generators, gentle process of aerosolization. The TSI atomizer, although shown to decrease viability of bacteria, may be a good alternative to the SLAG for virus aerosolization. The TSI atomizer has the possibility of recirculating the sample, to make sure that everything is aerosolized. This also applies a lot of stress to the organism, but if it can be shown that the aerosolization process does not inflict much harm to the viruses it can increase the generation efficiency remarkably.

The next thing to optimize would have been the sampling from the exposure tube, as a lot of aerosolized material simply just goes to waste. Removing the dilution flow would have increased the concentration of collected material, but then the possibility to change the environmental settings in the exposure tube is eliminated. Other than that, not too much can be done to improve the sampling as the flows through the analysis instruments and BioSampler are set. However, the method of creating a controlled environment in the exposure tube can be improved upon. In this thesis, only the humidity was changed. The bubbling of air through water to create humid air was not the best method and a suggestion to have more controlled air parameters is to couple the dilution flow to a climate air generator. The climate air generator can produce air of various temperature and humidity. The flow connections of the setup would need configuration, but the modification is deemed highly possible.

The sampling efficiency of the BioSampler for small particles would need some investigation. If it turns out that the sampling efficiency is below 50 % for particles at

100 nm, a change of sampling device need consideration. The BioSampler cannot be improved much, but the usage of a filter sampler instead may work better for viruses. The main reason behind not using a filter sampler was that the survival of bacteria have been shown to be decrease for longer sampling periods (Hinds, 1999). However, if viruses do not show a loss of viability on filters, it would be an excellent option to the BioSampler as the filter has higher efficiency for smaller particle sizes. Bonifait et al. showed a good sampling efficiency without too much viability loss using a specially designed filter sampler.

The setup was in this work characterized and tested with *P. syringae* and *Norovirus*, but is not limited to the use of only these two organisms. For instance, the setup could be used to investigate transmission of tuberculosis, which is caused by the bacteria *Mycobacterium tuberculosis*. There exist model organisms for *Mycobacterium tuberculosis* that are harmless, and would be well suited for studies with this setup. The transmission of tuberculosis could then be studied under different controlled circumstances. The setup constructed can work for many organisms where there is an interest in determining their properties and survival in air, e.g. influenza virus.

The generation and analysis section of the setup could also be coupled to a larger exposure section, e.g. an exposure chamber. That would allow simulation of spreading in a room, investigation of survival in a more realistic environment and imitation of a more natural aerosolization process. A more natural aerosolization process could be a sneeze, cough or vomiting and with some modification to the generator, it could potentially be imitated. A study with controlled aerosolization and analysis, but in a realistic setting would then give better answers to questions regarding transmission of disease.

Another possibility is the connection of the generation and exposure part of this setup to an external measurement instrument. Examples of such instruments are the air-liquid interface (ALI) instrument (Jeannet et al., 2015) and the ambient *Norovirus* air sampler with integrated microfluidics (Pardon et al., 2015). The ALI simulates deposition of inhaled particles into the lung and measures the toxicological effect on epithelial cells. By coupling such an instrument to the setup of this thesis, the effect of inhaled bioaerosol on the lung cells may be studied. However, this would be of most interest for harmful organisms (or potentially high concentrations of others) and the safety of both the lab and the setup would need to be improved. In addition, the inventors of the ALI-system have only characterized it for nanoparticles and not bioaerosols. Nevertheless, it is an interesting prospect where the controlled generation and exposure comes to use in an applicable way.

The ambient air sampler is for sampling of *Norovirus* specifically and collects its material into a small volume of liquid. The liquid can then be extracted for microbiological analysis. The thought behind this sampler is to sample *Norovirus* from the ambient air to detect presence of the virus. The setup could be coupled to this sampler to create virus aerosol of different concentration to test and validate the sampler. Once the sampler is validated, it can be used as a regular sampler for the setup as it collects the virus into a small volume and thus effectively reducing the sampling time required.

A drawback of both these samplers are the fact that they make use of an electrostatic precipitator to collect the aerosol. The electrical field may very well harm or even kill the organism, and therefore proper validation of the electrostatic precipitator needs to be performed.

5.2 Reflections on ethics and sustainability

The main focus of an engineer tends to always be on the development and result of their project, leaving little or no time for reflection on the work. Your responsibility as an engineer is towards the society, not yourself or your own recognition. Your work should aim at facilitating the struggles of people's everyday life or enabling the participation in society of those otherwise unavailable. However, not all progress is or have been used solely for positive purposes. While being developed with good intentions, some work may be used in a harmful or destructive manner. It is therefore the responsibility of the engineer to beforehand reflect on the risks contra benefits of the work, if it was to be used with bad intentions.

The study of airborne transmission of infectious agents are not an exception to this need for reflection. In fact, it is rather on the contrary. Many of the greatest epidemics and pandemics in the history have been caused by airborne transmission of the pathogen. The lives of millions of people have been lost due to naturally occurring diseases or diseases caused by the urbanization of civilization. With the progression of science and engineering in many cases being driven by military purposes, the emergence of biological warfare was only a matter of time. In this area, the biology of infectious agents is studied with the purpose of increasing the spreading and transmission to create a more effective weapon. A horrifying example of this would be the usage of the bacteria *Bacillus anthracis* to spread the disease anthrax. Luckily, the usage of biological weapons is prohibited by the United Nations, but it does not remove all concerns. Acts of terrorism have in recent years included the usage of biological weapons, with one example being the mail-bombing of several postal offices with anthrax across the United States in 2001 (Johnson, 2005). As the usage of these weapons are hard to prevent, scientists and engineers need to evaluate if and how their findings may be used with bad intentions.

The investigation of the effect of aerosolization on *Norovirus* was made with the intention of increasing the knowledge of the routes of transmission to allow for better prevention methods in the public health care. With this in regard, the author recognizes that someone with the wrong intentions may use the findings in a harmful manner. By knowing the optimal environment for transmission, an ideal location for spreading can be chosen by someone intentionally desiring to spread the disease. Such a case could be, as the example above, a terrorist attack as a biological weapon may inflict enormous damage and instill fear in a community. Although *Noroviruses* are very pathogenic and during the right circumstances may evolve into an epidemic, it is for the large majority not a lethal disease. As acts of terrorism often are used as a way to spread fear, a mostly

non-lethal infection can be considered a low priority. Therefore, the benefits of having the prospect of lowering health care cost, reduce nosocomial infection and relieving the burden on the health care workers are considered to outweigh the risks. Even so, the effective spreading of the *Norovirus* makes it appalling and an infectious agent that definitely deserve a spot on the watch list. When publically publishing findings of bioaerosol, the author needs to evaluate what results he/she presents and what it could be used for.

The work in this thesis was designed to be completely experimental and in vitro based. Virus cultivation and analysis were both conducted in vitro, eliminating any animal studies. The addition of animal studies would not have contributed any essential information and therefore was not conducted to spare the suffering of the animals. As mentioned before the findings in this thesis may result in increased understanding of the infectivity of norovirus in different air conditions, leading to decreased health care costs, reduced events of nosocomial infection and a relieve of the strain of health care workers. It is thus clear that this thesis may improve the sustainability of the health care both socially, by reducing stress on workers and reducing the amount of infections, and economically, by reducing the costs of treatment and sick leave. Nurses is a group of workers with high stress and strain related to their work and any progress improving their work conditions may reduce sick leaves and early retirement.

6 Conclusions

The setup presented in this thesis have been shown to, in a controlled manner, generate a bioaerosol, expose it to varying environmental parameters and collect it for biological analysis. The setup was built and initially validated with test aerosol, with further tests with both the bacteria *P. syringae* and *Norovirus*. *P. syringae* was aerosolized and exposed to air with a relative humidity of 25 % and 60 %, analyzed survivability of the bacteria accounted to 58 % and 40 %, respectively. This confirmed that in the studied setup, changed RH conditions influence survivability of the bacteria, i.e. decreased survivability was observed at higher RH. Throughput of the system (i.e. ratio of the final concentration in comparison to initial) for bacteria was measured to 0.2%. Aerosolisation of the *Norovirus* has also been performed successfully. A measureable concentration of *Norovirus* collected in a sample after aerosolisation has been quantified with PCR. The obtained throughput of the system was the same as for bacteria, i.e. 0.2 %. The results of this thesis present a platform for future work in the field of bioaerosol by producing a method for controlled studies. The main advantage of the setup lies in its versatility, allowing for changes between different generation instruments or collection instruments used. A proof-of-concept, i.e. that controlled laboratory studies on airborne microorganisms can be done, has been experimentally confirmed in this thesis. Based on obtained results, further optimization of the setup has been suggested focusing mainly on increasing the throughput of the system. Examples of improvements to be done are: parameter optimization of the SLAG, possible change of the aerosol generator and collection instruments. Coupling of the generation and exposure section to external analysis instrument can enable interesting studies on for example lung deposition and toxicology.

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Appendix A Risk analysis (in Swedish)

A.1 Risk assessment (Riskbedömning enlig 4-5 §§ i AFS 2005:1 Murint *Norovirus*)

Murint(mus) *Norovirus* har, till skillnad från sin humana variant, inte påvisats vara human patogen. Humant *Norovirus*, som orsakar vinterkräksjuka, har visats kunna infektera människor med doser ner till 20 virus vilket gör att viruset får anses kraftigt infektiöst. Viruset har även visats kunna överleva länge utanför sin värd, vilket gör att den indirekta exponeringen kan bli stor. *Norovirus* är orsaken till större delen av all vinterkräksjuka-utbrott i Sverige som i det flesta fall är en sjukdom som kroppen själv tar hand om. Inga av den humana strängens negativa effekter har kunnat visas hos den murina strängen.

Utifrån vad vi kan bedöma av AFS 2005:1 kan vår hantering av murint *Norovirus* hänföras till riskklass 1 [1]. Viruset är inte smittsamt för människor och vi har inte heller någon djurverksamhet. Men eftersom vi arbetar med aerosolprocesser och vill säkerställa god rutin har för säkerhets skull valt att genomföra rapportering till AMV enligt riskklass 2.

[1] Till riskklass 2 hör biologiska agens som kan orsaka infektioner som kan ge upphov till sjukdomar av olika allvarlighetsgrad, som antingen går att bota eller förebygga eller som normalt självläker utan några allvarliga men. Till riskklass 2 räknas virus som isolerats från människor, och som inte tillhör någon högre riskklass. Till riskklass 2 räknas också biologiska agens som misstänks kunna orsaka cancer hos människa, men där sannolikheten för att exponering leder till cancer är mycket liten t.ex. därför att det krävs många samverkande faktorer.

A.2 Facilities (lokaler och utrustning för mikrobiologiskt arbete)

Aerosollaboratoriet vid IKDC uppfyller alla krav för säkerhetsklassning 2 för biologiskt material och får behandla biologisk agens av riskklass 2 (se Tabell 1, AFS 2005:1). Viruset kommer att aerosoliseras i ett slutet system med avsikt att minimera möjligheten för exponering.

- Utrustning och lokaler vid aerosollaboratoriet, Lunds universitet:
- Anordning för handtvätt och desinfektion
- Exponeringen av biologiskt material sker i avslutet rum eller system.
- Skylt med symbol för smittrisk placeras vid utrustning eller utrymmen när tillämpligt.
- Bänkyta och golv resistent mot vatten, syra, lösningsmedel och desinfektionsmedel.
- Tillgång till autoklav för sterilisering finns.
- Säkerhetsbänk med HEPA-filtrerad luft med larmsystem vid funktionsbrist finns tillgänglig för arbete med infekterat material eller vid risk för spridning.
- Skydd mot skadedjur finns genom byggnadsförvalatern.
- Tillträde begränsat till endast personer med kunskap om riskerna tillämpas. För personligt tillträde till aerosollaboratoriet krävs genomgången riskkurs.
- Lämplig skyddsklädsel under arbete med bioaerosol används.
- Biologisk agens förvaras i särskilt rum för att minska risken för exponering.
- Material och utrustning är utformade för att underlätta dekontaminering: avtorkbara ytor; behållare och utrustning som går att autoklavera.
- Aerosolexperiment med virus (klass 2) sker i slutna system med HEPA-filtrerat utflöde. Filtret är autoklaverbart.
- Inga djuranläggningar finns i laboratoriet eller angränsande byggnad.

A.3 Control and prevention (Kontroller och skyddsåtgärder för mikrobiologiskt arbete vid aerosollaboratoriet, Lunds universitet)

A.3.1 Bakgrund

Bioaerosol omfattar alla slags luftburna partiklar med biologiskt ursprung, såsom bakterier, virus, sporer eller pollen. Bioaerosol skiljer sig från andra aerosolpartiklar genom att materialet ibland är levande. En ensam bakterie kan, om den hamnar i rätt miljö, föröka sig och växa i antal. Virus har visserligen inte förmåga att föröka sig på egen hand men kan göra det när de kommer i kontakt med levande organismer. Pollen kan ge hälsoeffekter i händelse av allergisk reaktion.

Bakterier, sporer, virus och pollen är ständigt närvarande i vår livsmiljö och vanligtvis orsakar de inga större bekymmer. Det är helt avgörande om partiklarna har en sammansättning som gör dem sjukdomsframkallande. Därför är det viktigt att känna till egenskaperna för varje typ av bioaerosol som hanteras.

Vi hanterar normalt endast mikrobiologiskt material ur klass 1 vid aerosollaboratoriet. Det är värt att notera att biologiska agens ur riskklass 1 potentiellt kan orsaka ohälsa genom överkänslighet eller toxinpåverkan. Virus isolerade från människa tillhör vanligtvis minst riskklass 2 såvida det inte finns belägg för att de sannolikt inte är sjukdomsframkallande. Vi använder endast virus isolerade från djur och icke-patogena bakterier. De hanterade volymerna överstiger nästan aldrig 1 L. Eftersom vi aerosoliserar mikroorganismerna och har studenter i verksamheten vill vi säkerställa god laboratorierutin enligt riskklass 2.

A.3.2 Riskanalys

A. *Riskidentifiering*

Förhållanden som gynnar tillväxt av bakterier förekommer i mycket låg grad i laboratoriet och koncentrationerna är små. Däremot finns risk för exponering då bakterierna görs luftburna. De personer som drabbas är framför allt de som befinner sig i närheten av pågående experiment.

B. *Exponeringens art*

Inga kända infektionsrisker (för människor) finns kring de biologiska ämnen som hanteras. Dock har flera av undersökta bakteriearter och virus släktingar som är humant patogena.

C. *Exponeringsmätning*

Viruset kommer att aerosoliseras i ett slutet system med avsikt att minimera möjligheten för exponering. Vi mäter i princip alltid luftburen partikelkoncentration i anslutning till våra experiment.

A.3.3 Skyddsåtgärder vid mikrobiologiskt arbete i aerosollaboratoriet

- System för incidentrapportering finns.
- Biologiskt material och dess behållare ska dekontamineras genom autoklavering innan det diskas/slängs.
- Handskar/skyddsglasögon bör bäras i allt arbete med risk för kontaminering.
- Ingen förtäring av mat eller dryck innanför det avgränsade området i labbet där experiment med biologiskt material utförs.
- Ordentliga handdesinfektions- och handrengöringsmöjligheter ska finnas tillgängliga.
- Avsedd yta för biologiskt material ska hållas fri från verktyg och dylikt för att minska korskontaminering. Ytan ska vara lätt att rengöra.
- Läs instruktioner till autoklaven eller be någon som har kunskap om autoklaven om hjälp före användning.
- Kylskåp/behållare/förvaring av biologiskt material ska märkas tydligt med ansvarigs namn. Undvik onödig kontakt med dessa behållare.
- Använd gula behållare för biologiskt avfall (inkluderar pipettspetsar, använda handskar, provrör etc) med tydlig markering för biologiskt avfall (symbolen för smittrisk, se nedan).
- Om ingen riskbedömning finns för organismen som ska användas, börja med att utföra en sådan analys.
- Torka av och använd desinfektionsmedel på ytor och instrument som använts om: 1) spill har skett, 2) planerade experiment avklarats eller 3) instrumentet ska flyttas utanför experimentzonen.
- Använd labbrock för att minimera spill på kläder och spridning på så sätt. Labbrocken ska förvaras vid labbstationen vid alla tillfällen.
- Ha alltid burkens lock åtskruvat och provrör stängda när dessa förflyttas eller inte används.

A.3.4 Planerade och genomförda tekniska kontroller

- Utrustning åtgärdas vid felrapportering
- Ventilation och filter kan kontrolleras med apparatur som finns ständigt tillgänglig i aerosollaboratoriet (partikelräknare, luftflödesmätare, tryckmätare).

A.3.5 Övriga åtgärder och anmärkningar

Ingen odling av virus eller bakterier sker i aerosollaboratoriet. Dessa transporteras från andra laboratorier (i allmänhet avdelningen för bioteknik vid kemicentrum [angränsande byggnad] eller vid medicinska fakulteten). Transport sker i slutna behållare med lämplig märkning. Den mikrobiologiska hanteringen vid aerosollaboratoriet är därför i princip helt begränsad till luftmätningar.

A.4 Practical laboratory guidelines (Rutiner vid arbete med biologiskt material i aerosollabbet)

- Tydlig golv-/skyltmarkering för att avgränsa arbetsområdet
- Ingen förtäring av mat eller dryck innanför det avgränsade området i labbet där experiment med biologiskt material utförs.
- Använd handskar/skyddsglasögon om riskbedömningen av det biologiska materialet anger så.
- Ordentliga handdesinfektions- och handrengöringsmöjligheter ska finnas tillgängliga.
- Läs instruktioner till autoklaven eller be någon som har kunskap om autoklaven om hjälp före användning.
- Kylskåp/behållare/förvaring av biologiskt material ska märkas tydligt med ansvarigs namn. Undvik onödig kontakt med dessa behållare.
- Använd gula behållare för biologiskt avfall (inkluderar pipettspetsar, använda handskar, provrör etc) med tydlig markering för biologiskt avfall (symbolen för smittrisk, se nedan).
- Om ingen riskbedömning finns för organismen som ska användas, börja med att utföra en sådan analys.
- Torka av och använd desinfektionsmedel på ytor och instrument som använts om: 1) spill har skett, 2) planerade experiment avklarade eller 3) instrumentet ska flyttas utanför experimentzonen.
- Använd labbrock för att minimera spill på kläder och spridning på så sätt. Labbrocken ska förvaras vid labbstationen vid alla tillfällen.
- Ha alltid burkens lock åtskruvat och provrör stängda när dessa förflyttas eller inte används.
- Använd avsedd yta för hantering av biologiskt material i samband med försök. Denna yta skall hållas fri från utrustning etc. för att begränsa spridning och korskontaminering.
- Biologiskt avfall i vätskeform ska autoklaveras innan hälls ut/slängs.

Figure 24. Symbol för att varna om smittrisk från biologiskt material.

Appendix B Standard curve qPCR murine *Norovirus*.

The highest concentration of virus was estimated, from comparison to similar samples, to be $2x10^6$ copies per ml. A dilution series was prepared with each step diluting the sample 10 times down to 20 copies per ml. The number of amplification cycles in the qPCR to reach a fluorescent signal above the set threshold were counted and connected to concentration. The result is visualized in Figure 25. The linear fit had a R^2-value of 0.993 which indicates a good fit.

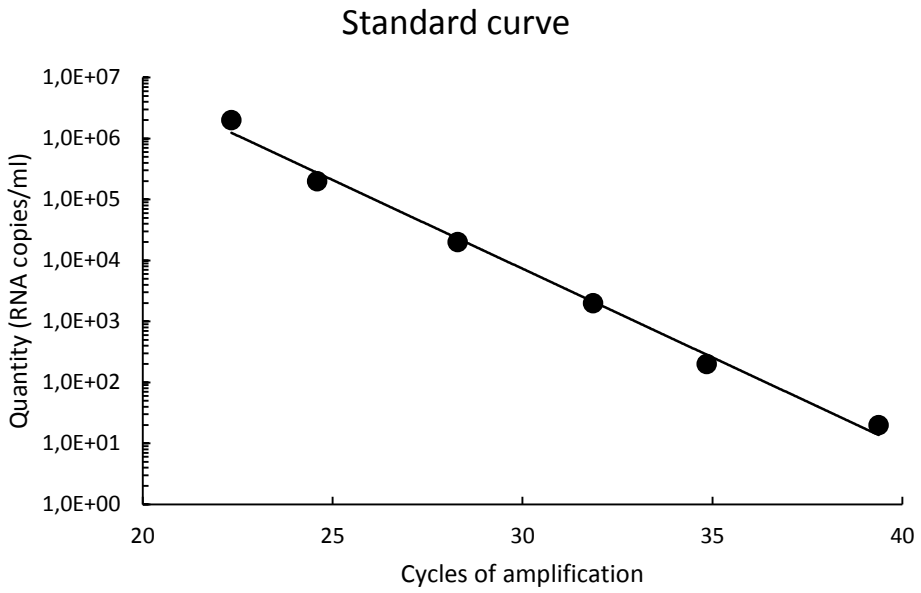

Figure 25. The standard curve for murine *Norovirus* analyzed with the qPCR. Note the logarithmic y-axis.