Survival and Re-aerosolization in Dust of *Mycobacterium smegmatis* - a Surrogate for *Mycobacterium tuberculosis*

By

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Declaration of Originality

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights, and that I have not previously, in its entirety or in part, submitted it for obtaining any qualification.

March 2017

Abstract

Background

Mycobacterium tuberculosis (*M. tuberculosis*) is essentially an airborne pathogen transmitted via aerosols. It remains viable in the soil and outside its hosts for extended periods of time. It has been suggested that *M. tuberculosis* cannot be re-aerosolized therefore it cannot cause disease once it has landed outside the body. This project aimed answering indirectly several questions relating to *M. tuberculosis* and the possibility of it being able to cause disease after re-aerosolization: Can *M. tuberculosis be* re-aerosolized? If it can, does *M. tuberculosis* remain viable? If it is viable, can it cause infection? Finally, can environmental bio-burden be reduced using copper surfaces?

Methodology

This two-phased prospective *in vitro* study preceded by a pilot study used as surrogate for *M. tuberculosis*, *Mycobacterium smegmatis (M. smegmatis),* a fast growing, non-pathogenic mycobacterium. A completely sealed Plexiglas® (Polymethyl methacrylate) cabinet was used as aerosol chamber where 125 mg of sterile dust was spread prior to nebulization of 20 ml of 10^6 CFU/ml of *M. smegmatis* pCHERRY3. The sampling was performed with a six stage Andersen cascade impactor (ACI) and settle plates before and after the dust lift-up using two small fans. Plates were incubated for three to ten days at 37ºC. The numbers of CFU were estimated based on viable plate count. The first phase of the study, the Plexiglas® phase, tested the survival of *M. smegmatis* in dust. The second phase evaluated the impact of copper surface on the survival of *M. smegmatis* in similar conditions.

Results

M. smegmatis survived in dust for more than nineteen days on settle plates after re-aerosolization in the presence of Plexiglas®. In copper presence, *M. smegmatis* survival rate was approximately fifteen days after nebulization in dust, almost 25% less than on Plexiglas®. Starting with an inoculum of 10^6 cells/ml nebulized on Day 0, on Day 1 there were 44.01% lower numbers of *M. smegmatis* isolated in copper presence compared with Plexiglas® surface 24 hours after nebulization. There was a significant decrease in the number of mycobacteria picked up from both copper (100%) and Plexiglas® surfaces (35.02%) on Day 2; copper showed significantly lower levels of mycobacteria ($p=<0.05$). From Day 3 onwards there was no statistically significant difference in *M. smegmatis* survival between the two surfaces as determined by the ACI level A5 & A6 (0.6-2.1µm) which equates to particle sizes that can reach the alveoli and terminal bronchi.

Conclusion

M. smegmatis, the model for *M. tuberculosis*, survived in dust and remained viable after re-aerosolization more than 19 days on Plexiglas® but 15 days on copper. This is particularly relevant in low to middle income countries with high *M. tuberculosis burden*, where dust is common and sweeping and the use of fans in health care facilities are frequent. It also illustrated that the anti-microbial property of copper surface remains effective in presence of dust. Copper can be used as a touch surface to reduce the bioburden of microbes including mycobacteria that could be re-aerosolized.

Key words

Mycobacterium smegmatis, Mycobacterium tuberculosis, survival, re-aerosolization, dust, air sampler, settle plates.

Opsomming

Agtergrond

Mycobacterium tuberculosis (*M. tuberculosis)* is in wese ‘n luggedraagde patogeenen word deurmiddel van aerosols oorgedra. Dit bly lewens vatbaar vir lang tyd perke in die grond en buite die gasheer. Daar is al voorgestel dat *M. tuberculosis* nieweerge-aerosoleerkan word nie en duskandit nie siekte veroorsaak wanneer dit buite die liggaam in die omgewing stof beland het nie. Die doel van hierdieprojek was om ‘n paarvraetevraoor *M. tuberculosis* en die moontlikheid dat dit weer kan aerosoleer en siekte veroorsaak. Hierdievrae was: Kan *M. tuberculosis* weeraerosoleer word? Indienwel, bly *M. tuberculosis* lewens vatbaar? Indien dit lewens vatbaar bly, kandit infeksie veroorsaak? Enlaastens, kan die bio-las in die omgewing deur koper oppervlaktes verminder word?

Metodologie

Hierdieprospektiewe*invitro*studie was in twee fasesvoltooi, voorafgegaandeur ‘n loodsstudie. *Mycobacterium smegmatis (M. smegmatis),* ‘n vinnig-groeiende, nie-patogeniese mikobakterie was gebruik as ‘n surrogaatvir *M. tuberculosis.* ‘n Dig verseelde Plexiglas® (polimetielmetakrilaat) kabinet van 90 x 70 x 90 sentimeter was gebruik as aerosolerings kamerwaarin 125mg sterielestofversprei was voor die nebulisering van 20 mL van 10^6 kolonie vormendeeenhede per milliliter*M. Smegmatis* pCHERRY3. Die monsters was geneem met behulp van ‘n gekombineerdeses-stadium Andersen lugmonsternemer, asookneerslagplatevoorenna her-aerosolering van die stofdeur twee kleinwaaiers. Die plate was virdrie tot tiendae teen 37°C geïnkubeer. Die geskatteaantalkolonievormendeeenhede was gebasseer op die lewensvatbareplaattellings. Die eerstefase van die studie (in Plexiglas®) het die oorlewing van *M. smegmatis* in stofgetoets. Die tweedefase het die impak van koperoppervlaktes op die oorlewing van *M. smegmatis* in soortgelykeomstandighedegetoets.

Resultate

M. smegmatis oorleefvirmeer as 19 dae in stof op neerslagplatenadatditweer in die Plexiglas® ge-aerosoleer is. In die teenwoordigheid van koper was die oorlewingnanebulisering van *M. smegmatis* ongeveervyftiendae – bykans 25% minder as op die Plexiglas®. Beginnende met ‘n inokulum van 10^6selle per milliliter op Dag 0, was 44.01% minder *M. smegmatis* op Dag 1 geisoleer in die teenwoordigheid van koper in vergelyking met Plexiglas® oppervlaktes, 24 uurnanebulisering. Daar was ‘n beduidendeafname in die aantalmikobakterieëopgetel van beidekoper (100%) ennie-koper (35.02%) oppervlaktes; die koper het aansienlikelaergetallemikobakterieëgetoon (p-waarde<0.05). Vanaf Dag 3 was daargeenstatistiesbeduidendeverskiltussen die twee oppervlaktesnie – soosbepaaldeur die Andersen se kaskadeimpakteerdervlakke A5 en A6 (0.6-2.1µm), wat

gelykstaande is aan partikelgroottes wat in geval van oorlewing van *M. smegmatis* die alveoli en bronchi soukonbereik.

Gevolgtrekking

M. smegmatis, die surrogaatmerker vir *M. tuberculosis* , het tot 20 dae nadat dit weer aerosoleer was op Plexiglas® oorleef en lewensvatbaar gebly in stof, maar vir ‘n korter tydperk in die teenwoordigheid van koper. Hierdie is veral relevant in lae- tot middel-inkomste lande met 'n hoë *M. tuberculosis* las, waar stof algemeen is en daar gereeld (met ‘n besem) in gesondheidsfasiliteite gevee word en waaiers algemeen gebruik word. Dit het ook aangedui dat die anti-mikrobiese eienskappe van koper in die teenwoordigheid van stof doeltreffend bly. Koper kan as tas-oppervlak gebruik word om die las van mikrobes, insluitend mikobakterieë, te verminder.

Sleutelwoorde

Mycobacterium smegmatis, Mycobacterium tuberculosis, oorlewing, re-aerosolering, stof, lugmonster, neerslagplate.

Dedication

I dedicate this dissertation:

- To the Master of Time and Circumstances for all opportunities given to conquer ignorance;
- To all of you who have sacrificed health, life, means and time to make the world much more friendly;
- To family and relatives who endured hard times in order for me to acquire skills and get a new degree;
- To all who are striving to overcome the frontiers of ignorance;
- To all who work for the advancement of knowledge and the dissemination of truth.

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Finally and above all, I would like to thank God for His presence in everything I encountered during this hard and very challenging journey and for having brought me unexpectedly to Infection Control and South Africa.

Conflict of Interest Statement

I hereby declare that I have no financial or non-financial interests which may inappropriately influence me in the conduct of this research study or in the publication of its results; moreover, I am not aware of any other competing interests with respect to this project, which may present a potential conflict of interest.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

^{0}C	degree centigrade
A	ampere
A1…A6	Andersen level one … Andersen level six
ACI	Andersen cascade impactor
Al	aluminium
ANOVA	analysis of variance
Ave	average
BFP	biofilm formation potential
Ca	calcium
CAF	Central Analytical Facilities
CDA	Copper Development Association
CFU	colony forming unit
Cl	chlorine
cm	centimetre
Cu	copper
D1…D20	day one...day twenty
DNA	Deoxyribo-nucleic acid
EDS	Electronic Data System
EPA	Environmental Protection Agency
EPS	Extracellular Polymeric Substance.
Fe	iron
Fig	figure
HBO	hyperbaric oxygen
hpa	hectopascal = 10 pascal
IPC	Infection Prevention and Control
ISO	International Standardization Organisation
IUPAC	International Union of Pure and Applied Chemists
K	kalium or potassium
Kpa	Kilopascal = 1000 Pascal
KV	kilovolt = 1000 Volt
L	litre

log10	decimal logarithm
Max	maximum
MDR-TB	multidrug resistant tuberculosis
Mg	magnesium
Min	minimum
nA	nano ampere = 10^{-9} ampere
Na	natrium or sodium
NHLS	National Health Laboratories Service
Nm	nanometre= 10^{-9} metre
O	oxygen
OADC	oleic acid dextrose catalase
OD	optic density
PBS	Phosphate-Buffered Saline
PFR	Particulate Filter Respirator
pH	potential of hydrogen
S	sulphur
S1	dust sample number one
S2	dust sample number two
SEM	scanning Electron Microscope
Si	silicium
SP or sp	settle plate (e.g. Sp1…sp3)
Std.Dev	standard deviation
TB	tuberculosis
UIPC	Unit for Infection prevention and control
USA	United States of America
µm	micrometre= 10^{-6} meter
Wt	weight

Chapter 1: Introduction

The end of 2015 marks a transition from the Millennium Development Goals established by the United Nations in 2000, to a post-development framework. The World Health Organization (WHO) has developed a post-2015 global tuberculosis (TB) strategy, the End TB strategy. The End TB strategy was approved by all Member States of the United Nations in May 2014 and it aims to end the global TB epidemic. Starting in 2015, the aim is to achieve a 95% reduction in TB deaths by 2035 and a 90% reduction in the incidence of TB. The strategy also includes a target of zero catastrophic costs for TB-affected families by 2020 (WHO, 2014).

In support of TB curative treatment, a major programme of TB infection prevention and control, as advocated by the WHO (WHO, 2006; WHO, 2015) has been developed and it includes routine immunisation and attempts to reduce the environmental burden of *Mycobacterium tuberculosis* (*M. tuberculosis).* It is has been suggested that *M. tuberculosis* cannot be re-aerosolized at the size of droplet nuclei to reach alveoli therefore it cannot cause disease once it has landed outside the body in dust or the environment. Tierney and collaborators wrote the following in the MSD Manual Professional Version: ‘‘Droplet nuclei (particles < 5 μ in diameter) containing tubercle bacilli may remain suspended in room air currents for several hours, increasing the chance of spread. However, once these droplets land on a surface, it is difficult to resuspend the organisms (eg, by sweeping the floor, shaking out bed linens) as respirable particles. Although such actions can resuspend dust particles containing tubercle bacilli, these particles are far too large to reach the alveolar surfaces necessary to initiate infection” (The Merk Manual, Professional Edition, 2014). Quoting a statement of the *ad hoc* committee on the treatment of tuberculosis patients in general hospitals, Dermot Maher wrote in *Tuberculosis*, a comprehensive clinical reference, that since the particles containing tubercle bacilli on the clothing, bedcovers, or belongings of a TB patient cannot be dispersed in aerosol, they do not play a significant part in the infection (Maher, 2009). There appears to be no clear published evidence for this concept. The aim of this project was to answer several questions relating to *M. tuberculosis* and the possibility of it being able to cause disease after re-aerosolization. These questions are: Can *M. tuberculosis be* re-aerosolized? If it can, does *M. tuberculosis* remain viable with the potential to cause infection? The reduction of the airborne *M. tuberculosis* can be achieved by mechanically removing the pathogen by facilitating increased circulation and the renewal of the air in rooms, but this has neither bacteriostatic nor a bactericidal effect on the pathogen (Centers for Disease Control and Prevention, 2005).

Unlike the mechanical removal of pathogens, copper and its alloys have been shown to have anti-microbial properties against *M. tuberculosis in vitro* (Mehtar S *et al.*, 2008; Gould WSJ &

Fielder M D, 2009). A concentration of > 55% in copper has been shown to be effectively anti-microbial for bacteria and yeasts. It has been documented that pure copper decreases the bacterial load four times faster than its alloy (Gould WSJ & Fielder M D, 2009; James Michel *et al.*, 2009). If re-aerosolized *M. tuberculosis is* infectious, can we use metals such as copper to reduce the environmental viability of *M. tuberculosis and* its bio-burden?

In this study we used *Mycobacterium smegmatis (M. smegmatis)* as a surrogate for *M. tuberculosis,* the latter being a slow growing pathogenic mycobacterium. *M. smegmatis* is preferred in short term experimental studies based on the fact that it has several similarities to *M. tuberculosis* and it has the advantage of being a fast-growing mycobacterium, and it is considered to be non-pathogenic (Akinola, *et al.*, 2013; Cordone *et al.*, 2011).

If copper antibacterial property is effective on *M. smegmatis*, the surrogate for *M. tuberculosis in* the current study, it is proposed that the combination of copper touch surfaces with controlled air renewal can significantly improve the reduction of the burden of *M. tuberculosis in* the environment. Therefore, this study attempted to answer the following questions focused on *M. smegmatis* rather than specifically on *M. tuberculosis:*

- What is the survival time of *M. smegmatis* in a dusty environment?
- Can *M. smegmatis* be re-aerosolized from the dust?
- Is it viable when it is re-aerosolized?
- What is the effect of copper on the survival of *M. smegmatis* in a dusty environment?

The current project discussed also the possibility of extending the findings with *M. smegmatis* to *M. tuberculosis*. Based on previous studies (animal experimental models) which demonstrated long-term survival of *M. tuberculosis* in soil and its infectiousness after twelve months (Ghodbane , et al., March 2014), the present study on re-aerosolization of the surrogate for *M. tuberculosis* and its survival in a dusty environment tried to establish whether mycobacteria can remain viable after re-aerosolization from dust. The current study was done in two phases preceded by a pilot study. The latter assessed the survival of *M. smegmatis* in dust, its re-aerosolization and the consolidation of the timeframe for several operations encountered in the two following phases of this study. The first phase of the study aimed to test the survival of *M. smegmatis* and establish a death curve in a dusty environment while the second phase evaluated the impact of a copper surface on the survival of *M. smegmatis* in the presence of dust. Practically the second phase of this study identified the role of anti-microbial properties of copper in reducing transmission of *M. smegmatis* (or *M. tuberculosis)* by decreasing the number of days it remains viable.

Chapter 2: Literature Review

2.1 *Mycobacterium tuberculosis*

2.1.1 Definition and epidemiology of tuberculosis

Tuberculosis (TB) is an infectious disease caused by species of the *M. tuberculosis* complex. The latter consists of closely related bacterial species and subspecies sharing 99.9% of the DNA sequence identity that causes tuberculosis in both humans and animals, but differing in their primary host range (*M. tuberculosis, M. africanum, M. bovis, M. pinnipedii, M. microti, M. mungi, M. caprae, M. orygis or Orygis baccilus, M. suricattae, M. canettii, Dassi bacillus*). Some of these pathogens are human pathogens (*M. tuberculosis and M. africanum*), whereas others are primarily animal pathogens, but are potentially zoonotic (Brites & Gagneux, 2016; Galagan, 2014; Forrellad *et al*., 2013; Brosch *et al.*, 2002).

Progress towards achieving global targets in reducing cases of TB continues; the targets set by the Millennium Development Goal to reduce the TB epidemic by 2015 have already been achieved in some countries (WHO, 2012). Worldwide, new cases fell by up to 2.2% between 2010 and 2011 and at an average rate of around 1.5% between 2000 and 2013 (WHO, 2014). The TB mortality rate has reduced by 41% since 1990 (WHO, 2012). Globally, the TB mortality rate fell by an estimated 45% between 1990 and 2013 and the TB prevalence rate fell by 41% during the same period (WHO, 2014). Access to TB care has expanded substantially since the mid-1990s. Despite this, the TB burden remains enormous (8.7 million new cases with 13% co-infected with human immunodeficiency virus (HIV) in 2011 (WHO, 2012). The number of cases of multi-drug resistant TB (MDR-TB) notified in the 22 high MDR-TB burden countries is growing, reaching almost 600 000 worldwide in 2011 and this represents only 19% of the notified cases estimated to have MDR-TB (WHO, 2012). In South Africa HIV, AIDS and tuberculosis contribute significantly to the burden of disease and 73% of TB patients are HIV positive (DOH/SA, 2012). The cost of TB treatment for multiple drug resistant TB (MDR-TB) is high.

Nosocomial tuberculosis is a huge concern in resource-restricted countries (Gandhi *et al.*, 2013; Bamford & Taljaard, 2010), especially among health care professionals (Casey *et al.*, 2015; Tudor *et al.*, 2014; Delft *et al.*, 2015). The annual incidence of TB infection among health care workers in low- and middle-income countries varies from 3.9% to 14.3%. This TB risk infection is attributable to occupational exposure (between 2.6% and 11.3%) (Baussano *et al.*, 2011). In South Africa the standardised incidence ratio of smear positive TB in primary health care workers indicated an incidence rate of more than double that of the general population

(Claassens *et al.*, 2013) and the highest incidence of TB disease was found in housekeeping staff and security staff, although in a review conducted by Nicol and her collaborators (Nicol *et al.*, 2014) no significant association was found between the occupation of health care workers and the risk of TB infection. Following the WHO recommendations on strategies to prevent TB transmission in health care facilities, congregate settings and households (WHO, 1999), South Africa drafted a National Infection Prevention and Control Policy for Tuberculosis, Multidrug Resistant and Extended drug resistant TB. This National IPC (Infection Prevention and Control) policy includes:

1. Work practices and administrative control measures which include: setting an infection and control plan; putting administrative support procedures (with quality assurance) in place, and training staff;
2. Environmental control measures including natural ventilation, air filtration and ultraviolet germicidal irradiation. (DOH, 2007);
3. Personal protection: Use of surgical masks for patients, N95 respirators for health care workers.

Health care facility design plays a major part in the effectiveness of the environmental control measures insofar as the IPC team role is to make recommendations on building design taking into account airborne transmission, the size of wards, air movement and on the placing of IPC provisions like hand wash basins, sluice rooms and treatment rooms (Mehtar, 2010).

Studies carried earlier revealed that infection prevention and control is still inadequate in health care facilities (Delft *et al.*, 2015; Tudor *et al.*, 2014), and that the strengthening of TB control and health care facilities infrastructure is required as TB infection prevention control (IPC) in the majority of South African public health care facilities does not meet the minimal health care standards or legal requirements (Casey *et al.*, 2015; WHO, 2015; Tudor *et al.*, 2014; Ogbonnaya *et al.*, 2011; Nicol *et al.*, 2014; Sissolak *et al.*, 2010).

There is still a need for further understanding of TB transmission in order to help improve infection control measures both in the health care facilities and the home (Schnippel *et al.*, 2013; Sissolak *et al.*, 2011).

2.1.2 Survival of *M. tuberculosis*

M. tuberculosis is essentially an airborne pathogen and is transmitted via aerosols and rarely by accidental inoculation. It is one of the bacteria of particular concern as it can be transmitted in the health care setting, especially where there is inadequate ventilation. Some physico-chemical factors such as temperature, pH, oxygen concentration, redox state, organic matter,

salinity and humidity may influence the survival of *M. tuberculosis in* the environment (Cook GM *et al.*, 2009). Mycobacteria possess an extraordinary ability to adapt to and survive under adverse conditions, including nutrient deprivation, hypoxia and various exogenous challenging conditions (Cook GM *et al.*, 2009).

A recent study on the long term survival of the tuberculosis complex showed that the three species tested survived for 12 months in soil with a final inoculum of 150 $CFU.g^{-1}$ for *M. bovis, 2 x* 10^4 $CFU.g^{-1}$ for *M. canettii* and 2 x 10^3 $CFU.g^{-1}$ for *M. tuberculosis* from a starting inoculum (Schnippel *et al.*, 2013) of 10^8 CFU. g^{-1} in the soil for each of them. This study presented evidence that *M. tuberculosis* remains viable while in the soil, outside its hosts for extended periods of time. It can still multiply once it encounters ideal conditions for growth after a long term survival and it is still virulent when introduced in its hosts (Ghodbane *et al.*, 2014). In addition to the slow growth, naturally occurring aerosolized environmental mycobacteria may not be viable and those that are viable may not be able to be cultured by routine laboratory methods (Tobias *et al.*, 2005).

Several studies experimented with the natural infection of guinea pigs exposed to TB patients in order to explore the infectiousness of patients in various conditions. In some of these studies, ward exhaust air containing patient-generated infectious droplet nuclei was delivered to guinea pigs in TB exposure chambers. Outcomes of these studies revealed that some of the exposed guinea pigs could become infected by TB bacilli through this process and the infective patients were exactly determined (Dharmadhikari *et al.*, 2012; Dharmadhikari *et al.*, 2011;Escombe *et al.*, 2008). Infectious particles that reached exposure chambers were totally assumed to come directly from ward patients. There was no idea that even a tiny fraction could have been generated by re-aerosolization of some particles deposited at any level in the patient ward as there were different movements in patients' surroundings that could have lifted up some of the settled infectious particles previously generated by the patients in these wards.

2.2 Aerosol and *M. tuberculosis*

2.2.1 Definitions

The presence of *M. tuberculosis* in dust may be explained by the fact that when a person with TB coughs, TB laden droplets are expelled into the air and become part of aerosols. These then settle in the environment and in dust, particularly in hot, dry countries as the air movement is easier when the atmosphere is dry and hot than when it is cold and wet.

According to the International Standardization Organization (ISO, 4225:1994), the definition of dust is as follows: *"*Small solid particles, conventionally taken as those particles below 75

µm in diameter, which settle out under their own weight but which may remain suspended for some time".

However, according to the "Glossary of *Atmospheric Chemistry Terms"* (IUPAC, 1990) dust is described as*:*

> Small, dry, solid particles projected into the air by natural forces, such as wind, volcanic eruption, and by mechanical or man-made processes such as crushing, grinding, milling, drilling, demolition, shovelling, conveying, screening, bagging, and sweeping. Dust particles are usually in the size range from about 1 to 100 µm in diameter, and they settle slowly under the influence of gravity. (Table 2.1)

Table 2.1: Size classification of aerosols (Herman *et al*., 2006)

Particle type	Size range (µm diameter)	Settling velocity (cm/min)
Droplet	100-400	1800-15,200
Dust	1-100	18-1,800
Droplet nuclei	1-10 0.1-1	0.2-18 0.005-0.2

Aerosols are airborne particles and can be defined in their simplest form as a collection of solid or liquid particles suspended in a gas. Aerosols are also defined as two-phase systems consisting of particles and the gas in which they are suspended. In a tranquil settling where there is no motion of the gas (air), each particle's motion is solely due to its gravitational settling in the stagnant atmosphere. Therefore, after a certain time there will be an upper stratum of the air with no particles and a lower stratum containing aerosol with a flat upper boundary having the original amount of concentration. Where there is air movement there is diffusion, re-suspension and deposition of particles on various surfaces including walls, ceiling and floors. The concentration of particles decreases with time, but the rate of removal also decreases because it is proportional to the number of particles left suspended in a chamber or a room (Hinds, 1999).

2.2.2 Diffusion, deposition, re-suspension and presence of particles

It has been considered that *M. tuberculosis transmission* is favoured by dust and overcrowding (Chigbu & Iroegbu, 2010). *M. tuberculosis* can readily be isolated from air, aerosols, dust (Herman *et al*., 2006); dust is found among conditions favourable for the multiplication and transmission of mycobacteria. Thatcher and Layton measured the re-suspension of particles under several different conditions and found that re-suspension was significantly dependent

on the indoor particles concentration (Thatcher & Layton, 1995). In 2011 Boor, Seigel and Novoselac were in agreement with Lazaridis and Drossinos (2007) that the re-suspension rate of the surface layer was generally less than of the outer layer (canopy) because of greater adhesive forces between a particle and a surface than between particles as seen in Figure 2.1. Thus, particle re-suspension was found to be significantly greater for multilayer deposits compared to monolayer deposits because higher friction velocities are necessary to re-suspend surface layer particles compared to particles deposited along the outer layers (Boor *et al*., 2011).

In 2009 Xie *et al*. confirmed Wells and Riley's findings that when droplets are expelled into the air, large ones (50-100 µm) would quickly drop out of the air and impact on surfaces near to their sources, while small ones (less than 50 µm) would evaporate into droplet nuclei which could remain suspended in the air and travel long distances from their sources of generation (Xie *et al*., 2009).

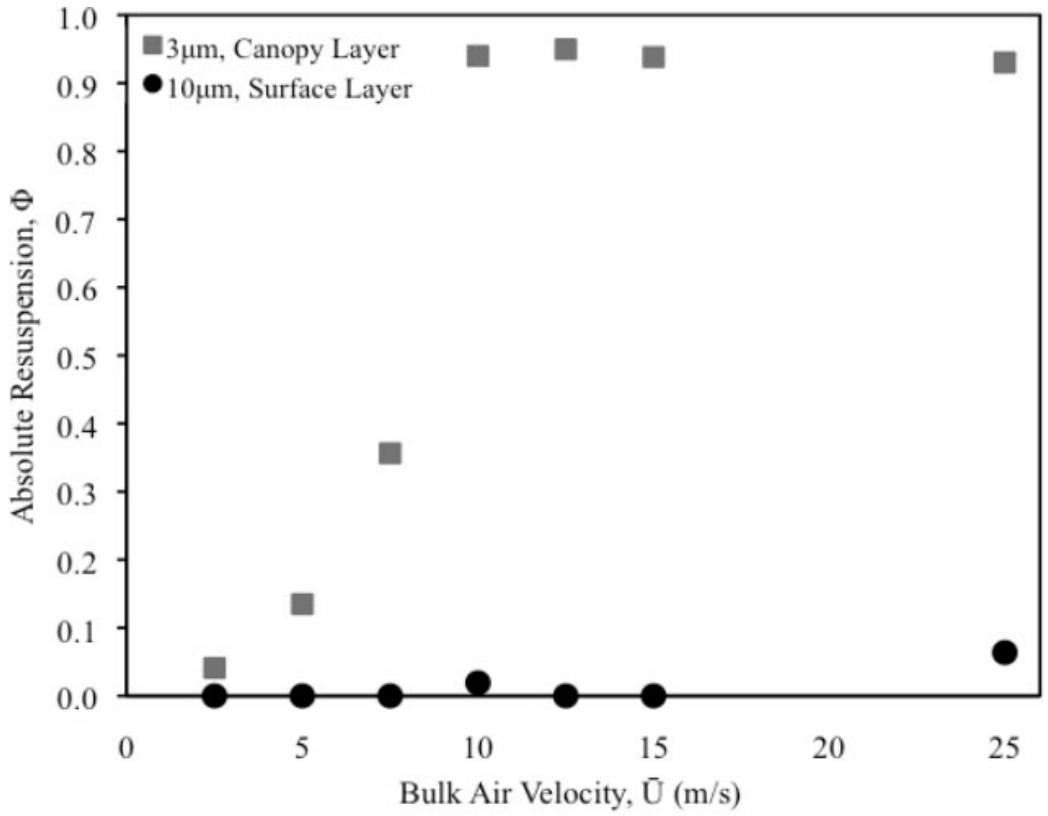


Figure 2.1: Multitude deposit absolute re-suspension fractions for canopy (3µm) surface (10µm) layers

Source (Boor *et al.*, 2011)

Herman reported in the Belgian Biosafety Server that the greater the settling velocity the less the operator is exposed and at risk. He also stated that bacteria or any other biological agent

in droplets remain in a dried state as droplet nuclei (infected airborne particles) which can be moved around rooms and buildings by air currents generated by ventilation and the movement of people. The smaller they are the greater their potential for travelling long distances (Herman *et al*., 2006). Therefore, being an airborne pathogen, *M. tuberculosis* is not exempt from these principles. Alex Müller in “How TB is spread” reported that beside the direct transmission from an infected person to an uninfected person, the TB bacilli can be transmitted by dust. The bacilli settle in dusty and dark areas; when the dust gets swirled up (by sweeping, wind, or when children play on the ground), TB bacilli can then fly and may be inhaled (Müller, 2011). While *M. tuberculosis* is a non-spore forming bacterium, this bacterium can survive for weeks in dust, on carpets or clothes, or on animal carcasses and for months in sputum (Walther & Ewald, 2004; Kunz & Gundermann, 1982).

2.2.3 Infectious droplet nuclei

Almost all infections with *M. tuberculosis* occur via inhalation of droplet nuclei aerosolized by coughing, sneezing or talking. A cough can generate 3000 infectious droplet nuclei; talking for five minutes can produce the same number; singing for one minute and sneezing can produce far more than that and a single sneezing can generate 10^6 bacilli or release up to 40.000 droplets (Mandell *et al*., 2010). One droplet nucleus contains no more than three bacilli. Droplet nuclei are so small that they can remain airborne for extended periods of time (Todar, 2008-2012). Although theoretically one droplet nucleus may be sufficient to cause infection, prolonged exposure and multiple aerosol inocula are usually required (Mandell *et al*., 2010). When a person inhales air that contains infectious droplets, most of the larger droplets are trapped in the upper respiratory tract (nose and throat), where infection is unlikely to occur. Large drops of respiratory secretions and fomites are unimportant in transmission, and special housekeeping measures for dishes and bed linen are unnecessary. Verreault *et al.* reported in Methods for Sampling of Airborne Viruses that evaporation of a liquid expelled can concentrate the non-evaporative content of a droplet until the latter is converted into a droplet nucleus (Verreault *et al*., 2008). However, smaller droplet nuclei may reach the alveoli, where infection begins (Todar, 2008-2012); (Mandell *et al*., 2010) when larger particles can only reach the upper respiratory tract. Re-aerosolization of micro-organisms or solid particles can occur in several circumstances such as when servicing an aerosol sampler (Byers, *et al*., 2013) or like the one noted by Fisher and his collaborators in simulating coughing on an N95 filtering face-piece respirator loaded with droplet nuclei containing MS2 bacteriophage (Fisher *et al*., 2012).

2.2.4 Sampling methods of air or bio-aerosols

Two methods of air or bio-aerosol sampling are often used in a clean room; they can be active or passive (Loddon Mallee Region Infection Control Resource Centre, 2003; Andon, 2006;Sutton, 2008).

1. Air samplers (active air sampling). Air samplers draw in predetermined volumes of air. The air is sucked over a sterile media plate, which is later incubated to reveal the number of viable organisms per cubic meter or litre of air. Agar impaction is the method of choice throughout the industry. This method uses a specially designed and calibrated piece of equipment which holds the media plate under a perforated lid and draws in a known amount of air so that one can accurately measure the number of viable bacteria in the air. The Andersen cascade impactor (ACI) is one these tools used in active air sampling. It has several versions among which is the six-stage air sampler. The six-stage viable particle sampler consists of a vacuum pump that draws 28.3 litres per minute and a mountable canister of six stages drilled with four hundred small round high velocity orifices per stage. The diameter of jets is constant at each stage but diminishes downwards from stage one to six. The air to be sampled enters the inlet cone and cascades through the succeeding orifice levels from stage one to stage six. Smaller particles inertially impact on agar plates. Viable particles are retained on the agar plates and the exhaust air is sucked out through the outlet at the base of the canister and the vacuum hose to the pump or in house vacuum system.

2. Settling plates (passive air sampling). Petri dishes containing sterile growth media are exposed to the environment for a specific period of time, usually between 30 and 60 minutes, but can be exposed up to four hours before compromising the integrity of the media itself. Viable microorganisms which settle onto the media surface will grow after the plates are incubated. However, passive air sampling is tending to be phased out because it does not correlate microbial contamination to an accurately measured volume of air.

Table 2.2. Comparison between active and passive (settle plates) air monitoring in routine environmental monitoring programme (adapted from Romanoa *et al.*, 2015; Andon, 2006).

	Active sampling (air sampler)	**Passive sampling (settle plates)**
Strength	• Qualitative and quantitative • Most official guidelines refer to CFU/m^3 • Suitable for situations with low microbial concentration • shorter sampling period • more accurate specificity and reproducibility	• Simple and inexpensive • Not aggressive towards microbes • Available everywhere • No longer sterile, once opened, • Many samples can be taken in different places at the same time • Meaningful samples (for the contamination of critical surfaces) • Useful for screening and baseline studies • The airflow is not disturbed • Reproduce real conditions
Weakness	• Laborious and expensive • Aggressive towards microbes and a certain number of microbes are inactivated by the impact on the nutrient • Device difficult to sterilise • Expensive • Noisy • The same sampler gives different results • Fallout of microorganisms is not evaluated • The sampler must be frequently calibrated • The air exhaust must be removed • The airflow is disturbed	• More qualitative than quantitative • May miss microbes • Lengthy sampling periods • Not always accepted by official guidelines

The above table shows a comparison between active and passive air sampling methods. Each of them has some strong and some week points. Though laborious, the combination of the two methods in an environmental study increases the qualitative strength of the environmental exploration in association to the quantitative component allowing the correlation between the CFU and the volume of air sampled. This is suitable especially where the bio-aerosol concentration is low.

Respiratory passage airways have different roles where the nose plays non-negligible functions among which are three distinct functions known as the air conditioning functions of the upper respiratory (Hall, 2016):

1. The air warming by the extensive surface of the turbinates and septum (nearly 160 cm^2). The air temperature rises to within two to three per cent of the body temperature before it reaches the trachea.
2. The air humidification is raised up two to three per cent of the full saturation with water prior to its passage through the trachea.
3. The air filtration is performed by the hairs at the entrance of the nostrils, turbulent precipitation, the mucus secreted by the mucous membrane covering the surfaces and by the cilia beats towards the pharynx. In fact, the hairs remove large particles from the air and some particles are separated from the air when the air hits many obstructive vanes (turbinates, septum, and pharyngeal wall). The air changes direction when encountering these obstacles, but some suspended particles, which have far more mass and momentum, cannot change their travel direction as rapidly as the air can and will continue striking the surface of the obstacles. The epithelium of the respiratory tract comprises ciliated cells from the nose to the bronchioles. The particles entrapped in the mucus secreted by the lining membrane of the nose (or other parts of the respiratory tract) are moved at a rate of about 1cm per minute by the constant beat of the cilia towards the pharynx from where they can be expectorated or swallowed.

The cough keeps the passageways of the lungs free of foreign matter. The bronchi and the trachea are so sensitive that any foreign matter or any cause of irritation initiates the cough reflex. During a cough, the exploding air actually passes through bronchial and tracheal shifts. The rapidly moving air carries with it any foreign matter that passes into the bronchi and the trachea.

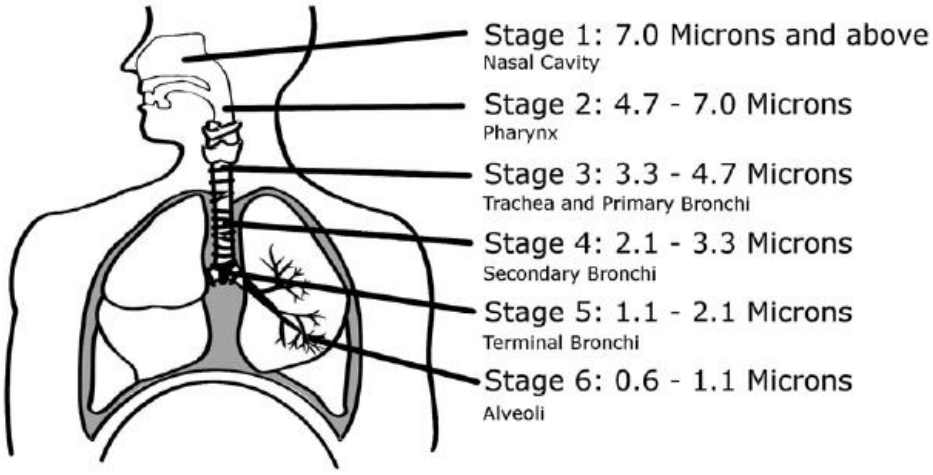


Figure 2.2: Andersen sampler simulates human respiratory system

(From Thermo Fisher, 2007)

According to Thomas and his collaborators, the site of deposition in the respiratory tract within humans and animals and the survival of the microbe in the aerosol are influenced by particle size. The latter determines the length of time aerosolized microorganisms remain suspended within the atmosphere (Thomas *et al*., 2008). Small particles about the size of a bacterium (<5 µm) deposit in the alveoli, while larger particles (> 10 µm) deposit in the upper respiratory tract and particles measuring between 5 µm and 10 µm are deposited in the central airways (Löndahl *et al.*, 2014). Stages 5 and 6 of the Andersen air sampler represent the size of breathable particles that can reach alveoli and respiratory bronchioles where most of the aerosolized particles settle in the agar plates. This corresponds to the most vulnerable exposed area of the respiratory tree and airborne particles such as bacterial bio-aerosols, are therefore the most exposed to the *M. tuberculosis* burden (Figure 2.2). If particles of 10 to 2.1 µm corresponding to 4-1 stages of the Andersen air sampler are inhaled, they will reach the respiratory tract above the terminal bronchi, and they can result in germs being carried. These germs can move to the deeper stages of the respiratory tract or cause an infection in the case of an imbalance between the host defences and the germs' virulence.

Use of *M. smegmatis* as a model for *M. tuberculosis* and other considerations

2.3.1 Similarities between *M. smegmatis* and *M. tuberculosis*

Some mycobacteria subspecies grow slowly in culture (*M. tuberculosis*) while others, such as *M. smegmatis,* grow rapidly. *M. tuberculosis* takes three to four weeks to form colonies on a plate; whereas *M. smegmatis* takes two to three days.

M. smegmatis is a 0.3 to 0.5 µm long bacillus considered to be non-pathogenic, although some cases of infections have been reported in human beings (Best & Best, 2008). It has, however, several similarities with other mycobacterial species (Figure 2.3) and more than 2000 homologies with *M. tuberculosis,* including a similar cell wall structure (Akinola, *et al*., 2013).Therefore this fast growing mycobacterium has been used as a model to study other mycobacteria including *M. tuberculosis* (Gupta & Chatterji, 2005). *M. tuberculosis* and its model, *M. smegmatis* can extraordinary withstand and replicate in an extremely hostile environment. Wong and his collaborators used these particular similarities of *M. smegmatis* and the Bacillus Calmet-Guerin, member of *M. tuberculosis* complex (an attenuated *M. bovis* strain) in high temperature drying and spraying processes. They demonstrated that spray dried BCG showed better viability on the only drying process on mycobacteria as an alternative means to improve on the current lyophilized TB vaccine.

Lauten and his collaborators (Lauten *et al.*, 2010) found that repeatedly exposing bacteria to stresses involving the process of spray drying and dry state containment can select bacteria populations with greater biochemical and biophysical ability to survive. They concluded from

their study that repeated spray drying and selective pressures in dry powders may enrich the capacities for strains which can persist in harsh conditions (like in *M. smegmatis*) and this can be used as a new approach in the formulation of a live whole-cell vaccine (using the BCG-*Mycobacterium bovis* strain).

M. smegmatis is considered to pose a low biosafety risk, and therefore a biosafety level 1 laboratory provides adequate operator protection (Singh & Reyrat, 2009).

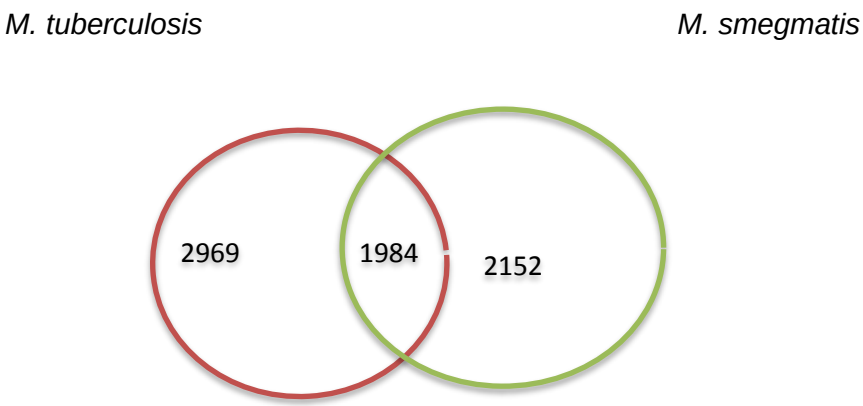


Figure 2.3: Venn diagram showing the orthologs shared and numbers of unique proteins in the two organisms (Adapted from Akinola *et al.*, 2013**)**

2.3.2 Comparison between the wild type and the mutant of *M. smegmatis*

Flint and his collaborators demonstrated that the wild-type cells of *M. smegmatis* can suppress the elevated transfer phenotype of mutant donors, which is consistent with the secretion of a factor that suppresses conjugation. In other terms, a mutant mycobacterium is more effective at conjugation than a wild-type mycobacterium, therefore, mycobacterium mc^2155 or a derivative is a preferred strain to the wild-type. Using the hyper-transformability of this particular strain mc^2155 of *M. smegmatis* they additionally showed that the RD1 region of *M. tuberculosis* complements the conjugation phenotype of the RD1 mutants in *M. smegmatis* with functional equivalence between the *M. tuberculosis* and *M. smegmatis* RD1 regions. Therefore, this provides good perspectives on the role of this critical secretion apparatus (Flint *et al.,* 2004). Mo and his colleagues corroborated Flint's findings in observing a significant increase in the level of plasmid transfer to eukaryotic cells infected with *M. smegmatis* hyper-conjugation mutants compared to a wild type control (Mo *et al.*, 2007). Wu and his collaborators compared the wild-type *M. smegmatis* with a *sigE* mutant strain and found a decreased survival in the mutant under conditions of high-temperature heat shock, acidic pH, exposure to detergent, and oxidative stress (Wu *et al.*, 1997).

Using the fluorescent proteins expression (Green Fluorescent Factor or Optimized Far-Red Reporters) as a means of visualising and quantifying the efficiency of DNA transfer the fast-growing *M. smegmatis* has been transformed with plasmids carrying the reporter genes under the control either under control of bacterial or eukaryotic promoter (Carroll *et al.*, 2010). In the transformation of mycobacterial species using antibiotics as selectable markers, hygromycin resistance vector was found to be more efficient than kanamycin resistant vectors for transformation of *M. smegmatis* (Garbe *et al.*, 1994).

2.3.3 Spectrometric quantification of M. smegmatis

Bacteria quantification can be done using various different methods, among which are: colony forming unit (CFU) count; absorbance; microscopy; flow cytometry and methylene blue dye reduction test. A comparison between CFU count and absorbance (Optic density) shows that the first can make a determination of absolute bacterial number but fail to make a difference between clumping colonies, while the latter cannot make a difference between viable cells and non-viable or their fragments although it is quicker and follows growth curves. It has been shown that, mCherry can be used under hypoxic conditions and can also be a reporter of cell death since its fluorescence decreases in the presence of a bactericidal compound and remains stable in the presence of a bacteriostatic one (Cordone, *et al.*, 2011). The spectrometric turbidity measured as an absorbance cannot be used for pathogenic mycobacteria. The use of OD_{600} of 0.2 is equivalent to 2 X 10^7 CFU/ml or OD_{600} of 1 measure 10^8 CFU/ml. It is recommended to double check this absorbance result with CFU count (Parish & Stoker, 1998).

2.3 Copper and anti-microbial properties

2.4.1 Anti-microbial properties of copper

In March 2008, following a year of comprehensive testing in an independent US laboratory, the USA Environmental Protection Agency (EPA) approved the registration of copper as an anti-microbial agent to reduce specific harmful bacteria linked to potentially deadly microbial infections. There are 275 copper alloys that can now be marketed in the USA as anti-microbial surfaces. The tests showed that 99.9% of the bacteria on copper alloy surfaces (with 65% or greater copper content) were eliminated within two hours of exposure (CDA, 2009).

Copper and its alloys are the first and only solid materials to acquire this status. Typically, this type of registration has been granted to liquids (or aerosols) and gases under the categories of sanitizers and disinfectants (CDA, 2009).

A recent study on the anti-microbial efficacy of copper touch surfaces in reducing the environmental bio-burden, including *M. tuberculosis* in a health care facility, showed the benefit of using copper touch surfaces over standard materials (Marais *et al.*, 2010) (Mehtar S, *et al.*, 2008). Once more a review of metallic copper as an anti-microbial surface conducted by (Grass *et al.*, 2011) reviewed studies on metallic copper as an anti-microbial surface and cited some of the mechanisms involved in the killing process (Grass *et al.*, 2011).

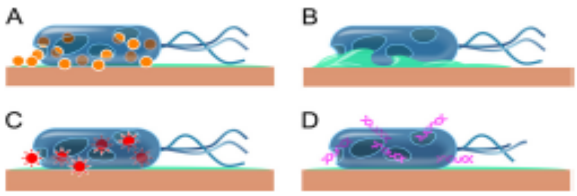
2.4.2 Mechanisms of anti-microbial copper in touch surfaces

The mechanism by which copper kills bacteria is complex (Copper Development Association, 2014). Cristina and her collaborators reported that the killing of bacteria by copper surfaces involves dissolved copper (ions) (Molteni *et al.*, 2010). Copper in touch surfaces kills bacteria via a multifaceted attack (Grass *et al.*, 2011). It affects bacteria in two sequential steps: first, direct interaction between the surface and the bacterial outer membrane, causing the membrane to rupture, and second the occurrence of holes in the outer membrane, through which the cell loses vital nutrients and water, causing a general weakening of the cell. The occurrence of these holes can be explained by a few mechanisms as follows:

> The often called "trans-membrane potential" is a voltage difference between the inside and the outside of a cell. This stable electrical micro-current characterizes every cell's outer membrane. It is strongly suspected that when a bacterium comes in contact with a copper surface, a short circuiting of the current in the cell membrane can occur (Figure 2.4). This weakens the membrane and creates holes (Grass *et al.*, 2011).
>
> Another way to make a hole in a membrane is by localized oxidation or "rusting" which happens when a single copper molecule, or copper ion, is released from the copper surface and hits a building block of the cell membrane (either a protein or a fatty acid). If the "hit" occurs in the presence of oxygen, we speak of "oxidative damage", or "rust." An analogy is rust weakening and making holes in a piece of metal (Stevenson *et al*., 2013; International Copper Association, 2012).

The outer envelope, which is the cell's main defense, is now breached and there is an unopposed stream of copper ions entering the cell. This endangers several vital processes inside the cell. Copper literally overwhelms the inside of the cell and obstructs cell metabolism (i.e. the biochemical reactions needed for life). These reactions occur thanks to cell enzymes' action. When excess copper binds to these enzymes, their activity grinds to a halt. The bacterium can no longer "breathe", "eat", "digest" or "create energy" (Lemire *et al.*, 2013).

After membrane perforation, copper can inhibit any given enzyme that "stands in its way" and stop the cell from transporting or digesting nutrients, from repairing its damaged membrane, from breathing or multiplying (Copper Development Association, 2014; Elguindi *et al.*, 2011).



(A) Copper dissolves from the copper surface and causes cell damage. (B) The cell membrane ruptures because of copper and other stress phenomena, leading to loss of membrane potential and content. (C) Copper ions induce the generation of reactive oxygen species which cause further cell damage (D) genomic and plasmid DNA becomes degraded (Grass *et al* ., 2011)

Figure 2.4: Illustration of the tentative events in contact killing

2.4.3 Obstacles to copper anti-microbial property

Grass and his co-workers mentioned in their review that some factors increased the efficacy of copper contact killing: higher copper content of alloys, higher temperature, and higher relative humidity, whereas this efficacy is lowered by some treatments that lower corrosion rates (application of corrosion inhibitors or a thick copper oxide layer) (Grass *et al.*, 2011). Corrosion inhibitors altered the concentration of copper ions (Cu+/Cu2+) released from copper surface in binding them and subsequently affect bacterial survival (Elguindi *et al.*, 2011). It is also reported that the formation of patina (oxidized copper) does not affect this property of copper because oxidized copper surfaces maintain sufficient corrosion rates and are effective for contact killing (Copper Development Association, 2014; Elguindi *et al.*, 2011).

A biofilm is referred to as a thin layer of microorganisms adhering to a surface of a structure, which may be organic or inorganic, together with the polymers they secrete (Saunders & Newman, 2007). Fouling refers to the undesirable formation of deposits on equipment surfaces, which significantly decrease equipment performance and/or its useful life. Several types of fouling and their combinations may occur, including biological, corrosion, particulate and precipitation fouling. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) (Bailey, 2011).

Many authors reported biofilm formation in studies on copper alone or on the latter compared to other materials. Morvay and his collaborators found that biofilms formed faster on stainless steel than on polyvinyl chloride (PVC) or copper with minimal biofilm formation on the copper surface during the first 30 days of exposure to chlorinated drinking water (Morvay *et al.*, 2009).

Some studies found that to a certain extent biofilm formation reduces the anti-microbial properties of copper (Schwake *et al.*, 2015; Waines *et al.,* 2011; Fox & Abbaszadegan, 2013; Carilloa *et al.*, 2010; Airey & Verran, 2007). In some conditions, like the use of some inhibitors of copper corrosion, and the quality of moisture content, other authors reported the development of the so called “copper resistant bacteria” (Elguindi *et al.*, 2011; Santo *et al.*, 2008). In fact, some organisms developed mechanisms to withstand toxic concentration of metals. This is the case in *Solfobolus solfataricus* and *Enterococcus hirae* which are equipped with two P-type ATPases, acting as specific transporters (copA and copB) coupling the energy of ATP hydrolysis to the ion transport across the cytoplasmic membranes. Thus copA and copB act as resistance factors to copper ions at overlapping concentrations (Völlmeck *et al.*, 2012).

The anti-microbial property of copper has also found use not only in water systems (Yu, Kim, & Lee, 2011) but also in air-conditioning systems as it eliminates biofilm formation and build-up on the heat-exchanger surfaces and reduces the concentration of contaminants in the air (Creamer Media (Pty) Ltd, 2015; Schmdit *et al.*, 2012).

Taking into account the above anti-microbial properties of copper and the bio-aerosols generation, this study was devised in two phases to examine the role of copper in reducing environmental contamination especially with *M. tuberculosis.*

Chapter 3: Research Design and Methodology

3.1 Study Design

There are currently no definitive studies related to the re-aerosolization of *M. tuberculosis* or its surrogate*, M. smegmatis*; therefore, a pilot study was required to assess and optimise the re-aerosolization of *M. smegmatis* and its survival in dust. This project aimed to answer several questions relating to *M. tuberculosis* and the possibility of it being able to cause disease after re-aerosolization: Can *M. tuberculosis be* re-aerosolized? If it can, does *M. tuberculosis* remain viable? If it is viable can it cause infection? Finally, can environmental bio-burden be reduced using copper surfaces?

An enclosed experimental box was used to facilitate the control of the environment for dosage of the microorganism and sampling following agitation of the dust**.** Following a pilot study to assess the survival of *M. smegmatis* in dust and its re-aerosolization, this prospective *in vitro* study was conducted in two phases. The first phase was to test the survival of *M. smegmatis* in the presence of dust and establishing a death curve, and the second phase which evaluated the impact of copper touch surfaces on the survival of *M. smegmatis* under similar conditions. The pilot study is described in the appendices (10A to 10G).

3.2 Study Site

A small isolated room in the Faculty of Medicine and Health Sciences, Stellenbosch University, was used to house the cabinet in which all the experiments were carried out. A completely sealed cabinet of 90 x 70 x 90 cm (693.07 litres in volume) made of Plexiglas® was used as the aerosol chamber to ensure the biosafety of the experiment (Figures 3.1 and 3.2). Holes for inlets from the nebulizer and the air filter and the hole for outlets to the Andersen sampler were on the top face of the aerosol chamber. On one of the lateral faces of the aerosol chamber were a hole for electrical cables and the big sealed opening for the cleaning of the inside of the chamber. An airtight glove port was built on another lateral face of the chamber to allow handling of material, particularly dust and to move the two small fans, each on four wheels in the aerosol biosafety cabinet. A cylinder with an inner and an outer sealed round openings was built aside the glove port to introduce and remove small material including settle plates from the aerosol chamber during experiments. These openings were never simultaneously opened to minimise leaks from the aerosol chamber during experiments. The inside of the cylinder served as resting station for material in transit from outside to the inside of the chamber, and vice-versa. On the top face of the chamber was installed a Kleenpak Emflon® PFR filter having a microbial removal rating of 0.2 µm in liquids and a particulate

removal rating of 0.003 µm in gases for equalisation of pressure inside and outside the aerosolization chamber during the experimentation (Figures 3.1 and 3.2).

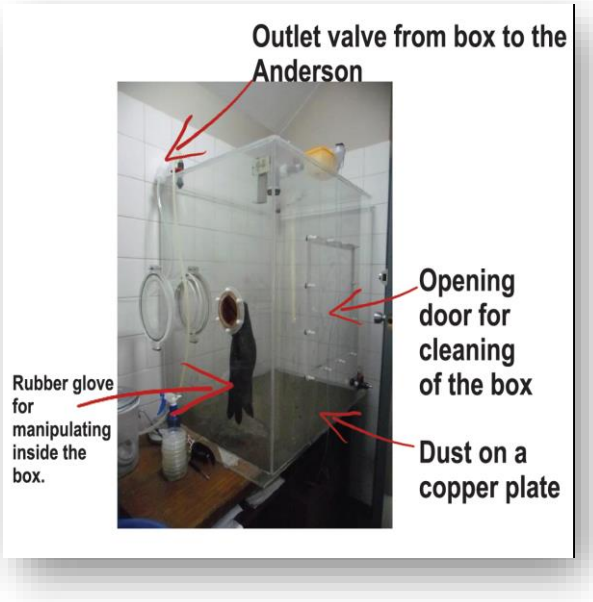


Figure 3.1: An aerosol chamber with dust inside and a filter at the exhaust end of the Andersen outlet tubed inside

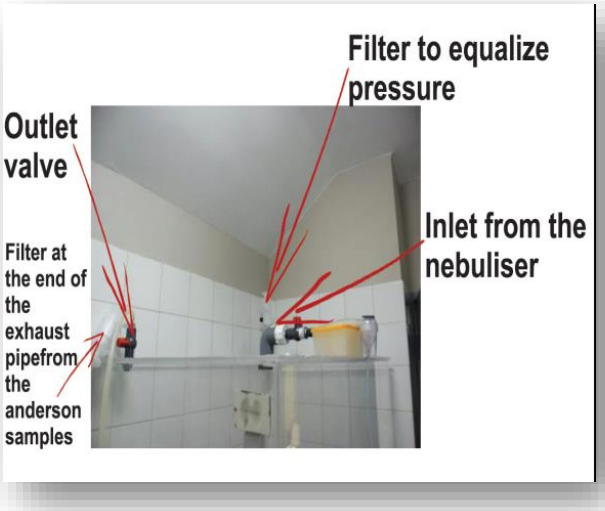


Figure 3.2: The top of the aerosol chamber with the nebuliser jar and the inlet tube from the nebuliser beyond which is a filter to equalise pressure between the external atmosphere and the inside of the box

Microbiological analysis was performed in the laboratory of the National Research Foundation Centre of Excellence for Tuberculosis Research situated at Tygerberg campus of Stellenbosch University, which has extensive experience of working with mycobacteria *M. tuberculosis*.

3.3 Methodology

In the current study the driving force of the dust for one small fan was at 2 Kpa and the velocity of the airflow was at average 2.035 m/s with a maximum of 2.47 m/s for the upwards airflow fan and a minimum of 1.60 m/s for the downwards airflow fan.

3.3.1 Preparation of 7H11 medium

To prepare the Middlebrook 7H11 Agar 19 g of 7H11 powder was suspended in 900 ml of purified water. The solution was swirled to obtain a smooth suspension and autoclaved at 121^{o} C x 10 minutes, then cooled to approximately 50^{o} C. One hundred mls of Middle brook OADC enrichment, 10 ml of filtered and sterilized 50% glycerol and 1 ml of 50 µg/ml hygromycin (here hygromycin was used to select for *M. smegmatis* pCHERRY3.

(50% = 50 µg/ ml) were added aseptically to the cooled autoclaved solution. Finally, 25 mls of the fluid media was poured in each 90 mm petri dish and allowed to set.

3.3.2 Preparation of stock for nebulization

A stock solution of *M. smegmatis* was prepared for nebulization. An overnight culture of *M. smegmatis* was prepared by inoculating 500 µl of a frozen stock of *M. smegmatis* pCHERRY3 into 50 ml of 7H9 containing 10% OADC, 0.5% glycerol, 0.05% Tween-80 and 50 µg/ml hygromycin was added. The mixture was gently mixed and incubated at 37^{o} C for 24 hours, with shaking at 180 rpm. The following day, 50 ml of *M. smegmatis* pCHERRY3 culture was passed through a sterile 40-micron filter and was filtered into sterile a 50 ml tube to remove clumps of bacteria in case there was an overgrowth of *M. smegmatis* and that could be visible on the surface of the preparation. A 1/10 dilution of the previous filtrated solution (standard) was placed in the spectrophotometer (Ultrospec 405 LKB Biochrom®) to measure $OD_{600.}$ Ten milliliters of the solution were spun in a centrifuge (Eppendorf® Centrifuge 5810 R) at 4000 rpm x10 min at room temperature (22^{o} C) and washed once with 10 ml PBS + 0.05% Tween-80 (4000 rpm, 10 min) and then re-suspended in 10 ml PBS + 0.05% Tween-80. Finally, the solution was sonicated by plunging the tube twice for 30 seconds in a sonicator (mrc® Utrasonic cleaner DC 150 H) to disperse clumps of microbes in the solution.

A formula was applied to arrive at the correct inoculum optical density (OD). The OD_{600} was measured and a density obtained (X). An equivalent volume of that number (X) obtained at OD_{600} from 1 ml of the spun solution was used to re-suspend the culture, to effectively adjust the OD_{600} to approximately 1.0. We assumed that 1 OD_{600} corresponds to 10^{8} bacteria per ml. However, we also plated and counted colony forming units (CFU) to confirm the total number of bacterial CFU. Thus, the final inoculum (in CFU) was determined by aliquots being removed and diluted in PBS-T80, plated into 7H11+ hygromycin^{50} and incubated for two to five days. *M. smegmatis* culture was diluted in sterile PBS to a concentration of 10^{6} cells/ml prior to aerosolization. In this experiment, we extrapolated from the literature that this concentration simulates the mycobacterial charge released during sneezing compared to coughing.

3.3.3 Disinfection of the material in the experimental room

Ethanol (70%) solution was used to disinfect the Andersen sampler machine and the experimental box prior to its use (except the cylindrical opening of the box which was wiped with normal detergent to avoid cracking when in contact with an organic solvent such as isopropyl alcohol). The box was dried by means of oxygen flow from the oxygen concentrator which, while not necessarily sterile, did not contain *M. smegmatis* pCHERRY3.

3.3.4 Dust analysis method

Dust collected in plastic bags from ceilings using a paint brush and a scooper was sterilized at 120°C in dry heat for two hours. The sterilised dust was analysed, at the Central Analytical Facility (CAF) at Stellenbosch University, for mineral composition and dust particle size determination. BSE-EDX analysis and elemental mapping was accomplished using a Zeiss EVO® MA15 Scanning Electron Microscope. Prior to analysis the sample was coated with a thin layer of gold coating in order to establish conductivity. The different elements were analysed by means of quantitative ED analysis with a ZEIS EVO MA 15 Scanning Electron Microscope (SEM). Phase compositions were quantified via EDX analysis using an Oxford Instruments® X-Max 20 mm² detector and Oxford INCA software. Energy dispersive spectrophotometry is only suitable for determining major elements of mineral concentrations over 0.1 wt% for heavy elements, and over 0.01 wt% for light elements. Beam conditions during the quantitative analyses were 20 KV and approximately 1.0 A, with a working distance of 8.5 mm and a specimen beam current of -20.00 nA. For mineral analyses counting time was 10 seconds live-time. Natural mineral standards were used for standardization and verification of the analyses. The system is designed to perform high-resolution imaging concurrently with quantitative analysis, with errors ranging from ± 0.6 to the major elements using EDS.

3.3.5 Dust spreading in the experimental box

After installation and connection of the Andersen sampler and the V oxygen concentrator-nebulizer (herein and thereafter nebulizer will stand for the atomization option of the V oxygen concentrator/Nebulizer) both valves connecting the aerosol chamber to the Andersen sampler machine and to the V oxygen concentrator-nebulizer were turned off. Then 125 g of sterile (sterilization with dry heat done at 120ºC) dust were spread manually by shaking its container inside the aerosol chamber and the dust was allowed to settle for at least 15 minutes.

3.3.6 Operation steps for first time use of the oxygen concentrator/nebulizer

Because the experimental area was a closed box, it was not necessary to wear personal protective equipment unless the aerosol chamber was opened. The oxygen pipe was connected to the oxygen outlet, and then to the power socket and switched on. Finally, the oxygen output flow was adjusted according to need (adjusted to $\geq 5L/min$) as indicated by the green part at the bottom of the flow meter.

3.3.7 Loading of the nebulizer and aerosolization steps

After unscrewing the lid of the jar and rinsing it with isopropyl alcohol 70% or ethanol 70%, it was dried using the oxygen flow from the nebulizer and then the sterilized jar (Appendix 5) was filled with six ml of prepared bacterial culture.

The filled jar was screwed back and attached to the nebulizer tube on one side and to the valve connecting it to the aerosol chamber on the other side, and the airflow meter closed. The next step was the switch on the nebulizer and run it until the jar was empty.

Once the jar was visibly empty the nebulizer was switched off, the valve connecting it to the aerosol chamber closed, the jar refilled and the nebulizing process repeated until 20 ml of the prepared bacteria culture was aerosolized in the experimental box. Finally, the nebulizer was switched off and closed as was the inlet valve to the aerosol chamber. At the start of the experiment, the aerosol was generated using a modified V oxygen concentrator-nebulizer to deliver a vertical aerosol discharge for a total time of 40 minutes to deliver 20 ml to be nebulized at a dilution flow rate of 5 litres/min and at the pressure 0.08 KPa with an airflow rate of 5 litres/min. The aerosol from the nebulizer was released near the middle of the ceiling of the aerosol chamber using non-flexible tube. Aerosolization steps are briefly illustrated by the following:

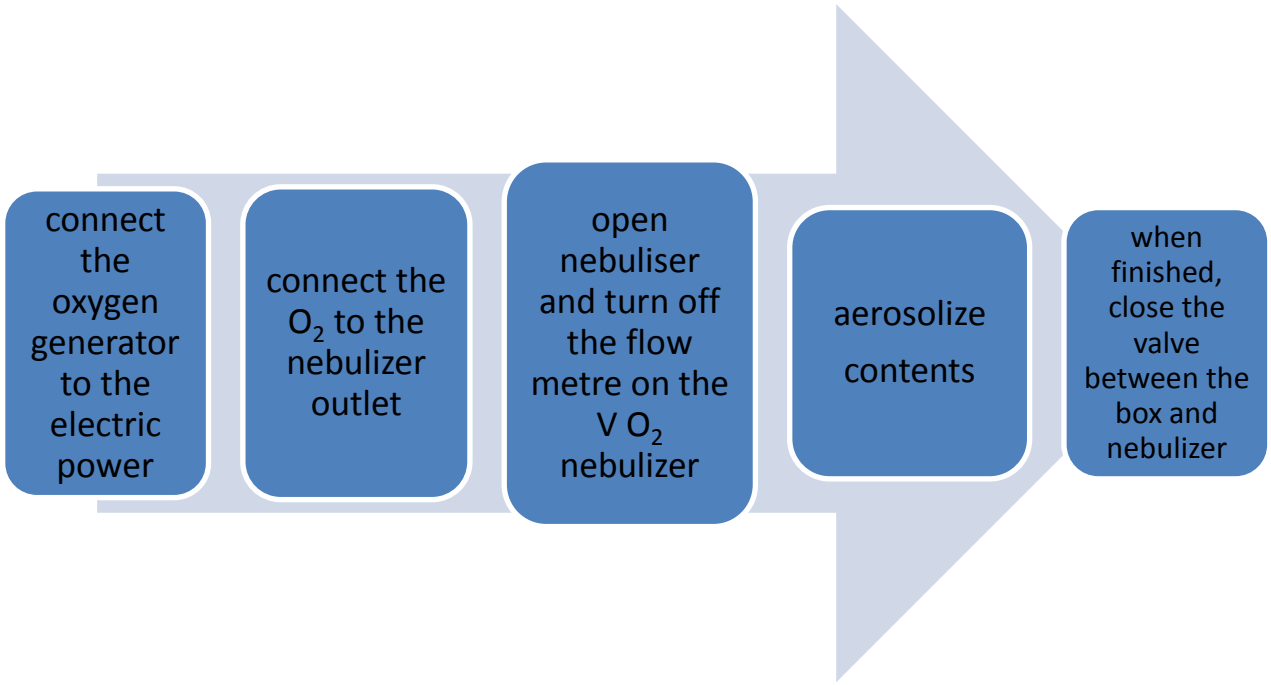


Figure 3.3: Steps of nebulization

3.3.8 Collection and incubation of samples and colony enumeration

We used a modified Andersen sampler six-stage cascade impactor (ACI, herein and thereafter) for air sampling of viable bacteria. The ACI, loaded with six plastic plates containing 7H11 agar with hygromycin (50µg/ml) placed into a 5 L plastic canister with a hinged top opening that could be clamped closed, was placed outside the aerosol area (Figure 3.2). A non-compressible 1 m extension tube was connected from the ACI to the middle of the aerosol

area and fitting through its wall. Outside the aerosol area, the ACI was connected to a vacuum air pump calibrated to pull 28.3 L/min into the sampler which was regulated using a calibrated flowmeter. A second Kleenpak Emflon® PFR filter was installed after the pump at the end of the exhaust tube from the aerosol chamber to enhance biosafety. Concurrently three uncovered agar settle plates were placed in a plastic box (175 x 120 x 65 mm) inside the aerosol chamber for direct sampling on the box; the sampling time for settle plates was five minutes. The combination of active and passive air sampling was done since the first method is useful for screening and baseline studies and the second one is suitable for low microbial concentration (Figure 2.1).

A pilot study was conducted prior to the main study to test the methodology (Appendix 10). This allowed us to consolidate a certain number of parameters as presented below.

The daily sampling was reduced to two main sequences (Figure 3.5). The sampling time was restricted to two minutes because our pilot study indicated that longer sampling times at the cell aerosol concentrations overloaded the agar plates, preventing the ability to quantify. This two-minute air-sampling time for the ACI was applied without having run the fans and two minutes after switching of the fans which were run for 10 minutes as the duration of the mycobacterium bio-aerosol generation period (Table 3.3). The concentration of the organism was modified to 10^6 CFU/ml. Furthermore, the size of the fan was reduced to the one that would re-aerosolise within the volume confines of the sampling box. The incubation period was defined for three to ten days based on the pilot study findings. The length of incubation *of M. smegmatis* transformant reported in mycobacteria protocols by Parish and Stoker is three to five days (Parish & Stoker, 1998). In the pilot study, the growth started being visible on day two of incubation. We prolonged the observation up to ten days as we noticed growth appearing a week later during the pilot study. The estimate of the number of CFU was based on the viable plate count.

Table 3.1: Scheme of Andersen sampling in relation with aerosolization or air movement

Time	ACI sample collection	
T_1	24 hours after aerosolization	**occurrence**
T_2	30 minutes from T_1	2 minutes after having run the fans for 10 minutes

The sequence of the two daily sampling episodes is shown in Figure 3.6 below.

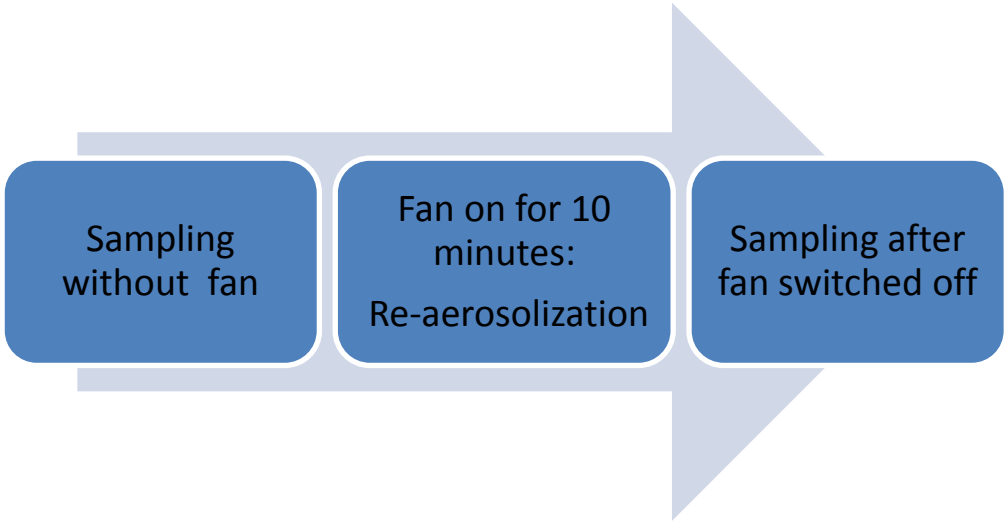


Figure 3.4: Representation of the daily sampling events after initial nebulization

3.3.9.1 Phase One: Plexiglas®

The first phase of the study consisted of three repeat experiments sampling (Anderson slit sampler) and passive air sampling using settle plates. All other procedures were the same as in the pilot study except the number of samples per day. Two daily samples using the ACI and settle plates were taken before and after re-aerosolization (using small fans) during the copper and the Plexiglas® phases of this study (Appendix 3). The first was without running the fan using the Andersen machine with settle plates on small boxes for three to five minutes to assess the presence of residual suspended microbes in the air from the previous day's operations. Following agitating of the dust using a 10-minute run of two small fans, the second sampling operation was done after cleaning of the Andersen sampler, replacement of connective tubes and reloading of the ACI and the experimental box with new agar plates. The second sampling occurred two minutes after having stopped the two small fans.

3.3.9.2 During Phase two: copper covered the floor surface.

The same methodology as in the Plexiglas® phase was repeated after fitting the chamber floor with anti-microbial copper sheeting made of 60% copper. When cleaning the aerosol chamber between the three repeats of 20 days sampling process, copper sheets were removed and cleaned and dried in the same way as the Plexiglas® box.

Twenty-one days after nebulization of *M. smegmatis* the dust was removed from the experimental box and using a hockey stick a certain quantity was plated daily and the plates were incubated for three to ten days to assess the bacterial viability following the copper phase. The results from the copper and the Plexiglas® phases were compared for viability of *M. smegmatis* under similar experimental conditions.

3.3.9 Checking of the aerosol chamber and post-experimental maintenance procedures

The connections were checked frequently and the team ensured that they were tight and that all connectors were tightly sealed.

After each sampling process, the valves between the aerosol chamber and the ACI were closed and all tubes changed. Used tubes were sent for autoclaving (121^0c for 10 min) after all sampling processes of the day. The post-experimental maintenance consisted mainly of removal of the dust from the aerosol chamber using a vacuum-cleaner and disconnection of all tubes. The nebulizer oxygen flow was used to blow all residual dust from valves connected to the box. The inner surfaces of the aerosol chamber were cleaned with a wet cloth and allowed to dry; then all inner surfaces were wiped with 70% ethanol. Cotton wool buds with 70% ethanol were used to wipe all valves connected to the box as well as all fans with an alcohol soaked cloth, and the box door was locked. A mild detergent and warm water were used for cleaning the ACI. The soap was removed by holding the stages under running water or by immersing them successively in baths of clean water. Each stage was examined for any foreign material in the stage holes (Appendix 4). A jet blast of dry air was used to clean them.

3.3.10 Measurement of humidity, pressure and temperature

Two combined barometer-hygrometer-thermometer units were placed in the experimental room with one sensor in the box and another sensor outside the box. This allowed us to measure pressure, humidity and temperature levels inside and outside the experimental box. These data allowed us to monitor the variability of these parameters impacting on the survival of microbes in the environment.

3.3.11 Biosafety

M. tuberculosis is a Biosafety Level 3 microorganism. In this study *M. smegmatis* was used as a surrogate for *M. tuberculosis* as it is a Biosafety Level 1 microorganism. We complied with Biosafety Level 1 practices and requirements for this study (see Appendix 6). Microbiological analysis was done in the Centre of Excellence for Tuberculosis Research. To minimise exposure to bio-hazardous and allergenic material, the researcher used a properly fitted disposable N95 respirator during collection and manipulation of the dust.

3.3.12 Data management and analysis

The agar plates were used to establish the viable colony counts. These were three settle plates sampled two times per day, for 20 days on three separate occasions or repetitions. The

Andersen 6 level plates that were used for air sampling of viable bacteria were also treated as individual observations, and analysed the same as the settle plates. Mixed model repeated measures ANOVA were used for data treatment. For post hoc testing, Fisher Least Significant Difference (LSD) testing helped to determine the effect of surface type (Plexiglas® or copper surface), time (days), temperature, humidity, Andersen level and pressure on the number of microbes cultured, with clustered robust standard errors on individual observations over days. An interaction term between surface type and days was also added to the model. Two separate regression models were run, one with the count of microbes determined by the settle plates and the other from the Andersen levels with particular focus on levels five and six. Levels five and six represent the alveoli and respiratory bronchioles where most of the aerosolized particles settle in the agar plates and this corresponds to the most vulnerable exposed area of the respiratory tree to airborne particles such as bacterial bio-aerosols, therefore most exposed to *M. tuberculosis* burden).

Maximum likelihood was used to estimate the negative binomial and dispersion parameters. A p-value< 0.05 was considered statistically significant; unknown parameters were estimated using 95% confidence intervals. Statistica 12.6 was used for data analysis. According to Statistica 12.6 letters are used in comparison of the two means or two parameters to assess the statistical difference. If at least one letter is found in the same position in both curves, we cannot reject the hypothesis that there is no statistical difference, in case there is no similarity in letters at the same position in both curves, the difference is statistically significant. All letters were removed from the comparison graphs of the two treatments and replaced by asterisks, where P value of less than 0.05% for easy reading.

3.3.13 Ethical considerations

The ethical approval for the study was obtained from the Faculty of Health Sciences and Medicine the University of Stellenbosch under #S13/09/173. There was no need in vitro study to get consent forms as there were no animals and no human beings involved as participants.

Chapter 4: Results

4.1 Chemical and physical characteristics of the study environment

The study was conducted in the aerosol chamber at the average temperature of 19.29^0 C (range 15.9 - 25.7^o C), average humidity of 59% (range 26% - 74%), and an environmental pressure of 1018.52 hpa, (range: 1001 - 1035 hpa).

In both the Plexiglas® and the copper phases the atmospheric findings were similar.

In the Plexiglas® phase, the average was 19.33 ^{0}C, (range 15.9 - 23 ^{0}C) for temperature, 58.92%, (range: 44 and 69%) for the humidity at the average pressure of 1021.27 hpa (average: 1012 and 1035 hpa). In the copper phase, the average, the min and the max were respectively 19.25, 17.2 and 25.7 ^{0}C for the temperature, 58.09, 26 and 74% for the humidity and 1015.78, 1001 and 1031 hpa for the pressure. In this phase, the environmental pressure averaged at 1015.78 hpa (range 1001-1031 hpa). There was no significant statistical difference in humidity, pressure or temperature between the conditions of two phases (copper and Plexiglas®), between the three repeats in each phase and among the 20 days of each series of repeat.

The mineral composition and results of granulometry analysis of two dust samples (Appendix 7) collected from the same ceiling were as follows:

Mineral composition

Table 4.1: Sample 1 Element analysis normalised to 100 wt%

Element	Na	Mg	Al	Si	S	Cl	K	Ca	Fe	Cu	O	Total
Average	1.98	1.28	5.66	23.59	1.24	2.30	1.29	12.09	7.06	0.87	42.63	100%

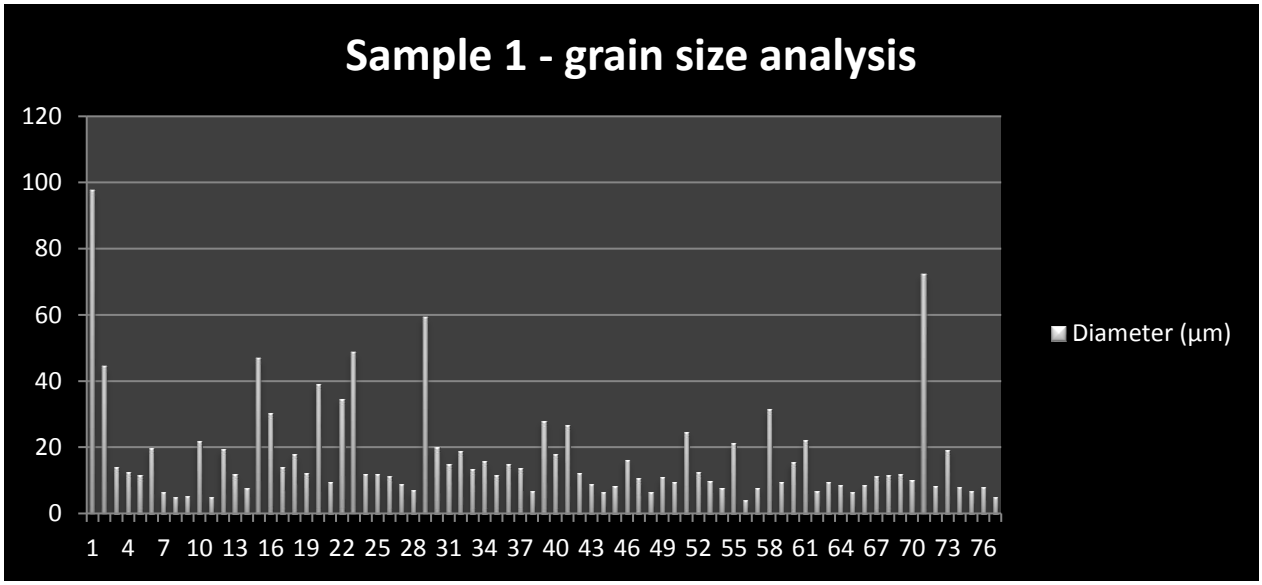
The percentage of copper in sample 1 is 0.87%

Table 4.2: Sample 2 Element analysis normalised to 100 wt%

Element	Na	Mg	Al	Si	S	Cl	K	Ca	Fe	Cu	O	Ti	Total
Average	1.39	1.25	4.68	22.38	2.25	1.90	1.15	14.18	6.07	1.23	42.78	0.73	100%

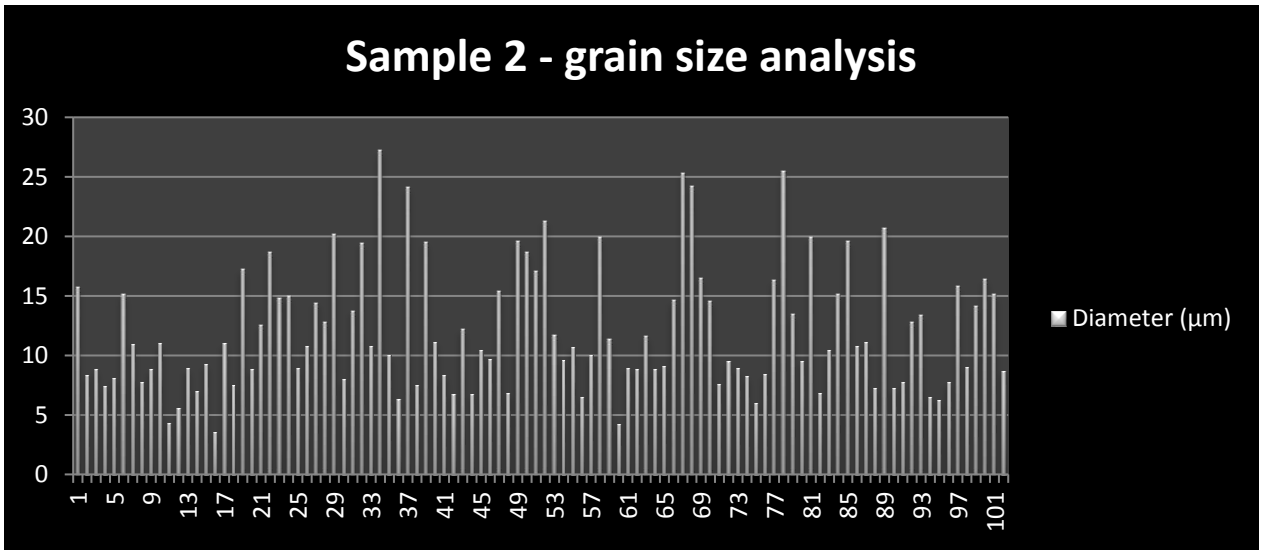
The percentage of copper in Sample 2 is 1.23%

The two samples included a variety of mineral grains which were interpreted as quartz, k-feldspar, biotite, calcite, gamet, sphene and staurolite.



Diameter (µm): AVE 17.09 MIN: 3.93 MAX 97.70

Figure 4.1: Grain size analysis of Sample 1 shows the diameter of the particles and the range



Diameter (µm): AVE 11.99 MIN: 3.56 MAX 27.24

Figure 4.2: Grain size analysis of Sample 2 shows the diameter of the particles and the range

The particles' sizes in sample S1 were generally larger than in sample S2 (Figures 4.1 and 4.2). The grain size of sample S1 was particularly variable. The modal abundance of elements and mineralogical composition for both samples were strikingly similar.

After each series of 20 days re-aerosolization and sampling, the dust was removed from the experimental box, and plates incubated for ten days to assess the viability of mycobacteria after the two sets of experiments on Plexiglas® and on copper. No growth of *M. smegmatis* was found after dust removal following Phase 2 triplicates, whereas cultures were still positive until the 24th day after nebulization in presence of Plexiglas®, therefore there was no return to positivity after the second phase of the current study. During the pilot study (Appendix 10), we observed some few growths from samples collected prior to re-aerosolization, 24 hours after nebulisation or re-aerosolization but no growth was noted in the two study phases 24 hours after nebulisation or re-aerosolization, no graph was provided. From each of the six levels of the ACI, samples with positive growths were encountered but our focus was mainly on samples collected from levels 5 and 6 and from settle plates.

4.2 Sampling in presence of Plexiglas®

4.2.1 Active sampling in presence of Plexiglas®

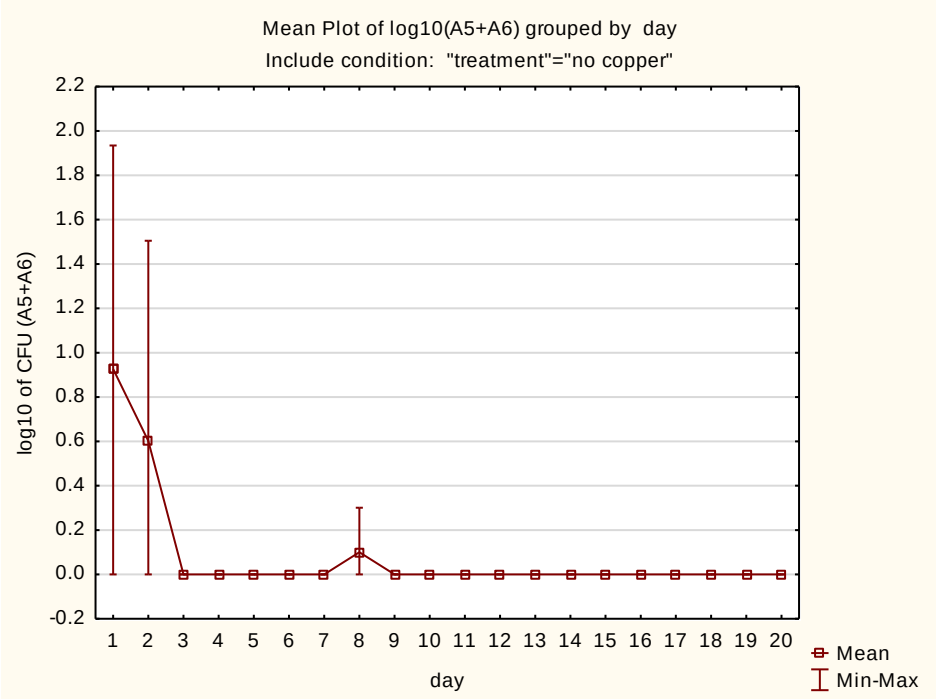


Figure 4.3: Mean plot of log10 (A5+A6) from active sampling grouped by day in Plexiglas® phase: a magnification of the post-inoculum

The combination of levels 5 and 6 of the Andersen in the Plexiglas® phase showed that $\log_{10}$ of the concentration of mycobacteria picked by active sampling was <1 on Day 1. *M. smegmatis* was not isolated between Day 3 and Day 7- a single survivor colony was noted on Day 8, after which there was no further growth.

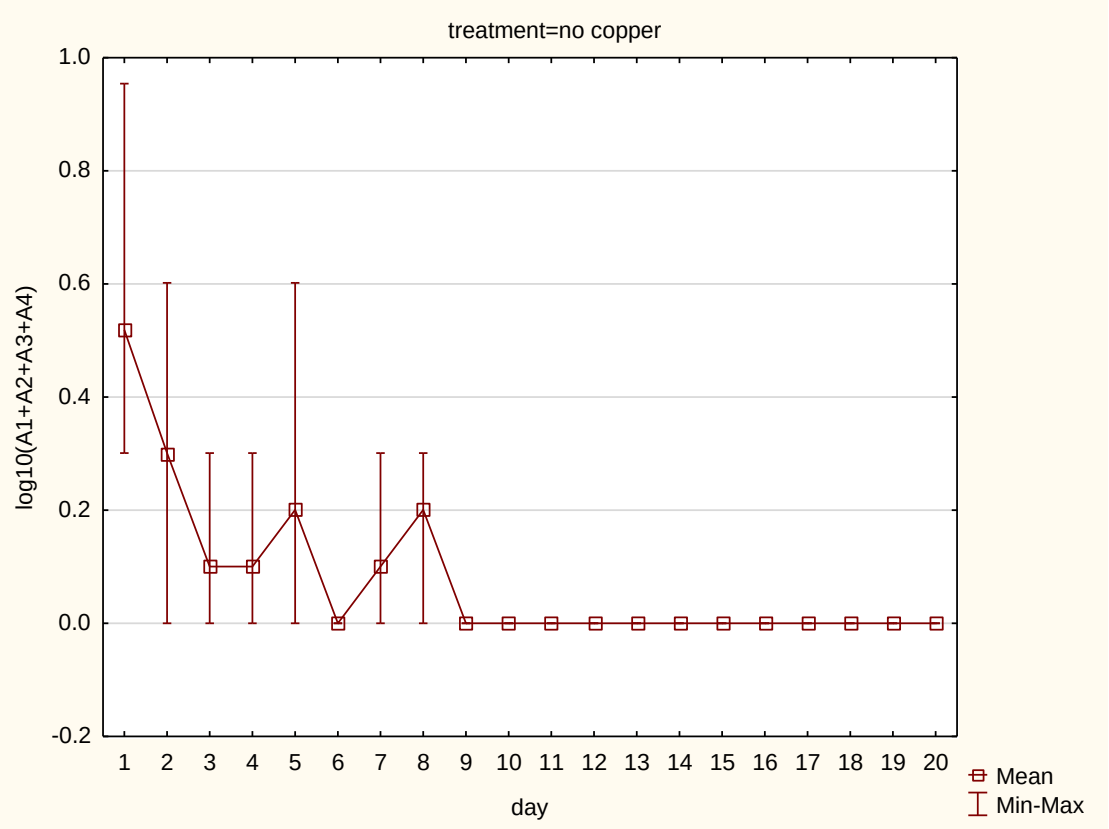


Figure 4.4: Mean plot of log10 (A1+A2+A3+A4) from active sampling grouped by day in Plexiglas® phase: a magnification of the post-inoculum

The combination of levels 1, 2, 3 and 4 of the Andersen in the Plexiglas® phase showed that the mean $\log_{10}$ of the concentration of mycobacteria picked by active sampling was <1 on Day 1. *M. smegmatis* was not isolated on Day 6 and Day 7; few survivor colonies were noted on Days 7 and 8, after which there was no further growth.

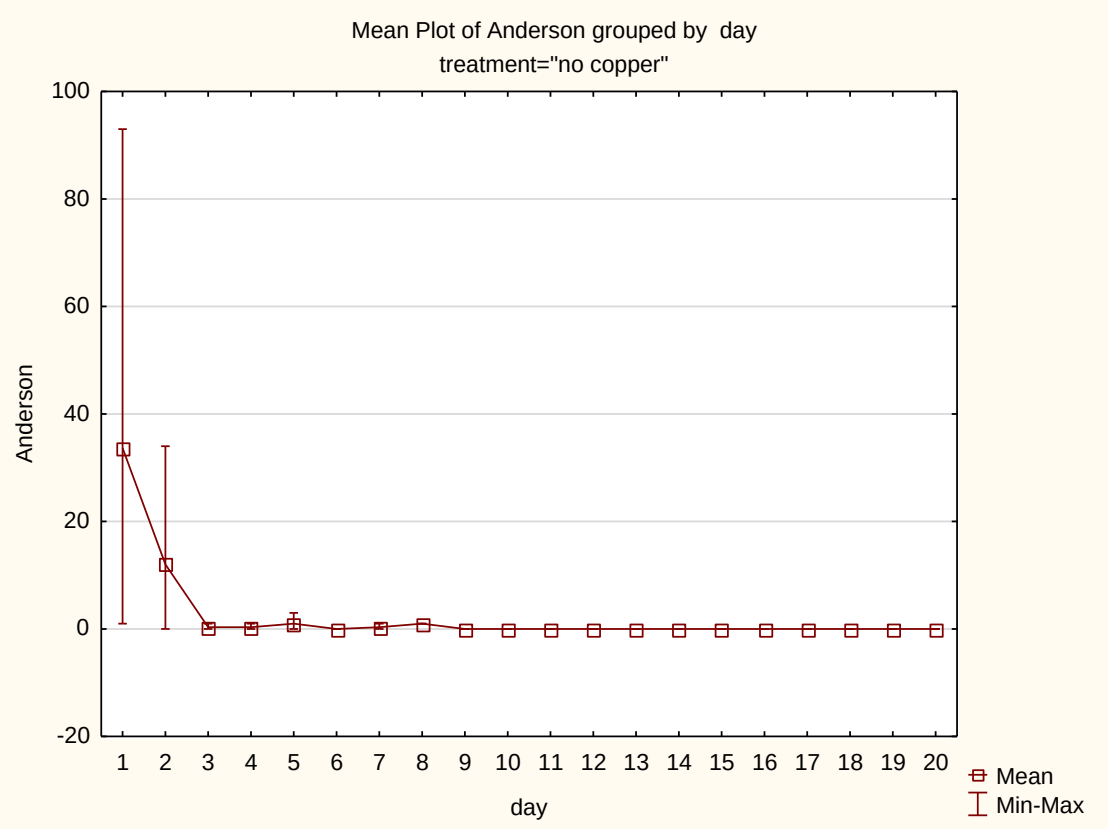


Figure 4.5: Mean plot of log10 (A1+A2+A3+A4+A5+A6) from active sampling grouped by day in Plexiglas® phase: a magnification of the post-inoculum

The combination of levels 1, 2, 3, 4, 5 and 6 of the Andersen in the copper phase showed that the mean $\log_{10}$ of the concentration of mycobacteria picked by active sampling was <1 on Day 1. The concentration of *M. smegmatis* dropped progressively until Day 9 when it became nil and there was no further growth.

4.2.2 Passive sampling in presence of Plexiglas®

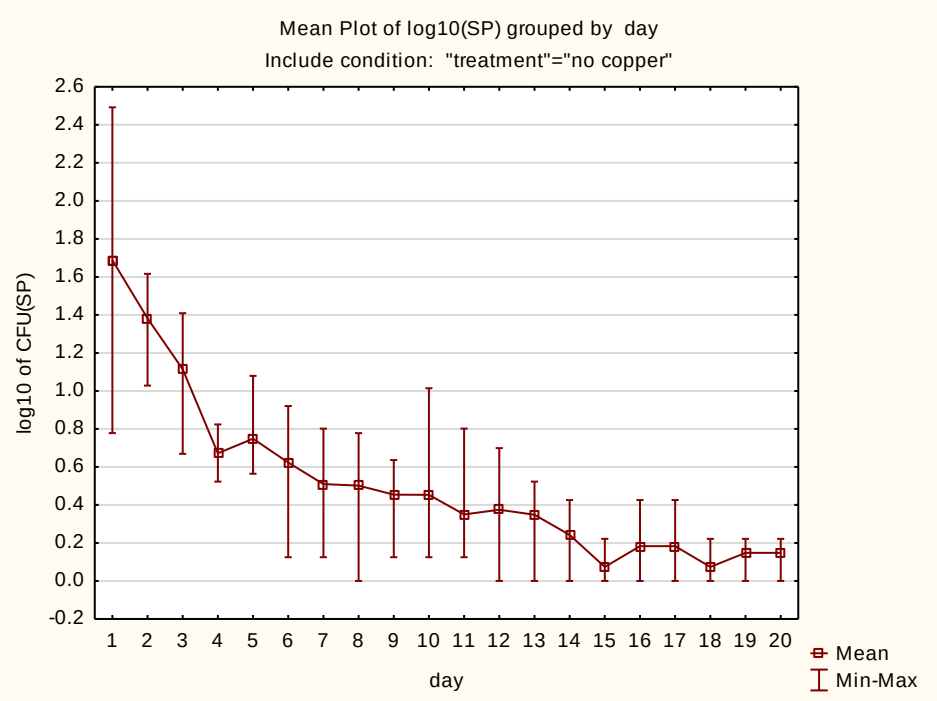


Figure 4.6: Mean plot of log10 for settle plates isolates from passive sampling in Plexiglas® phase: a magnification of post inoculum

The $\log_{10}$ mean concentration of mycobacteria on Plexiglas® from passive sampling from Day 1 was around 1.6 and gradually decreased to 0.7 at Day 4. However, *M. smegmatis* could be isolated up to Day 20 in this group of experiments.

4.3 Sampling with Copper

4.3.1 Active sampling with copper

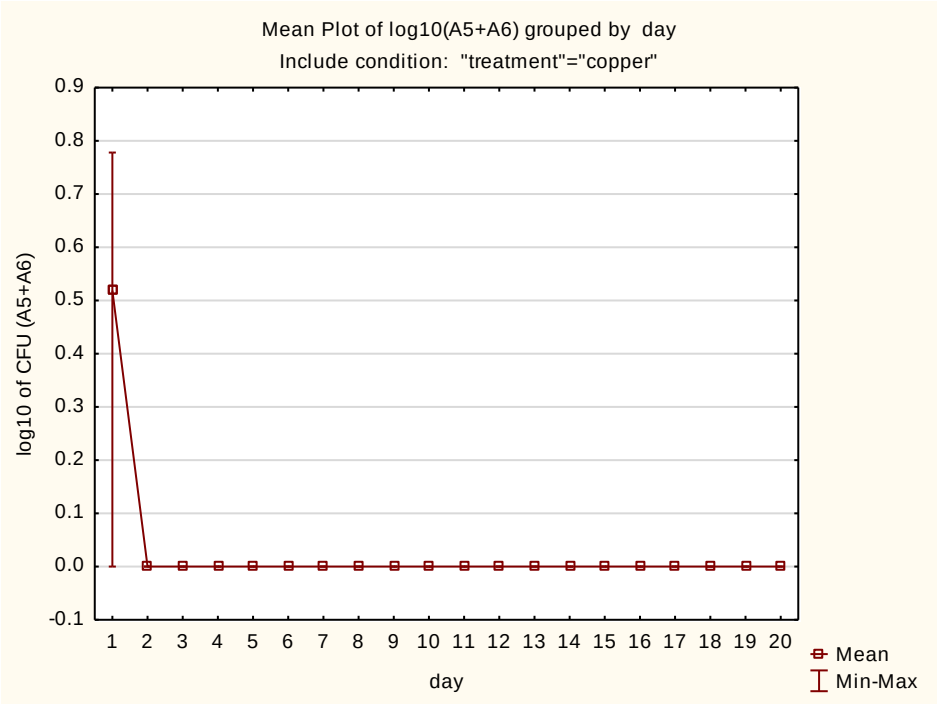


Figure 4.7: Mean plot of log10 (A5+A6) from active sampling grouped by day in copper phase: a magnification of the post-inoculum

In copper treatment, the trend of decrease of log10 means of the concentration of mycobacteria at ACI level 5 and 6 is abrupt and reached zero around Day 2 up to the end of the experiment.

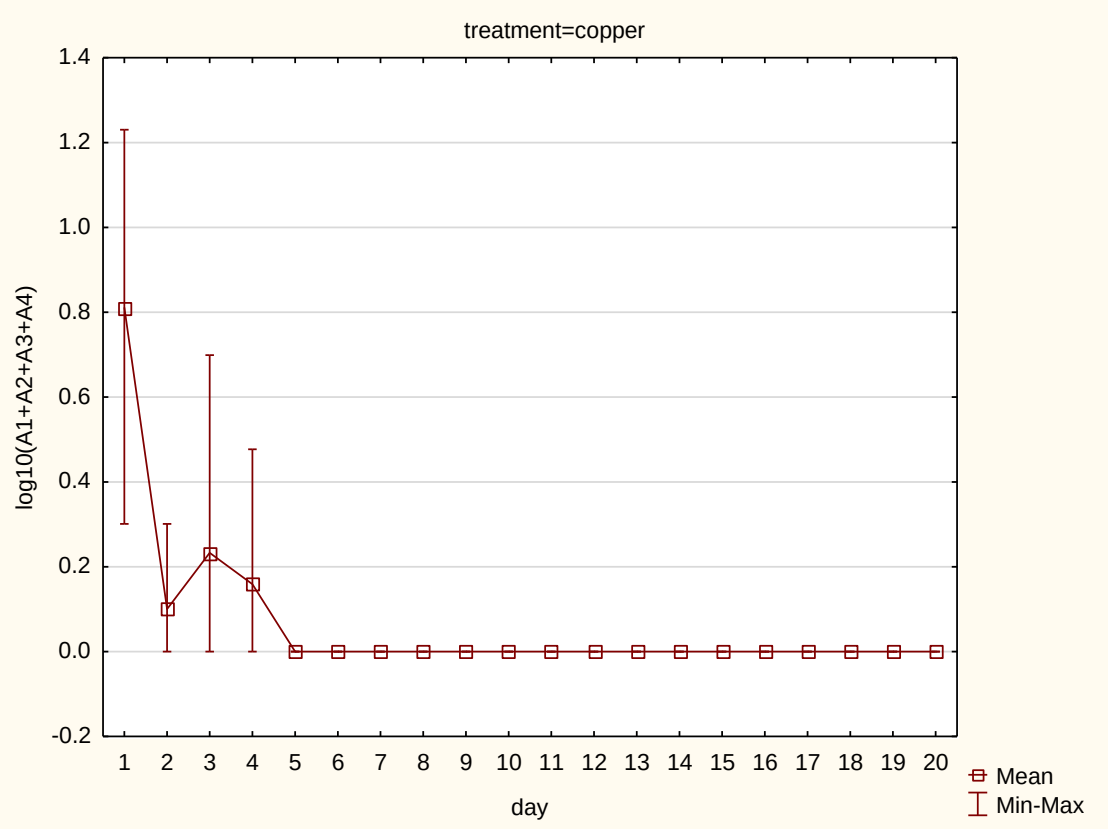


Figure 4.8: Mean plot of log10 (A1+A2+A3+A4) from active sampling grouped by day in copper phase: a magnification of the post-inoculum

In copper treatment, the trend of decrease of log10 means that the concentration of mycobacteria at ACI combined levels 1, 2, 3 and 4 was abrupt from Day 1 to Day 2 with rebound growth on Day 3, and dropped progressively to reach zero on Day 5, with no further growth up to the end of the experiment.

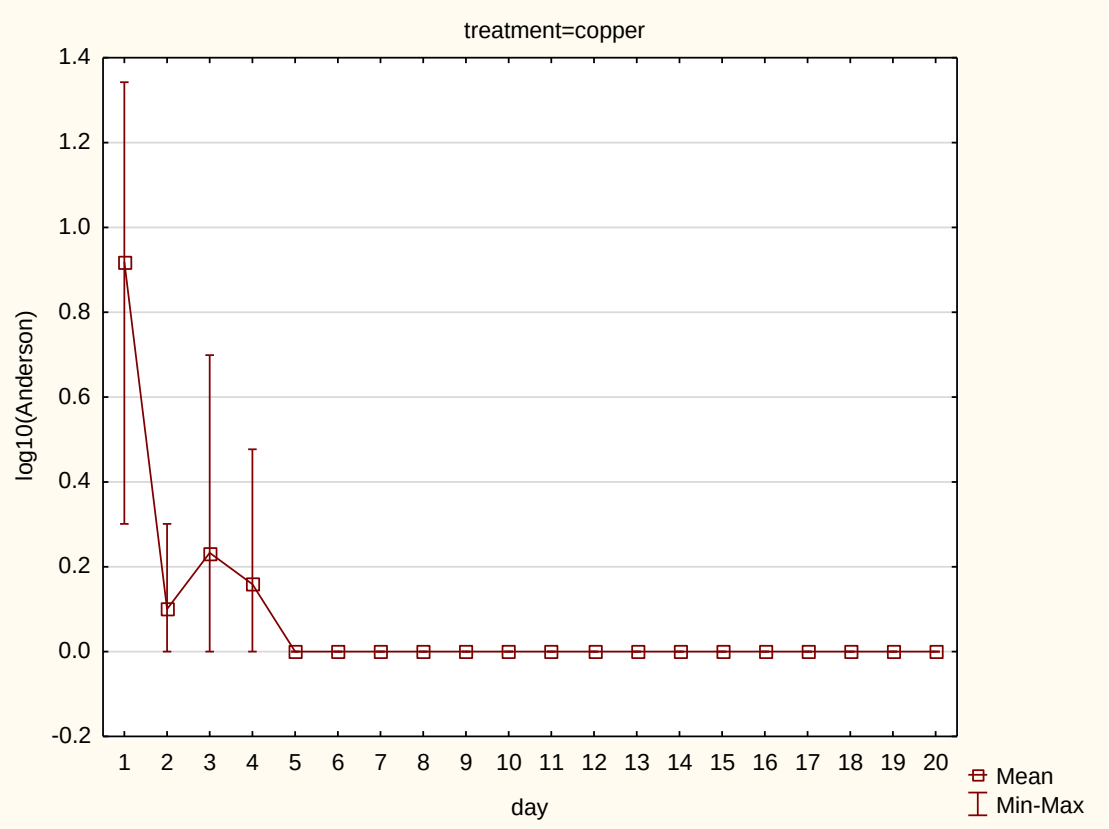


Figure 4.9: Mean plot of log10 (A1+A2+A3+A4+A5+A6) from active sampling grouped by day in copper phase: a magnification of the post-inoculum

In copper treatment, the trend of decrease of log10 means of the concentration of mycobacteria at ACI overall combined levels 1 to 6 was abrupt from Day 1 to Day 2 before it rebounded on Day 3, and dropped progressively to reach zero on Day 5 with no further growth up to the end of the experiment.

4.3.2 Passive sampling with copper

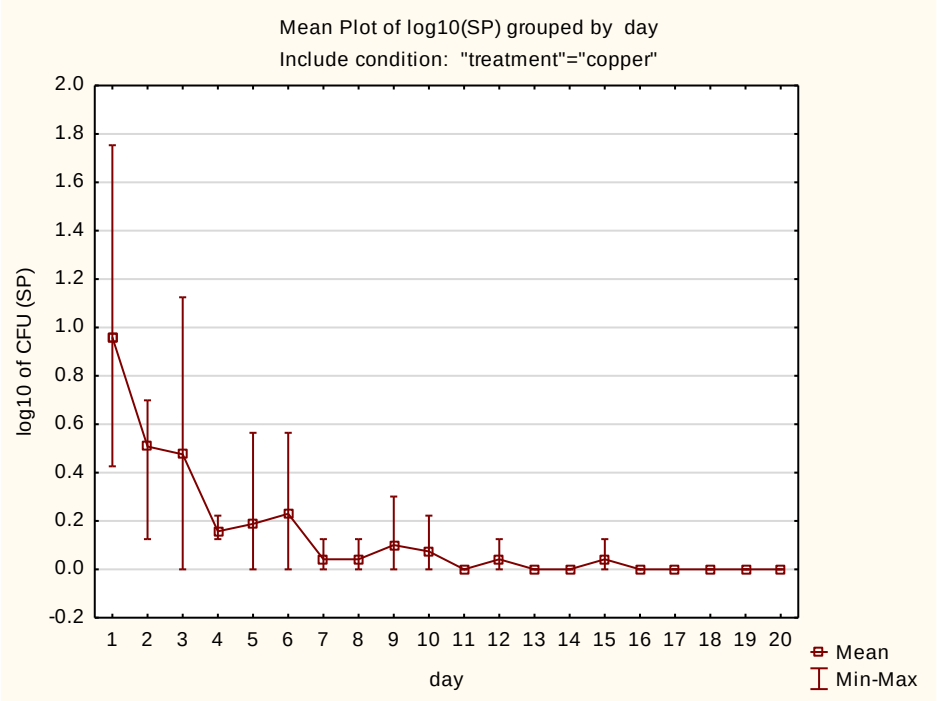


Figure 4.10: Mean plot of log10 for settle plates isolates from passive sampling in copper phase: a magnification of the post-inoculum

On settle plates in the presence of copper the trend of decrease on $\log_{10}$ concentration of mycobacteria from the initial inoculum dropped just below 1 and continued to decrease gradually until no further colonies were isolated by Day 16.

4.4 Comparative data copper and Plexiglas®

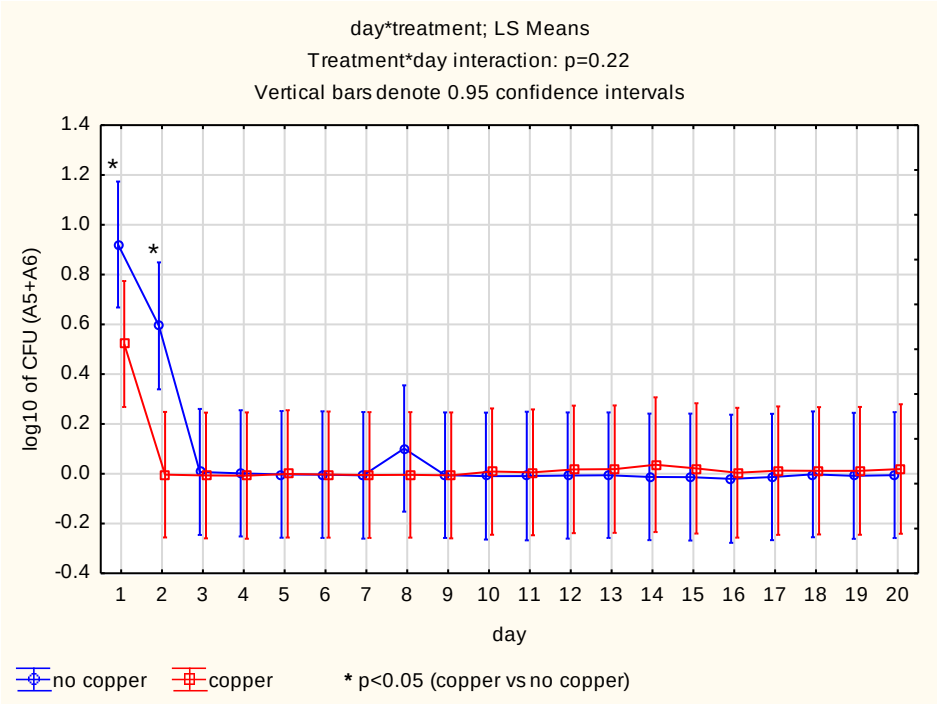


Figure 4.11: Comparison between copper and Plexiglas® mean plot of log10 (A5+A6) from active sampling grouped by day

The number of captured microbes from Day 0 to Day 1 was generally less in the presence of copper than in the presence of Plexiglas® with a difference which was statistically significant before Day 3 (presence of asterisks where p value < 0.05). This difference became statistically non-significant from Day 3 onwards. The overall p value was 0.22, which is > 0.05.

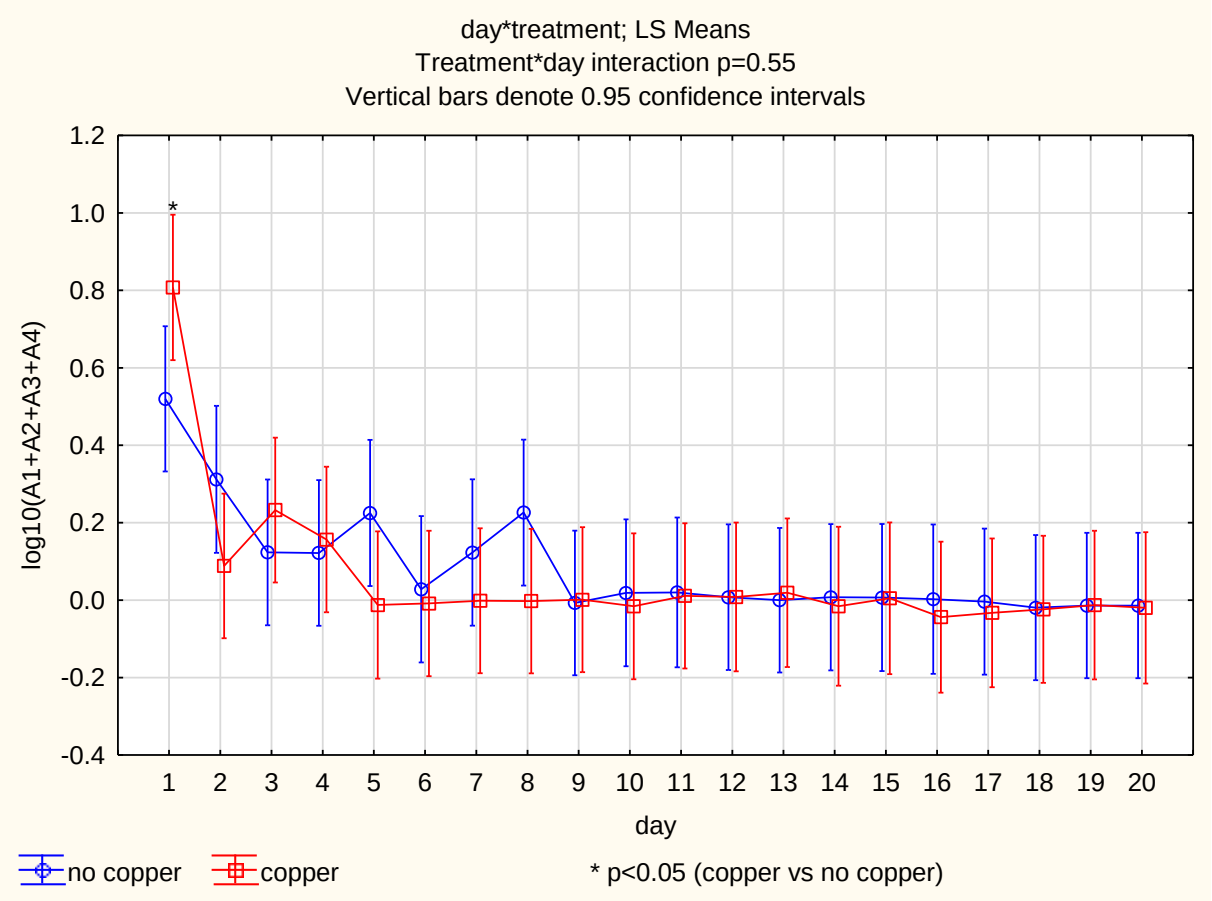


Figure 4.12: Comparison between copper and Plexiglas® mean plot of log10 of isolates from active sampling (A1+A2 +A3 +A4): a multivariate analysis

The number of captured microbes from Day 0 to Day 1 is generally less than on Plexiglas® in copper treatment, with a difference which is statistically significant (presence of asterisks Day 9 on the two graphs) on Day 1 before the combined number of mycobacteria captured at these four levels in copper treatment drastically dropped under the one captured at the same levels in Plexiglas® presence, with no significant statistical difference between the two treatments. The overall p value is 0.55, which is > 0.05.

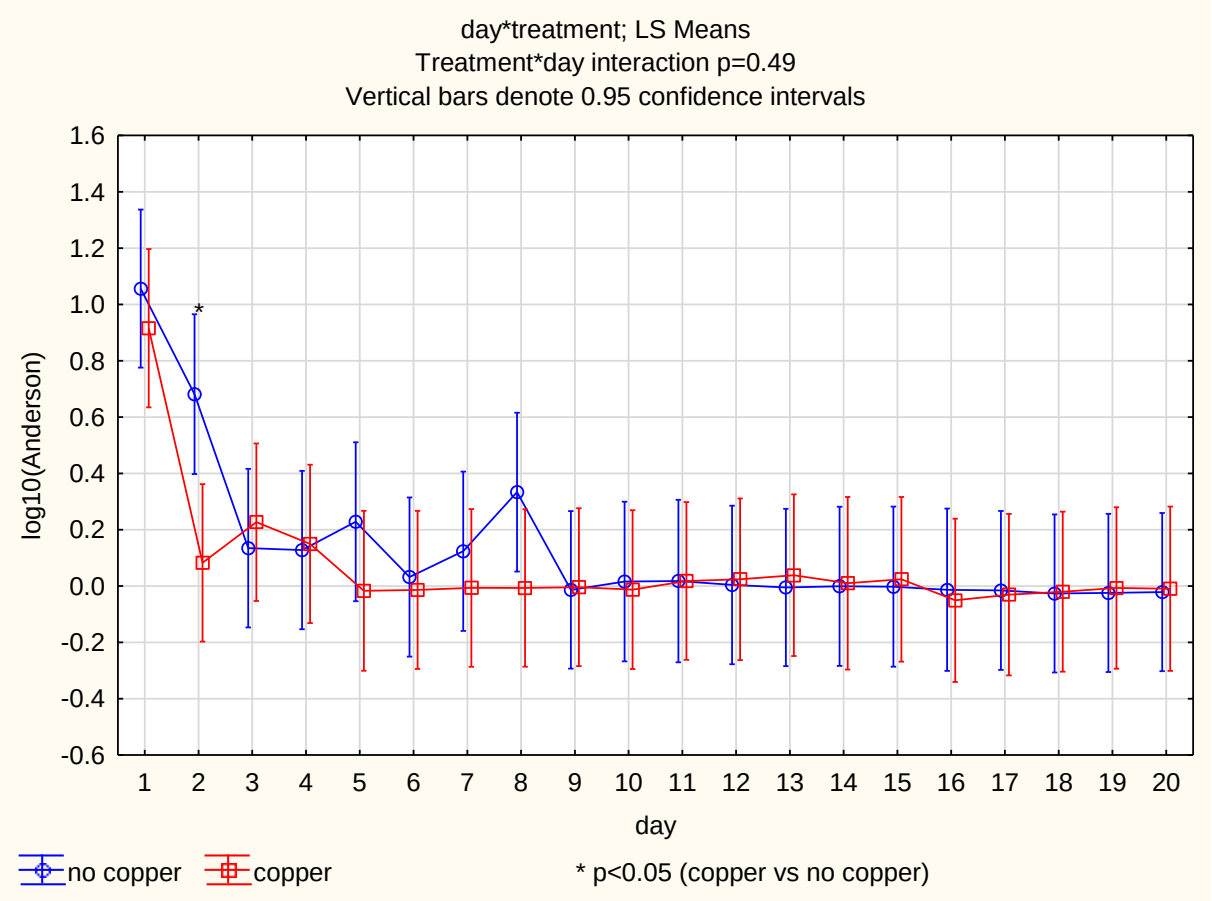


Figure 4.13: Comparison between copper and Plexiglas® mean plot of log10 of isolates from active sampling (Andersen): a multivariate analysis

The number of captured microbes from Day 0 onwards is generally less in the presence of copper than in the presence of Plexiglas® with a statically non-significant difference. The difference between the two treatments is statistically significant only around Day 2 (presence of asterisks where p value < 0.05). The overall p value was 0.49, which is > 0.05.

Table 4.3: Table representing the effect of treatment on the log $_{10}$ microbe number captured at levels 5 and 6 of Andersen from Day 1 to Day 2 for three repeats

Day	Treatment	log10(A5+A6) Mean	log10 (A5+A6) std.Dev.	% of reduction between Plexiglas® and copper
D1	Plexiglas®	0.926532	0.969817	44.01
D1	copper	0.518768	0.449266	
D2	Plexiglas®	0.602060	0.796451	100
D2	copper	0.000000	0.000000	

The reduction of captured viable microbes is exponentially reduced by 35.02% {(0.926532-0.602060)/ 0.926532} from Day 1 to Day 2 in the Plexiglas® phase whereas it is exponentially

reduced by 100% {(0.518768-0)/ 0.518768} in the copper treatment for the same period of time. The mean quantity of captured microbes has been reduced from Plexiglas® to copper treatment on Day 1 of 44.01% and 100% on Day 2.

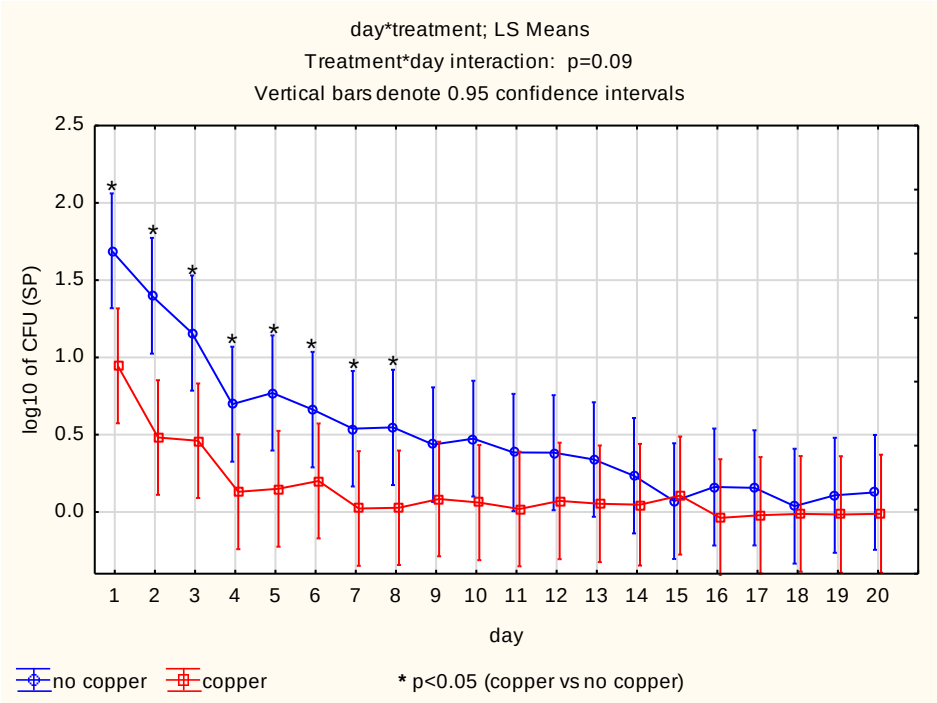


Figure 4.14: Comparison between copper and Plexiglas® mean plot of log10 for settle plates isolates from passive sampling: a multivariate analysis

The number of captured microbes from Day 0 to Day 1 is generally less in copper treatment than on Plexiglas® with a difference which is statistically significant from Day 1 to Day 8 (presence of asterisks Day 9 on the two graphs) before the difference became statistically non-significant from Day 9 onwards (absence of asterisks from Day 9 expressing no statistical difference between the copper treatment and Plexiglas® phase). The overall p value is 0.09, which is > 0.05.

This figure shows that *M. smegmatis* can survive for at least twenty days in a dusty environment in the presence of Plexiglas® and can be re-aerosolized whereas *M. smegmatis* can survive for only about fifteen days after nebulization in a dusty environment in copper treatment with significant statistical difference up to Day 8 between the two phases (presence of asterisks).

Table 4.4: Table representing the effect of treatment on log10 microbe captured by passive sampling from Day 1 to Day 9 for three repeats

Day	Treatment	log (SP) Mean	Log (SP) Std.Dev	% of reduction from no copper to copper
D1	Plexiglas®	1.688389	0.862004	43.18
D1	Copper	0.959422	0.700961	
D2	Plexiglas®	1.381644	0.311656	45.79
D2	Copper	0.507626	0.331417	
D3	Plexiglas®	1.113873	0.392132	57.32
D3	Copper	0.475323	0.582370	
D4	Plexiglas®	0.671931	0.150536	76.60
D4	Copper	0.157242	0.055951	
D5	Plexiglas®	0.748504	0.286997	74.87
D5	Copper	0.188090	0.325782	
D6	Plexiglas®	0.623222	0.434238	63.14
D6	Copper	0.229737	0.296374	
D7	Plexiglas®	0.509544	0.347704	91.83
D7	Copper	0.041646	0.072133	
D8	Plexiglas®	0.501717	0.435251	10.36
D8	Copper	0.454607	0.286030	
D9	Plexiglas®	0.454607	0.286030	77.93
D9	Copper	0.100343	0.173800	

Table 4.4 above illustrates the trend of the exponential difference between the numbers of viable microbes captured during the Plexiglas® phase compared to the copper treatment from passive sampling.

4.5 Comparative data active sampling versus passive sampling

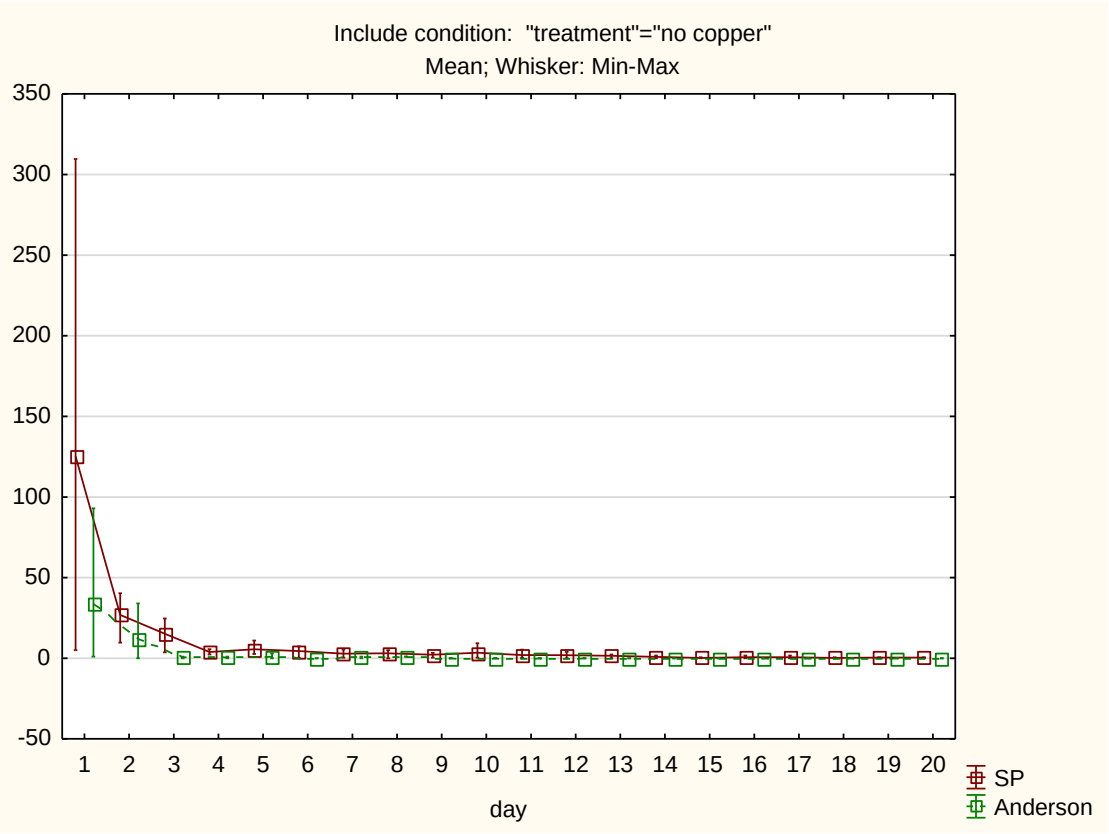


Figure 4.15: Comparison between active sampling and passive sampling in presence of Plexiglas®

Before Day 4, active sampling showed lower number of microbes captured comparatively to passive sampling in presence of Plexiglas®.

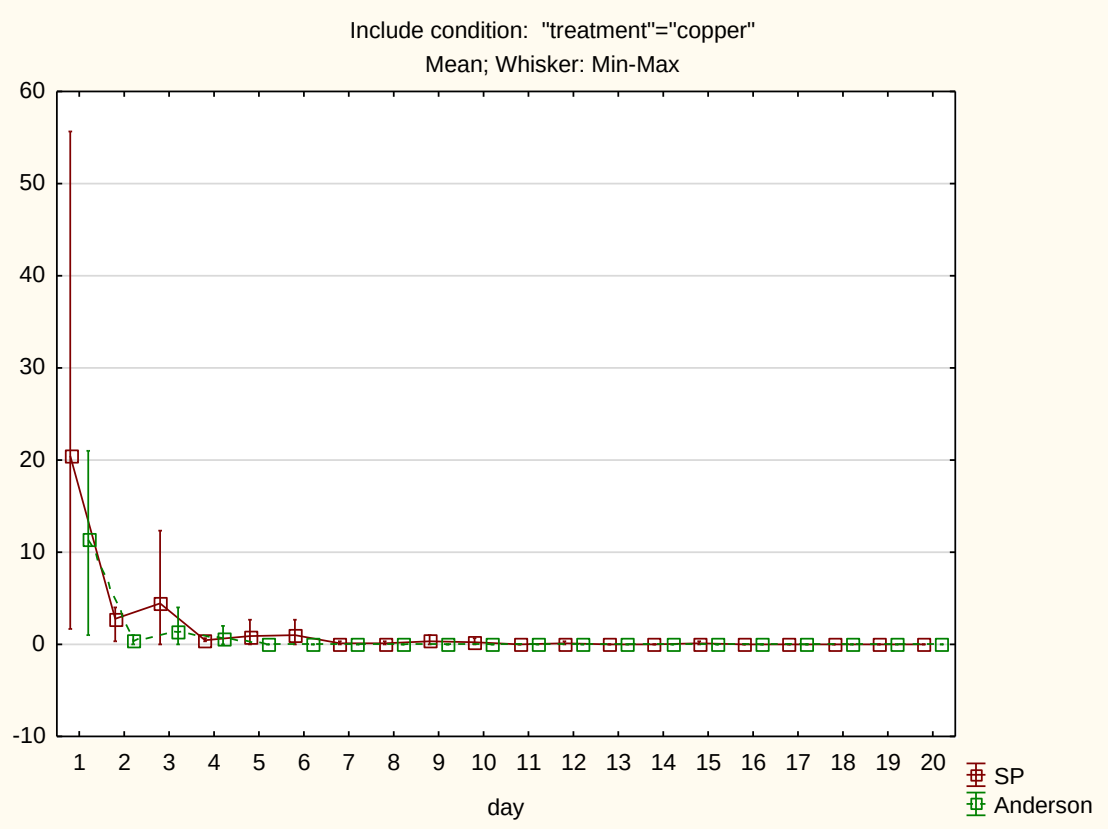


Figure 4.16: Comparison between active sampling and passive sampling in presence of copper

Before Day 4, active sampling showed lower number of microbes captured comparatively to passive sampling in copper presence.

Table 4.5: Table representing number of CFU captured on ACI plates during 2 minutes sampling from Day 1 to 20 of lifting the dust.

	CFU captured on plates during 2 min of ACI sampling or from 56.6 litres of air			
Day	Plexiglas A1-6	Copper A1-6	Plexiglass A5-6	Copper A5-6
1	25.25	8.5	22.75	2.5
2	9	0.25	8	0
3	0.25	1	0	0
4	0.25	0.5	0	0
5	0.75	0	0	0
6	0	0	0	0
7	0.25	0	0	0
8	0.75	0	0.25	0
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0
12	0	0	0	0
13	0	0	0	0
14	0	0	0	0
15	0	0	0	0
16	0	0	0	0
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0
21	0	0	0	0

This table shows that the number of CFU captured on ACI plates during the 2 minutes sampling or from 56600 ml is very few. Number of CFU recovered in copper environment is fewer than in the Plexiglas® one.

Chapter 5: Discussion, Conclusion and Recommendations

5.1 Discussion

The discussion of some of the results from the pilot study and the two phases of the current study attempted to answer the following questions:

- What is the survival time of *M. smegmatis* in a dusty environment?
- Can *M. smegmatis* be re-aerosolized from the dust?
- Is it viable when it is re-aerosolized?
- What is the effect of copper on the survival of *M. smegmatis* in a dusty environment?

The results from the experiments with *M. smegmatis*, a surrogate for *M. tuberculosis* aimed is to indirectly answer several questions relating to *M. tuberculosis* and the possibility of it being able to cause disease after re-aerosolization: Can *M. tuberculosis* be re-aerosolized? If it can, does *M. tuberculosis* remain viable with the potential to cause infection? If re-aerosolized *M. tuberculosis* is infectious, can we use metals such as copper surfaces to reduce the environmental viability of *M. tuberculosis* and its bio-burden?

The physical conditions of the environment in which the study was conducted were similar for the two sets of experiments. The difference between them was only the presence or not of the copper touch surface. Two episodes of sampling were done and were separated by the re-aerosolization process as illustrated in Figure 4.2 The mineral composition did not have any direct bactericidal effect on *M. smegmatis*, and therefore was a suitable vehicle for these studies. Dust was used to simulate the natural effect of sweeping and other aspects for agitating microbe-laden dust.

The results from incubation of plates after the sampling of dust prior to each nebulization of *M. smegmatis* into the experimental chamber revealed no growth and therefore confirmed no contamination of the dust used in our experiments. Also results from the sampling before re-aerosolization were null, this means that there was no growth on the plates (ACI plates and settle plates) and therefore no graph was done. However, during experiments, it was noted that the number of microbes recovered by active or passive sampling diminishes over time. This reduction in the number of re-aerosolized microbes can be explained by the reduction of the concentration of microbes in the dust due to the removal of some microorganisms from the box (via the ACI, or contact with settle plates) and due to natural death. This is in

accordance with Hinds findings (Hinds, 1999) that the re-aerosolized particles number depends on the concentration of those particles in the environment where re-suspension occurs. The growth on agar of the re-aerosolized *M. smegmatis* in both presence of Plexiglas® and copper treatment established that re-aerosolized mycobacteria can be viable. In our study this was possible in the presence of Plexiglas® for up to 20 and 8 days on settle plates and ACI plates respectively; and for a shorter period of time in the copper treatment (15 days for settle plates and 2 days on ACI plates).

As settle plates were situated 6.5 cm above the box floor from where dust was lifted by generated turbulence, the fans were placed in such a way that the airflow of one was vertically upwards and for the other the airflow was oriented downwards in the chamber. Some of the dust that could not be sucked by the ACI fell on the settle plates or on the aerosol chamber floor or walls. This scenario represents any elevated level where microbes could have landed and been driven by any force, then re-suspended in the air and could be inhaled in such an environment if the viable particles are airborne. Our focus was mainly on particles of 0.6 to 2.1 µm equated to levels A5 and A6 of the ACI corresponding to the alveoli and terminal bronchioles in the human respiratory tree noted in both Plexiglas® presence (Figure 4.3) and copper treatment (Figure 4.7). This showed that the number of captured microbes on ACI level 5 (0.6 to 1.1 µm) and 6 (1.1 to 2.1 µm) is less than the one collected from settle plates (Figures 4.6 and 4.10). This discrepancy can be explained by several factors: the number of microbes captured at levels 5 and 6 is only a fraction of the overall number of microbes captured by the ACI at all six levels combined (Figures 4.5 and 4.9). This overall number from the ACI is less than the one collected by settle plates as the latter could collect aerosols of any size that could settle on its agar **(**Figures 4.15 & 4.16). Moreover, the ACI outlet is situated in a corner of the ceiling of the aerosol chamber at a distance of 90 cm from the floor of the chamber and 83.5 cm higher than settle plates placed on small boxes of 6.5 cm high. The position of settle plates and that of the outlet to the ACI provide the opportunity for the slightest particles of dust to reach the exit to the ACI when both heavier and slightest particles can land on settle plates or fallout. Over time or days, the number of slightest particles decreased and the chance to have those carrying viable particles diminished as microbes died over days in the aerosol chamber. This could explain why viable particles could be noticed on settle plates for longer (20 days) than on ACI plates (8 days) in the presence of Plexiglas®. The air current is upwards in active sampling, while it is downwards in passive sampling. The passive sampling started before the active sampling and lasted longer when the active sampling occurred in the middle of it. The changing air current had an impact in somehow reducing the falling of particles on settle plates when it occurred, but this is of no major importance as far

as the settle plates still displayed higher concentrations of bacilli than the ACI at any level or at the overall combined levels of the ACI.

The distance between settle plates and the outlet to the ACI (83.5 cm) or the distance separating the floor where dust is from the same outlet to the ACI (90 cm) is sufficiently high to correspond to the position of the head or the nose of all seated patients and health professionals in health facilities so that they can inhale re-aerosolized dust. In the real situation, when we sweep, the dust settles not only on tables but also on cupboards. The dust collected from the ceiling for this study is a result of everyday deposition in the ceiling and even deposition of dust that travelled from far away to the ceiling. Humans, in the vicinity of sweeping are susceptible to inhaling dust re-aerosolized from the cupboard, the floor or the wall or directly when we sweep the area. If the outlet to the ACI could be moved down or extended inside the box to its middle, or towards the settle plates, more particles and more microbes could have been captured.

When nebulized in the aerosol chamber, the starting inoculum is dispersed in the chamber where some microbes settled mainly in the dust, others on the inner surface of ceiling and on the inner surfaces of the chamber. On Day one, the only re-aerosolized mycobacteria that could be captured via the ACI and settle plates are displayed on these graphs. The captured microbes are only fractions from the re-aerosolized number of microbes to an extend that the number of microbes captured during a two-minute run of the ACI is coming from two fold 28.3 litres (56.6 litres) withdrawn from 693.07 litres representing the total volume of the aerosol chamber. The volume of air that is taken for that two-minute run represents 8.17% of the chamber volume. Assuming that re-aerosolized microbes were equally and normally distributed in the air and correlating this percentage to the number of microbes re-aerosolized in the box, only up to 8.17% of the air microbes' burden could be driven into the ACI. From this amount, only a certain fraction captured at levels 5 and 6 has been reported on figures representing levels 5 and 6 of the ACI. Sucking a volume of 693.07 litres via the ACI would have taken 24.49 minutes in a continuous run. As the full amount of dust cannot be removed in one sampling operation or in a continuous run of the ACI to avoid overloading of agar plates, only a 2-minute run of the ACI could be operated as repetitively as possible to remove a maximum number of particles that could be re-suspended. In Table 4.5, the results are not presented as $\log_{10}$ of the number of CFUs but as true numbers of the CFUs recorded on the plates after incubation. The ACI plates allowed correlating between the volume of air and the number of colonies found on the plates. We added up the number of CFUs found on the plates for the duration of sampling (28.3 l/min for 2 minutes); it should be noted, however, that a certain number of mycobacteria escaped through the ACI exhaust without impaction on agar plates. Days to be considered include not only the first two days following aerosolization, but

also the rest of the days during both treatments, summing up the number of bacteria of all the six levels of the ACI.

When discussing pulmonary ventilation, it is important to define a number of pulmonary volumes and capacities. We focused mainly on inspiratory volumes rather than expiratory volumes. The volume of air inspired or expired with each normal breath amounts to 500 ml (the tidal volume). The inspiratory reserve volume is the extra volume of air that can be inspired beyond and over the normal tidal volume, and it is nearly 3000 ml. The minute respiratory volume is defined as the total amount of new air moved into the respiratory passages each minute. This equates the tidal volume times the respiratory rate, the latter is approximately 12 breaths per minute. The minute respiratory volume is approximately six litres per minute and can be increased under several circumstances, the respiratory rate can augment up to 30 or 50 per minute thus the tidal volume can reach 6000 ml. Table 4.5 shows that the number of CFU captured on ACI plates during the two minutes sampling or from 56600 ml is very few. This volume equates to nearly 113.2 times the normal tidal volume meaning that the volume sampled by the ACI can be inspired by a normal adult in nearly 114 minutes or two hours when sitting in such a dusty environment. In this case the person is at risk of inhaling mycobacteria mostly during the first two days after starting lifting the dust; the following days there is lower risk in presence of copper compared to Plexiglas®.

Three 9 cm × 1.5 cm petri dishes with agar received settling microbes on a surface area size of 58 cm^2 for each plate making a total surface of 174 cm^2 for the three settle plates. This total plate surface (3 petri dishes) represented only 2.76 % of the entire surface of the experimental chamber surface (90 x70 cm^2 =6300 cm^2). Assuming that the distribution of microbes was equal and normal in the aerosol chamber, the three settle plates could pick up only up to 2.76 % of total number of microbes that settle during the five minutes of collection of samples using settle plates.

In reality, the distribution of particles is not normal or equal, their falling speed and the impacting force would never be equally or normally distributed at all points in this chamber due to the difference in the distribution of driving forces lifting particles and to the variety of the size and the density of particles as described in the gravimetric analysis of the dust. This is also true for particles in the ACI within one sampling operation and over days' samplings. This would explain why the re-aerosolized concentration is far away from the initial inoculum, independent of natural death of mycobacteria. Finally, Figures 4.6 showed that *M. smegmatis* can survive up to 20 days after its nebulization in dust in presence of Plexiglas® and when re-aerosolized the organisms remain viable over that period of time. These results on the survival of mycobacteria in the environment should be seen under the light of some uncertainties

arisen by Bruno and his colleagues on pathogen survival in the external environment and the evolution of virulence. In fact, the importance of dust-borne infections could be underestimated if the pathogen can survive on dust far longer than the duration of the study, if the study was conducted under relatively dust-free conditions, or if the smallest dust particles were not represented in the study. Moreover, we noticed that some yeast growth occurred beyond two weeks of incubation of plates and on stored plates after 30 days in a cold room after our experiments, this contamination of plates by other microbes could be also a limitation factor to the survival of mycobacteria not only in vitro but also in vivo if they have to compete for the same nutrients or one is aggressed by chemical secreted by the other.

Graphs from Figures 4.3 to 4.16 revealed that *M. smegmatis* can be re-aerosolized in dust and can remain viable in this environment after aerosolization and re-aerosolization as demonstrated by the two air sampling methods for viable organisms, active sampling (ACI) as well as passive sampling (settle plates) in both Plexiglas® presence and copper treatment. These findings are in line with those of Lauten and his collaborators who demonstrated in a new approach to the formulation of live whole-cell vaccines that repeated spray drying and selective pressures in dry powders may enrich capacities for strains which can persist in harsh conditions. In this procedure, they used a Buchi B-290 mini spray dryer. The latter is based on the principle of spray drying involving the evaporation of moisture from an atomised feed by mixing the spray and the drying medium where the drying medium is typically air. The solution was spray dried at a feed rate of 7 ml/min with a drying air flow rate of 35 litres per hour and the outlet temperature between 42°C and 45°C and the inlet temperature varying from 115°C to 125°C. Wong and his collaborators previously used the same tools for *M. smegmatis* or for BCG, a member of *M. tuberculosis* complex, closer to *M. tuberculosis* species than *M. smegmatis*. The generated aerosol underwent the drying process and the drying proceeded until the desired moisture content was reached in the sprayed particles and the product was then separated from the air. In other words, the powder settled (meaning that the suspended particles were removed from the generated aerosol). The size of the particles generated by the Buchi B-290 mini spray dryer varies from less than 0.7µm (standard) to 200µm according to the type of the spray dryer (Buchi/Switzerland, 1997). The idea of mycobacteria surviving re-aerosolization is not new. It was already in the procedure used by the Lauten team. “Cycling” meant repetition of the following steps: culturing bacteria to stationary phase; then spray drying (aerosolization of mycobacteria centrifuged and re-suspended in low osmolyte excipient solution and drying of the initially suspended particles in the air); then collecting and vial filling of the dry powder followed by incubation of the vial at 40ºC in stability chambers until viable bacteria were mostly eliminated. The surviving bacteria were then cultured from the dry powder to the stationary phase. This cycle was repeated four

times, meaning that the powder of mycobacteria (*M. smegmatis* or BCG) was re-suspended and re-aerosolized three times (multiply cycled). This procedure enhanced the viability and survivability of the processed mycobacteria (Lauten, *et al*., 2010).

The Lauten and Wong teams demonstrated that both *M. smegmatis* and BCG can survive spray and dry process at higher pressure and temperature than that encountered in our study and still be culturable. Lauten's team demonstrated that *M. smegmatis* can survive multiple spray dryings at higher temperatures (42°C to 45°C), pressures (5.10^5-8.10^5 Pa), velocities (7liters/min) than those of our experiments (15.9-25.7°C, 5-8 Pa and 5 litres/min). In their conditions the experimental mycobacteria could exhibit some phenotypical and gene expression differences denoting their adaptability to counter environmental challenges. These significant differences were noted in the genetic expression of *M. smegmatis* between log phase and stationary phase conditions, between naïve (non-spray dried) and multiply cycled dried *M. smegmatis* in log and stationary phase), and between *M. smegmatis* in the dry powder following a single spray drying operation and after four consecutive spray drying operations. Lauten and his collaborators noticed that the viability over time in desiccated state continued to increase as formulations were cycled through drying and heat-exposure processes and finally the mycobacteria showed a nearly ten-fold increase in stability with the ability to form colonies until 105 days when multiply cycled.

Our findings met at a certain point those in term of survivability of mycobacteria after re-aerosolization. Taking into account similarities between *M. smegmatis and M. tuberculosis,* in various extends including the stress response (among which the presence of the sigE protein); we can cautiously associate all these findings considering the fact that *M. tuberculosis* can replace *M. smegmatis,* although Tobias and his collaborators (Tobias, *et al*., 2005) found a consistent discrepancy between the *M. tuberculosis* H37Ra and *M. smegmatis* in number of cells measured using Bioaerosol Mass Spectrometry. This difference in number in favour of *M. tuberculosis* H37Ra over *M. smegmatis* for reasons not clearly established showed that there is more captured aerosolised pathogenic mycobacteria H37Ra than the non-pathogenic one *(M. smegmatis)*. When using the Andersen Cascade Impactor, the difference between the two mycobacteria is in favour of *M. smegmatis*, the two methods substantially deferring one from another at the fact that the ACI measured viable particles meanwhile the BAM measures both culturable and non-culturable cells. Thus, we can understand the additional role played by dust in TB transmission and the potential risk the lies in re-aerosolizing this pathogenic bacterium especially when it has acquired resistance to harsh conditions through various mechanisms.

In a comparison of copper treatment to the presence of Plexiglas®, Figures 4.11, 4.12, 4.13 and 4.14 display the significant decrease in surviving microbes in both copper and the presence of Plexiglas® and on both settle plates and ACI plates, but copper treatment still shows more significant lower levels of surviving *M. smegmatis* in the dusty environment. This suggests that copper is effective in its anti-microbial property on *M. smegmatis* in dust. This significant difference is already seen on Day 1 after nebulization in Figures 4.11 and 4.14 compared to Figures 4.7 and 4.8 on both settle plates and on levels 5 and 6 of the ACI, and globally on Figures 4.13 and 4.16. One could expect no difference on the first day after nebulization between the two phases as nebulized microbes could land on both the copper sheet and in the dust. But during this study dust spread in the chamber covered the entire copper surface (Appendix 1). As the solution had a certain humidity, droplets can settle on the dust and bring particles in contact with the copper plate or not, depending on the thickness of dust which might play the role of shield between microbes and the copper plate. If most of the aerosolized microbes fall on a thicker layer of dust, the pattern of re-aerosolized microbes captured by the ACI could be similar both in copper treatment and in presence of Plexiglas® (the thicker the dust layer, the less would be the contact and the longer the survival of bacteria in such an environment). The small number of repeats could have played a role in the non-difference or the difference noted on Day 1 after nebulization. Even though, as far as there is a significant difference between the two phases for ACI, the effectivity of anti-microbial properties of copper might have played a role in this difference. At levels of breathable particles plates and settle plates, this difference on Day 1 after aerosolization (nebulization) can be considered in favour of the anti-microbial property of copper. This difference between the two phases can be noticed easily on settle plates up to Day 8 after nebulization and over re-aerosolization when this significant difference does not last more than two days of re-aerosolization for levels 5 and 6 of the ACI. This non-significant statistical difference on respirable particles (levels 5 and 6) after the first 48 hours of re-aerosolization in the survival of *M. smegmatis* might be due to the small number of microbes captured by the ACI comparative to settle plates. The overall p value > 0.05 noted in the comparison of the Plexiglas® and the copper treatment and would lead one to conclude that there is no difference between the two phases; the end result in the survival of *M. smegmatis* being microbes death due to the natural cause and/or to the presence of copper, the difference between the two phases being only in their early days, afterwards there is no difference between the death due to the presence of copper and the natural death of *M. smegmatis* (Figures 4.11 and 4.14).

Tables 4.3 and 4.4 display the percentage of reduction in the mean number of captured microbes from each day where there is a significant difference between the two phases. The

percentage of reduction from Plexiglas® to copper is generally much higher on the following day comparative to the previous one in the ACI samples, whereas the same trend is observed in settle plates with an exception from Day 5 to Day 8. This difference may be due to the difference in the redistribution of the dust when settling down after its re-aerosolization; the quantity of dust lifted up the next day might have been less than the previous day, therefore providing fewer particles with viable microbes. The redistribution of dust in the chamber might be a factor to be taken into account in differences in the sampling process and between days. Would this variability in size, quantity and distribution of dust particles in the chamber day after day be statistically different between the two phases and between days within each phase? As days go by there was less and less dust in the chamber and more areas of direct touch between copper and microbes so that one could expect that more microbes would be killed. Even though there is a certain percentage of reduction from Plexiglas® to copper phase even if it is 10.36% on Day 8, the difference between the two phases is still statistically significant according to the corresponding graph in Figure 4.14. The starting percentage of reduction from the Plexiglas® presence to copper of surviving microbes on the ACI levels 5 and 6, in other words in respirable particles, is more than 40% on Day 1 and it quickly reaches 100% on Day 2. This noticeable reduction on Day 1 and the impressive reduction on Day 2 might be mainly due to the presence of copper touch surface.

No bacteria were recovered from dust removed for 10 days from copper treatment, even after extended incubation of plates, suggesting that the anti-microbial property of copper was bactericidal and not bacteriostatic in this case. This is in line with and suggested an alternative solution in combining the use of other means with copper touch surface in the management of drug and multi-drug resistant microbes. There was no viable microbe detected on settle plates or by the ACI 24 hours after aerosolization or re-aerosolization. This strongly suggested that in a tranquil environment the settling process happened to such an extent that there were no viable microbes suspended in the air (results not presented in any table as figures are only zeros).

Expelled droplet nuclei can directly, or after having travelled, be deposited on surfaces. It is believed, therefore, that droplet nuclei cannot be re-aerosolized or *M. tuberculosis* be re-aerosolized to reach alveoli after deposition and dryness because its generating process which is either through cough, sneezing or talk, cannot be reproduced out of the body and droplet nuclei size cannot be reproduced. As droplet nuclei carrying TB bacteria cannot be re-aerosolized once settled down, consequently it is believed that *M. tuberculosis* cannot be re-aerosolized in droplet nuclei (The Merk Manual, Professional Edition, 2014). One can understand that once settled droplet nuclei can be mixed with dust and dry. Any air movement with enough force or velocity to lift up the dust can bring back microbes in the air especially

for canopy particles (Figure 2.1), as it occurs with some allergens. Therefore, microbes can be transported closer or further away according to the density of their supportive particles. These findings contradicted the position of The Merck Manual on the re-aerosolization of mycobacteria, specifically of *M. tuberculosis*. We noticed a growth of mycobacteria from particles with certain variability in size including particles < 5 µm and specifically particles can be inhaled and if they are charged with viable particles they can reach alveoli and terminal bronchioles (levels 5 and 6 of the ACI).

The anatomy and functions of the airways may allow the separation of the mycobacteria from the dust particles which act as a vehicle in transporting the microbes into the airways. The increase in humidity (more water concentration) can ease the sliding of the microbe on the surface of the dust particles. The impaction of the dust particles may generate sufficient forces to separate the mycobacteria from the dust particles especially in the upper respiratory ways (Figures 4.4 and 4.8). It should also be borne in mind that the nasal turbulence mechanism is so effective that virtually no particles larger than 4 µm to 6 µm in diameter can penetrate the lungs through the nose (Hall, 2016).

The separation between the mycobacteria (size < 5µm) and dust particles (size > 5µm) deposited in the upper respiratory tracts can occur as it can happen or not when the dust particle carrying the mycobacteria impacts the agar surface on different levels of the ACI. Therefore, it should be taken into account not only levels 5 and 6 of the ACI corresponding to particles of respirable range but also the other levels of the ACI corresponding to the upper airways where mycobacteria can be transported by dust particles of > 5µm in size with a certain risk of separation between dust and microbes.

The driving force and speed of the wind, shaking bed sheets, walking and sweeping (TB online) can re-aerosolize dust and consequently TB bacilli. In the current study the driving force of the dust for one small fan was at 2 Kpa and the velocity of the air flow was at average 2.035 m/s with a maximum of 2.47 m/s for the upwards airflow fan and a minimum of 1.60 m/s for the downwards airflow fan. This minimum velocity is due to the rebounding effect of the downwards airflow against the aerosol chamber floor. This is far less than figures of wind speed in Cape Town. This wind speed varied from 15 Km/h to 28 Km/h from November 2000 to February 2015 according to Windfinder (Appendix 11). This range of wind speed includes the slot of 20-28 Km/h where the effect on the land is lifting of dust and loose papers while small branches begin to move (Windfinder, 2015). In low income and limited resources countries the means used to clean the floor in many areas, including hospital environments, may lift settle dust and be an opportunity to re-aerosolize some airborne pathogens. Since re-aerosolized microbes can be viable, the risk of transmission of diseases is still present if the

infectious doses are reached even after several expositions, there is a major risk for immune-compromised patients.

Ghodbane *et al.* found that *M. tuberculosis* remains viable while in the soil, outside its hosts for extended periods of time. It can still multiply once it encounters ideal conditions for growth after a long-term survival and still be virulent when introduced in its hosts (Ghodbane *et al.*, March 2014). Some studies correlated high survival with high virulence (Walther & Ewald, 2004) when others correlated high travelling distance with high virulence (Boots & Sasaki, 1999). This suggested that in the first case the longer a pathogen survives the more it becomes virulent and in the second case it implies that the higher virulence evolves in pathogens that are transmitted over long distance. As the high durability in the external environment reduces the dependence of the transmission on host mobility, the sit-and wait- hypothesis (where the pathogen does not travel but the host moves to it) predicts that virulence should be positively correlated with durability in the external environment. In their study, Walther and Ewald placed *M. tuberculosis* in high virulence high survival group.

Alex Müller in “How TB is spread” reported that besides the direct transmission from an infected person to an uninfected person, the TB bacilli can be transmitted by dust. The bacilli settle in dusty and dark areas; when the dust gets swirled up (by sweeping, wind, or when children play on the ground), TB bacilli can then fly and may be inhaled (Müller, 2011). We demonstrated that *M. smegmatis*, a surrogate for *M. tuberculosis*, can survive in dust, be re-aerosolized and still be viable.

M. smegmatis and *M. tuberculosis* can survive in dust and both can be re-aerosolized using dust particles as vehicles to reach animal or human airways.

In the results of our study copper showed the effectiveness of its antimicrobial properties in dust. In other words, recovered by a layer of dust of a certain thickness the copper surface was still bactericidal. In other studies, embedded copper in the new vermiculite-copper hybrid material showed strong antibacterial activity against *Staphylococcus aureus* (Drelich, et al., 2011). As the stealing of copper or copper alloys limited the wide use of copper on large surfaces in hospital design, we can figure that the use of paints with high concentrations of copper nanoparticles to cover surfaces in some specific areas of heath care facilities to broaden and revalue the applications the use of embedded copper where antimicrobial properties of copper are desirable.

The fact that copper has been bactericidal suggested the combination of copper touch surface and other preventive means for the prevention and control of TB.

The following weaknesses were encountered during the study:

- Failure to measure the number of microbes lost through the exhaust of the ACI after the canister containing plates, as well as the ones left in the tube through which the air was sucked from the aerosol chamber or on the walls of this experimental chamber and the one attached on the external surfaces of the plates. However, the experiments were conducted in triplicate and an average was used for the results.
- The number of repeat experiments for each copper treatment or Plexiglas® phase was restricted to three due to time constraints.
- Due to the clumping effect of mycobacteria, it was difficult to get individual colony counts from the agar plates.

5.2 Summary of Findings and Conclusion

Findings in the current study can be summarised as follows:

The dust used was composed of particles in conformity with the size of particles ranged in the category of dust by IUPAC (1-100 µm). The mineral composition of the dust was not aggressive and was friendly to the survival of *M. smegmatis* and strikingly similar in both Plexiglas® and copper. There was no significant statistical difference in physical conditions (humidity, pressure, temperature) between days of the study. Some of 10^6 nebulized *M. smegmatis* could be re-aerosolized over days with a decreasing trend in the number of mycobacteria landed on settle plates as well as those captured by the Andersen especially on respirable particles that can reach terminal bronchioles and alveoli (ACI stage 5 and 6). Copper showed the effectiveness of its anti-microbial properties in reducing the survival of mycobacteria, especially of *M. smegmatis.*

This study allowed us to establish that *M. smegmatis* can survive in a dusty environment more than 19 days and stay viable for days after re-aerosolization.

The findings of this study suggested the use of wet material to wipe surfaces, to wet the surface before sweeping, to use disinfectant sometimes, and the use of copper touch surface especially in surroundings where drug and multi-drug resistant microbes are encountered. Sweeping or the removal of bed sheets in patient wards should take into account the risk of re-aerosolizing airborne pathogens. This study elucidated the mechanism of transmission of TB via contaminated dust.

In addition to other preventive means of TB transmission, copper touch surface can be used to reduce the bio-burden of mycobacteria, specifically *M. tuberculosis* with at least a daily cleaning (or more) of copper surface depending on the surface usage. As the effectiveness of antimicrobial properties of copper has been shown in dust, and taking into account the stealing of copper where it is visible, a study on the effectiveness of copper nanoparticles in paints for large surface should be explored using BCG as experimental bio-aerosol.

The wearing of personal protective equipment is still to be recommended when cleaning dusty areas.

This study opened a way to the use re-aerosolized the BCG type, an attenuated strain of *M. bovis*, a member of *M. tuberculosis* complex (one of the causal agents of tuberculosis) in animal experimental studies or the use of *M. tuberculosis* in BL3 laboratories to assess its infectiousness after re-aerosolization.

Stellenbosch University has a Respiratory Research Unit. The unit, however, does not work on respiratory infectious diseases. It would be very beneficial to create a subunit that would provide for multidisciplinary collaboration between the Tuberculosis Desmond Tutu Institute, the Academic Unit for Infection Prevention and Control, the Department of Infectious Diseases and the Respiratory Research Unit. This would create an environment, forum and important opportunity for studies dealing with infectious aerosols.

List of References and Bibliography

Airey, P. & Verran, J., 2007. Potential use of Copper as a Hygienic Surface; Problems Associated with Cumulative Soiling and Cleaning. *Journal of Hospital Infection (2007) 67, 271e277,* Volume 67, pp. 271-277.

Akinola, R. O., Mazandu, G. K. & M, N. J., 2013. A Systems Level Comparison of Mycobacterium tuberculosis, Mycobacterium leprae and Mycobacterium smegmatis Based on Functional Interaction Network Analysis. *Journal of Bacteriology and Parasitology,* 4(4).

Andon, B. M., 2006. Active air vs. Passive air (settle plate) Monitoring in Routine Environmental Monitoring Programs.. *PDA Journal of Pharmaceutical Science and Technology,* 60(6), pp. 350-355.

Bailey, W. C., 2011. Biofilms: Formation, Development and Properties. s.l.:Nova Science Publishers.

Bamford, C. M. & Taljaard, J. J., 2010. Potential for Nosocomial Transmission of Multidrug-resistant(MDR) Tuberculosis in a South African Tertiary Hospital. *South African Medical Journal,* 100(100), pp. 438-441.

Baussano, I. *et al*., 2011. Emerging Infectious Diseases. *Emerging Infectious Diseases,* 17(3), pp. 488–494.

Boor, E. B., Siegel, A. J. & Novoselac, A., 2011. Development of an Experimental Methodology to Determine Monolayer and Multilayer Particle resuspension from Indoor Surfaces. *American Society of Heating, Refrigeratingand Air-Conditioning Engeneers (ASHRAE) Transactions,* 117(LV-11-C053).

Boots, M. & Sasaki, A., 1999. "Small worlds" and the Evolution of Virulence: Infection Occurs Locally and at a Distance.. *Proceedings of the Royal Society of London,* Volume 266, pp. 1933-1938.

Brites, D. & Gagneux, S., 2016. Co-evolution of Mycobacterium tuberculosis and Homo sapiens. *Immunological Reviews,* Volume 264, pp. 6–24.

Brosch, R. *et al*., 2002. A New Evolutionary Scenario for the Mycobacterium tuberculosis Complex. *PNAS,* 99(6), pp. 3684-3689.

Buchi/Switzerland, 1997. *B-290_Data_sheet_en_D.pdf.* [Online]
Available at: www.buchi.com/spray-drying [Accessed 1 August 2016].

Byers, R. J. *et al*., 2013. Transfer and Reaerosolization of Biological Contaminant following Field Technician Servicing of an Aerosol Sampler. *Journal of Bioterrorism and Biodefense.*

Carilloa, M. *et al*., 2010. In vitro Actinomyces israelii Biofilm Development on IUD Copper Surfaces. *Contraception,* Volume 81(3), pp. 261–264.

Carroll, P. *et al*., 2010. Sensitive Detection of Gene Expression in Mycobacteria under Replicating and Non-Replicating Conditions Using Optimized Far-Red Reporters. *PLOS One,* 5(3), pp. 1-17.

Casey, H. *et al*., 2015. Pulmonary Tuberculosis in a South African Regional Emergency Centre: Can Infection Control be Improved to Lower the Risk of Nosocomial Transmission?. *South African Medical Journal,* 105(10), pp. 862-865..

CDA, 2009. Reducing the Risk of Healthcare-Acquired Infections-Antimicrobial Copper-*the Scientific Evidence.* [Online] Available at: www.copperinfo.co.uk/
[Accessed 10 April 2013].

Centers for Disease Control and Prevention, 2005. Guidelines for Preventing the Transmission of Mycobacterium Tuberculosis in Health-Care Settings*, 2005.MMWR,* Atlanta: Coordinating Center of Health Information and Service,Centers for Disease Control and Prevention, US Department of Health and Human Services.

Chigbu, L. N. & Iroegbu, C. U., 2010. Incidence and Spread of Mycobacterium tuberculosis-Associated Among Aba Federal Prison Inmates in Nigeria. *Journal of Health, Population and Nutrition,* Issue ISSN1606-0997, pp. 327-332.

Claassens, M. M. *et al*., 2013. Tuberculosis in Healthcare Workers and Infection Control Measures at Primary Healthcare Facilities in South Africa. *PLOS,* 8(10).

Cook, G. M. *et al*., 2009. Physiology of Mycobacteria. *Advances in Microbiology,* 55(978-0-12-374790-7).

Copper Development Association, 2014. Reducing the Risk of Healthcare Associated Infections: The Role of Antimicrobial Copper Touch Surfaces. [Online]
Available at: http://www.antimicrobialcopper.com
[Accessed 16 March 2015].

Cordone, A. *et al*., 2011. Characterization of a Mycobacterium smegmatis uvrA Mutant Impaired in Dormancy Induced by Hypoxia and Low Carbon Concentration. *Biomed Central Microbilogy,* Volume 11, p. 231.

Creamer Media (Pty) Ltd, 2015. *http://www.engeneeringnews.co.za.* [Online]
Available at: http://www.engeneeringnews.co.za/article/copper-used-in-hvacsystems-to reduce-air-contamination-2015-02-06
[Accessed 24 Feb Feb 2015].

Delft, A. V. *et al*., 2015. Why Health Care Workers are Sick of TB. *International Journal of Infectious Diseases,* Volume 32, pp. 147–151.

Dharmadhikari, A. S. *et al*., 2011. Natural Infection of Guinea Pigs Exposed to Patients with Highly Drug-Resistant Tuberculosis. *National Institutes of Public Health,* 91(4), pp. 329-338.

Dharmadhikari, A. S. *et al*., 2012. Surgical Face Masks Worn by Patients with Multidrug-Resistant Tuberculosis, Impact on Infectivity of Air on a Hospital Ward. *American Journal of Respiratory Critical Care Medicine ,* 185(10), pp. 1104-1109.

Diel, R. *et al*., 2013. Costs of Tuberculosis Disease in the EU – a Systematic Analysis and Cost Calculation. *ERJ Express.*

DOH/SA, 2012. *www.doh.gov.za/docs/stratdocs/2012/app2012.pdf.* [Online]
Available at: Annual Performance Plan 2012/13-2014/15
[Accessed 22 June 2013].

DOH, 2007. The Draft National Infection Prevention and Control Policy for TB, MDRTB AND XDRTB. Pretoria: s.n.

Drelich, J. *et al*., 2011. Vermiculite Decorated with Copper Nanoparticles: Novel Antibacterial Hybrid Material. *Applied Surface Science,* Volume 257, pp. 9435-9443.

Elguindi, J., Moffitt, S. & Hasman, H., 2011. Metallic Copper Corrosion Rates, Moisture Content, and Growth Medium Influence Survival of Copper Ion-resistant Bacteria. *Applied Microbiology And Biotechnology,* Volume 89, pp. 1963–1970.

Escombe, A. R. *et al*., 2008. The Infectiousness of Tuberculosis Patients Coinfected with HIV. *PLOS/Medicine,* 5(9), pp. 1387-1397.

Fisher, E. M. *et al*., 2012. Reaerosolization of MS2 Bacteriophage from an N95 Filtering Facepiece Respirator by Simulated Coughing. *Annals of Occupational Hygiene,* 56(N 3), pp. 315–325.

Flint, J. L., Kowalski, J. C., Karnati, P. K. & Derbyshire, K. M., 2004. The RD1 virulence locus of Mycobacterium tuberculosis Regulates DNA Transfer in Mycobacterium smegmatis. *Proceedings of the National Academy of Sciences of USA,* 101(34), pp. 12598–12603.

Forrellad, M. A. *et al*., 2013. Virulence Factors of the Mycobacterium tuberculosis Complex. *Virulence ,* 4(1), pp. 3-66.

Fox, P. & Abbaszadegan, M., 2013. *Impact of Scale Formation on Biofilm Growth in Premise Plumbing,* Arizona: Arizona Sate University.

Frampton, R. *et al*., 2011. Toxin-antitoxin Systems of Mycobacterium smegmatis are Essential for Cell Survival. *Journal of Biological Chemistry,* 287(8), pp. 5340-5356.

Galagan, J. E., 2014. Genomic Insights into Tuberculosis. *Reviews,* Volume 15, pp. 307-320.

Gandhi, N. R. *et al*., 2013. Nosocomial Transmission of Extensively Drug-Resistant Tuberculosis in a Rural Hospital. *The Jounal of Infectious Diseases,* Volume 207, pp. 9-17.

Ghodbane, R. *et al*., March 2014. Long-term Survival of Tuberculosis Complex Mycobacteria. *Microbiology,* Volume 160, pp. 496-501.

Gould, W.S.J. & Fielder M. D., 2009. The antimicrobial Properties of Copper Surfaces Against a Range of Important Nosocomial Pathogens. *Annals of Microbiology,* Volume 59, pp. 151-156.

Grass, G., Rensing, C. & Solioz, M., 2011. Minereviews Metallic Copper as an Antimicrobial Surface. *Apllied and Environmental Microbiology,* 77(5), pp. 1541-1547.

Griffin, D. W. *et al*., 2010. Observations on the use of Membrane Filtration and Liquid Impingement to Collect Airbone Microorganisms in Various Atmospheric Environments. *Aerobiology,* 27(2011), pp. 25-35.

Gupta, S. & Chatterji, D., 2005. Stress Responses in Mycobacteria. *Critical Review,* 57(3), pp. 149 - 159.

Hall, J. E., 2016. Pulmonary ventilation. In: *Textbook of Medical Physiology.* Philadelphia: Elsevier, pp. 497-507.

Herman, P. *et al*., 2006. *Biosafety Recommendations for the Contained Use of Mycobacterium tuberculosis Complex Isolates in Industrialized Countries.* Brussels-Belgium: Royal Library of Belgium.

ISO, T. I. O. F. S., 4225:1994. *Online Browsing Platform (OBP):Air quality — General aspects — Vocabulary.* [Online] Available at: https://www.iso.org/obp
[Accessed 11 September 2013].

James, M., *et al*., 2009. Antimicrobial Benefits of Copper. *Advanced Material and Processes,* July(7. l, Wilton Moran, Adam Estelle, Kyle Sexton & Harold Michels r alloys in Advanced Materials and Processes July 2012: 27-29), pp. 27-29.

Kunz, R. & Gundermann, C. K., 1982. The Survival of Mycobacterium tuberculosis on Surfaces at Different Relative Humidities. *Zentralblatt Fur Bakteriologie, Mikrobiologie and Hygiene,* Volume 176, pp. 105-115.

Lauten, E. H. *et al*., 2010. Gene Expression, Bacteria Viability and Surviability Following Spray Drying of Mycobacteria smegmatis. *Materials,* Volume 3, pp. 2684-2724.

Lemire, J. A., Harrison, J. J. & Turner, R. J., June 2013. Antimicrobial Activity of Metals: Mechanism, Molecular Targets and Applications. *Nature Reviews Microbiology,* Volume 11.

Loddon Mallee Region Infection Control Resource Centre, 2003. *InfectionControl Manual for the Management of Construction, Renovation, Repair and Maintenace within Health Care Facilities: A Manual for Reducing Health Care Associated Infection by Dust and Water Borne Micro-organisms.* 2nd ed. Loddon Mallee : Loddon Mallee Region Infection Control Resource Centre.

Löndahl, J. *et al*., 2014. Measurement Techniques for Respiratory Tract Deposition of Airborne Nanoparticles: A Critical Review. *Journal of Aerosol Medicine and Pulmonary Drug Delivery,* 27(4), pp. 229–254.

Maher, D., 2009. The Natural History of Mycobacterium tuberculosis Infection in Adults. In: H. S. Schaaf, A. Zumla & J. M. Grange, eds. *Tuberculosis : a Comprehensive Clinical Reference.* Edinburgh: Saunders/Elsevier, pp. 129-132.

Mandell, G. L., Bennet, J. E. & Dolin, R., 2010. *Mandell, Douglass, and Bennet's Principles and Practice of Infectious Diseases.* 7th ed. Philadelphia: Churchill Livingstone Elsevier.

Marais, F., Mehtar, S. & Chalkley, L., 2010. Antimicrobial Efficacy of Copper Touch Surfaces in Reducing Environmental Bioburden in a South African Community Healthcare Facility in *Journal of Hospital Infection,* Volume 74, pp. 80-95.

Mehtar, S., Wiid, I. & Todorov, S. D., 2007. The Antimicrobial Activity of Copper and Copper Alloys against Nosocomial Pathogens and Mycobacterium tuberculosis Isolated from Healthcare Facilities in the Western Cape: an in vitro study. *Journal of Hospital Infections ,* Volume 68, pp. 45-51.

Mehtar, S., 2010. *Understanding Infection Prevention and Control.* 1st ed. Cape Town: Juta and Company Ltd.

Melvin, F. W. et al., 1998. The Nebulizer Characteristics for Certification of Biosafety Cabinets with Bacteria and Simulants. *Jounal of American Biological Safety Association,* 3(1), pp. 26-29.

Molteni, C., Abicht, H. K. & Solioz, M., 2010. Killing of Bacteria by Copper Surfaces Involves Dissolved Copper. *Applied and Environmental Microbiology,* 76(12), pp. 4099–4101.

Morvay, A. A., Decun, M., Sala, C. & Morar, A., 2009. *Dynamics of Microbial biofilm on Different Material in Drinking Water Systems.* s.l., s.n.

Mo, Y. *et al*., 2007. Genetic Alteration of Mycobacterium smegmatis to Improve Mycobacterium-Mediated Transfer of Plasmid DNA into Mammalian Cells and DNA Immunization. *Infection and Immunity,* 75(10), pp. 4804–4816.

Müller, A., 2011. *TBonline.* [Online]
Available at: http://www.tbonline.info/posts/2011/5/27/how-tb-spread
[Accessed 25 Feb 2015].

Nicol, L. *et al*., 2014. *A Systematic Review of the Epidemiology of and Programmatic Response to TB in Health Care Workers in South Africa,* Cape Town: EVISAT project.

Ogbonnaya, J. *et al*., 2011. The status of Tuberculosis Infection Control Measures in Health care Facilities Rendering joint TB/HIV services in “German Leprosy and Tuberculosis Relief Association” supported states in Nigeria. *Nigerian Journal of Clinical Practice ,* 14(3), pp. 270-275.

Parish, T. & Stoker, N. G., 1998. *Methods in Molecular Biology: Mycobacteria Protocols_Electroporation of Mycobacteria.* 101 ed. s.l.:Humana Press.

Romanoa, F. *et al*., 2015. Some Aspects on the Sampling Efficiency of Microbial Impaction Air Samplers. *Paricuology,* Volume 19.

Sala, A., Bordes, P. & Genevaux, P., 2014. Multiple Toxin-Antitoxin Systems in Mycobacterium tuberculosis. *Toxins ,* Volume 6, pp. 1002-1020.

Santo, C. E., Quaranta , D. & Grass , G., 2012. Antimicrobial Metallic Copper Surfaces Kill Staphylococcus Haemolyticus via Membrane Damage. *MicrobiologyOpen,* pp. 46-52.

Santo, C. E., Taudte, N., Nies, D. H. & Grass, G., 2008. Contribution of Copper Ion Resistance to Survival of Escherichia colion Metallic Copper Surfaces. *Applied and Environmental Microbiology,* 74(4), pp. 977–986.

Saunders, I. & Newman, W. A., 2007. *Dorland's Illustrated Medical Dictionary.* 31st ed. Philadelphia: Saunders/Elsevier.

Schmdit, M. G. *et al*., 2012. Characterization and Control of Microbial Community Affiliated with Copper or Alluminium Heat Exchangers of HVAC. *Current Microbiology,* Volume 65, pp. 141-149.

Schnippel, K. *et al*., 2013. Cost of Inpatient Treatment for Multi-drug Resistance Tuberculosis in South Africa. *Tropical Medicine International Health,* 18(I), pp. 109-116.

Schwake, D. O., Alum, A. & Abbaszadegan, M., 2015. Impact of Environmental Factors on Legionella Populations in Drinking Water. *Pathogens,* Volume 4, pp. 269-282.

Singh, A. K. & Reyrat, J. M., 2009. Laboratory Maintenance of Mycobacterium Smegmatis. In: J. W. &. Sons, ed. *Current Protocols in Microbiology.* s.l.:s.n., p. Chapter 10:Unit10C.1.

Sissolak, D., Bamford, C. M. & Mehtar, S., 2010. The potential to transmit Mycobacterium Tuberculosis at a South African Tertiary Teaching Hospital. *International Journal of Infectious Diseases,* Volume 14, pp. 423-428.

Sissolak, D., Marais, F. & Mehtar, S., 2011. TB infection Prevention and Control Experiences of South African nurses - a phenomenological study. *BioMed Central,* Volume 11.

Sutton, S., 2008. Qualification of an Environmental Monitoring Program. *Pharmaceutical Microbiology Forum,* August.14(8).

The Merk Manual, Professional Edition, 2014. [Online] Available at: http://www.merckmanuals.com/professional/infectious_diseases/mycobacteria/tuberculosis_tb.html [Accessed 27 March 2014].

Thermo Fisher, 2007. *Instruction Manual: Two and Six stage Viable Samplers.* s.l.:s.n.

Tobias, H. J. *et al*., 2005. Bioaerosol Mass Spectrometry for Rapid Detection of Individual Airbone Mycobacterium tuberculosis H37 Ra. *Applied Environmental Microbilogy,* 71(10), pp. 6086-6095.

Todar, K., 2008-2012. *Mycobacterium tuberculosis and Tuberculosis.* [Online]
Available at: www.textbookofbacteriology.net
[Accessed 27 November 2014].

Thomas, R. J. *et al*., 2008. Characterization and Deposition of Respirable Large- and Small-Particle Bioaerosols. *Applied and Environmental Microbiology,* 74(No. 20), pp. 6437–6443.

Tudor, C. *et al*., 2014. Tuberculosis among Health Care Workers in KwaZulu-Natal, South Africa: a Retrospective Cohort Analysis. *BioMed Central,* Volume 14, pp. 891.

Verreault, D., Moineau, S. & Duchaine, C., 2008. Methods for Sampling of Airborne Viruses. *Microbiology and Molecular Biology Reviews,* 72(3), pp. 413-444.

Völlmecke, C. *et al*., 2012. The ATPases CopA and CopB both Contribute to Copper Resistance of the Thermoacidophilic Archaeon Sulfolobus solfataricus. *Microbiology ,* Volume 158, pp. 1622–1633.

Waines, P. L. *et al*., 2011. The effect of Material Choice on Biofilm Formation in a Model Warm Water Distribution System. *Biofouling: The Journal of Bioadhesion and Biofilm,* 27(10), pp. 1161–1174.

Walther, B. A. & Ewald, P. W., 2004. Pathogen Survival in the External Environment and the Evolution of Virulence. *Biology Review,* Volume 79, pp. 849-869.

WHO, 1999. *Guidelines for Prevention of Tuberculosis in Healthcare Facilities in Resources-limited settings.* Biella: WHO.

WHO, 2006. *The Stop TB strategy, Building on and Enhancing DOTS to Meet the TB-related Millenium Development Goal.* [Online]
Available at: http://www.who.int/tb/publications/2006/stop_tb_strategy
[Accessed 26 Feb 2015].

WHO, 2012. *Global Tuberculosis Report,* Geneva: World Health Organization.

WHO, 2014. *Global Tuberculosis Report,* Geneva: WHO.

WHO, 2015. *End TB_The Strategy_The Global strategy and Targets for Tuberculosis Prevention, Care and Control after 2015.* [Online] Available at: http://www.who.int/tb/post2015_TBstrategy
[Accessed 19 March 2015].

WHO, 2015. *Global Tuberculosis Report,* Geneva: WHO.

Windfinder, 2015. *Wind & weather statistics Cape Town Airport http.* [Online]
Available at: http://www.windfinder.com
[Accessed 13 March 2015].

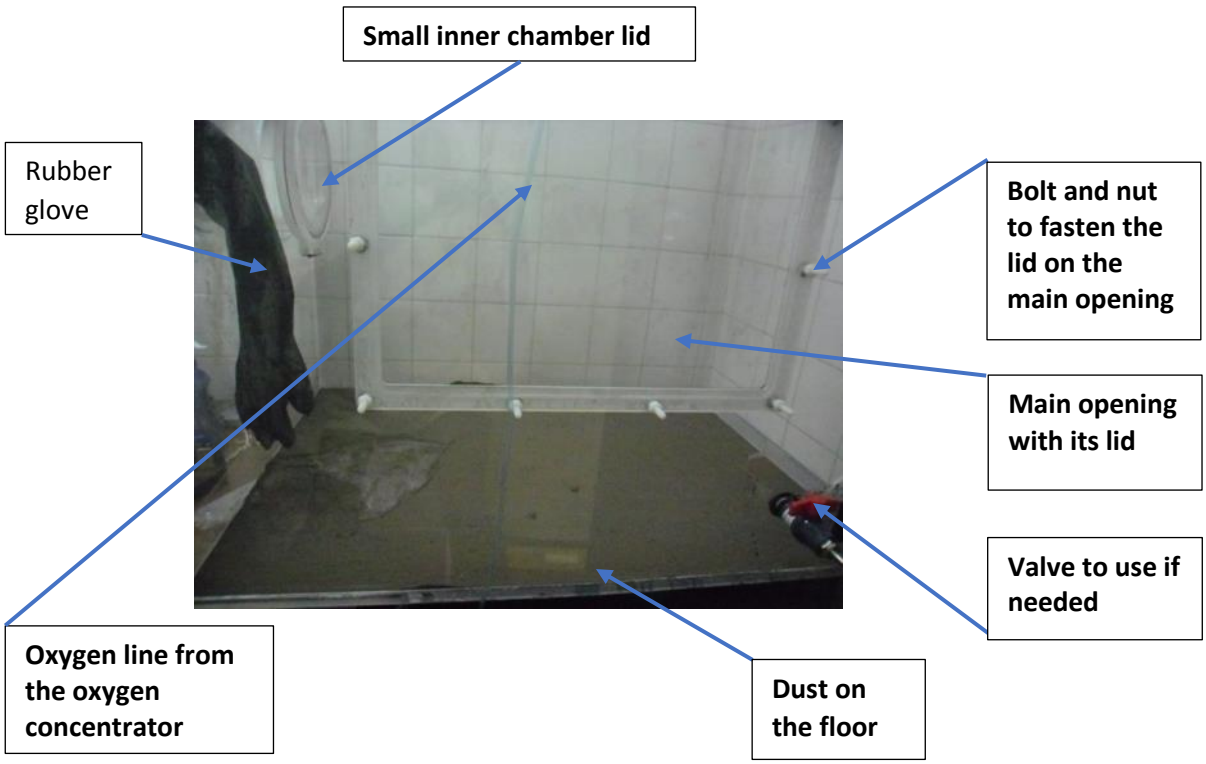
Wu, Q. L., Kong, D., Lam, K. & Husson, R. N., 1997. A Mycobacterial Extracytoplasmic Function Sigma Factor Involved in the Survival Following Stress. *Journal of Bacteriology,* 179(9), pp. 2922-2929.

Appendices

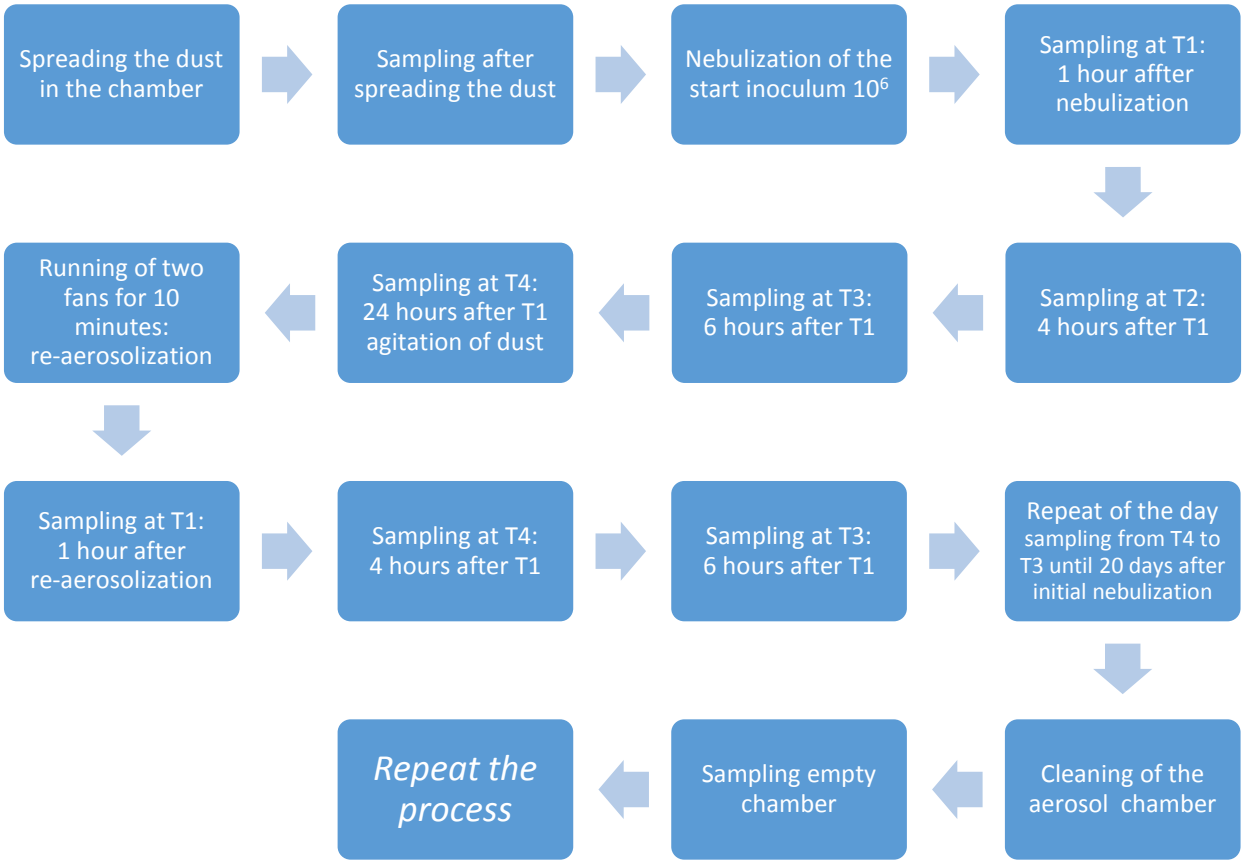
Appendix 1. Aerosol chamber

1. A. Aerosol chamber with the entire floor surface covered a copper plate before nebulization

1. B. Aerosol chamber showing dust covering the entire surface of the copper plate before nebulization

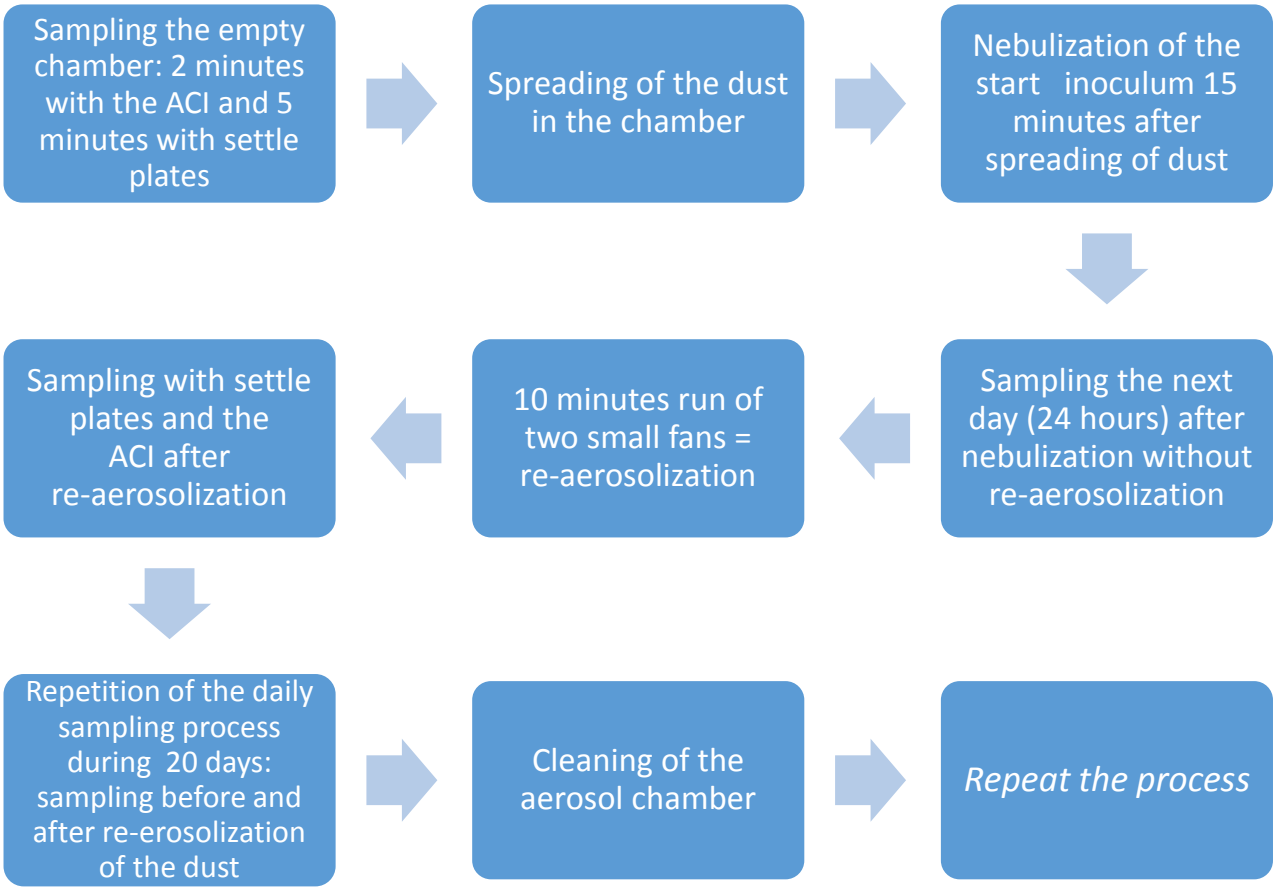


Appendix 2. Cycling sampling process during the pilot study



On the above figure, following the direction of arrows displays the succession of steps of the sampling process during the pilot study.

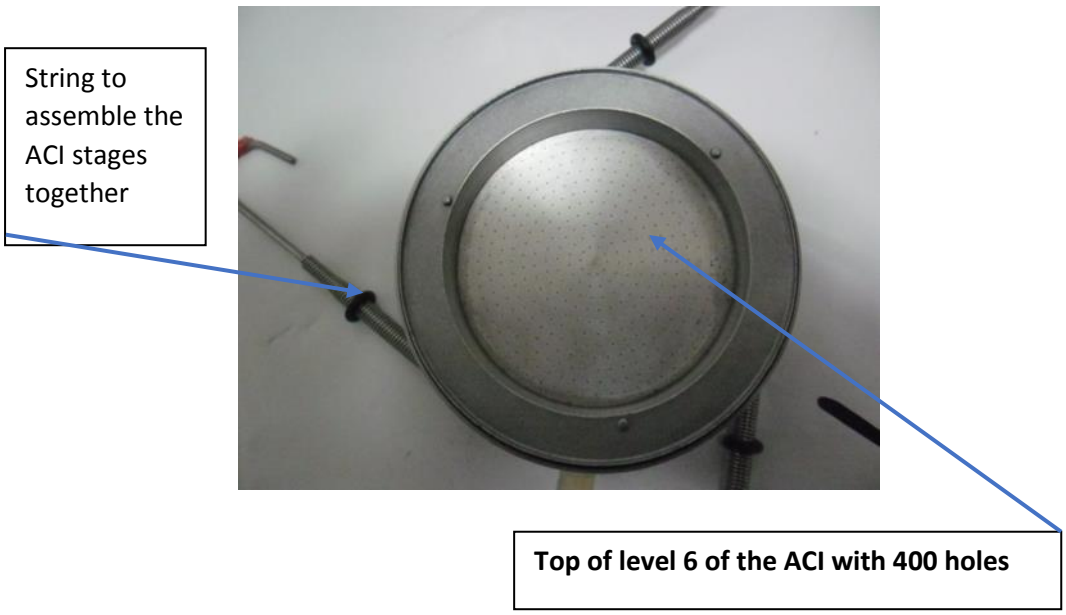
Appendix 3. Cycling sampling process during the study after consolidation of parameters during the pilot study



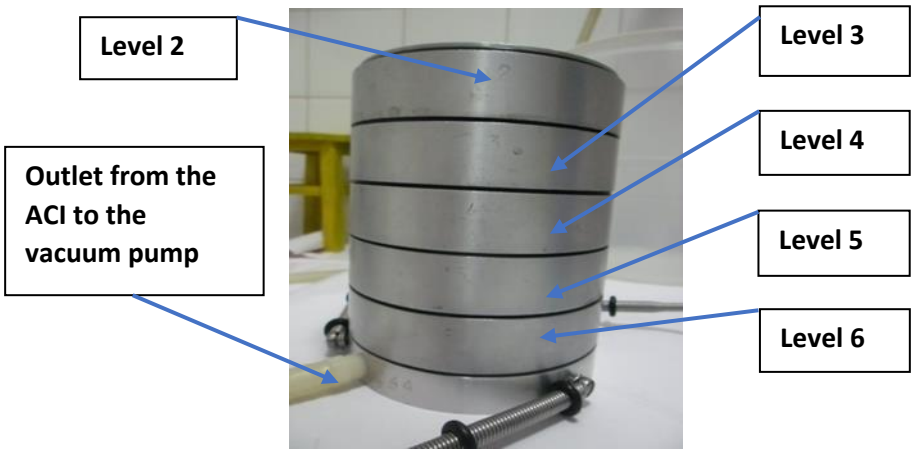
Succession of events is shows by the direction of the arrows

Appendix 4. The Andersen canister parts

4. A Top surface of one of the Andersen sampler levels with 200 holes



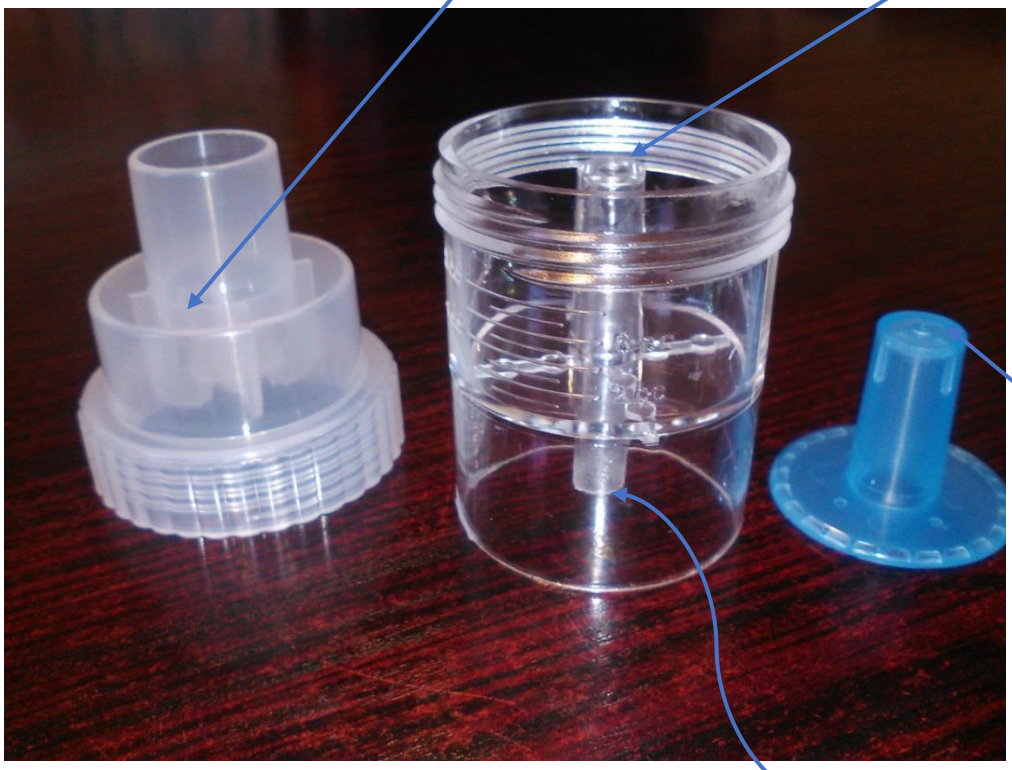
4. B The six- stage Andersen Air Sampler is comprised of five of the six aluminium stages (stage 2 to 6)



The six- stage Andersen Air Sampler with the six aluminium stages held together by three spring clamps and gasketed with O-ring seals

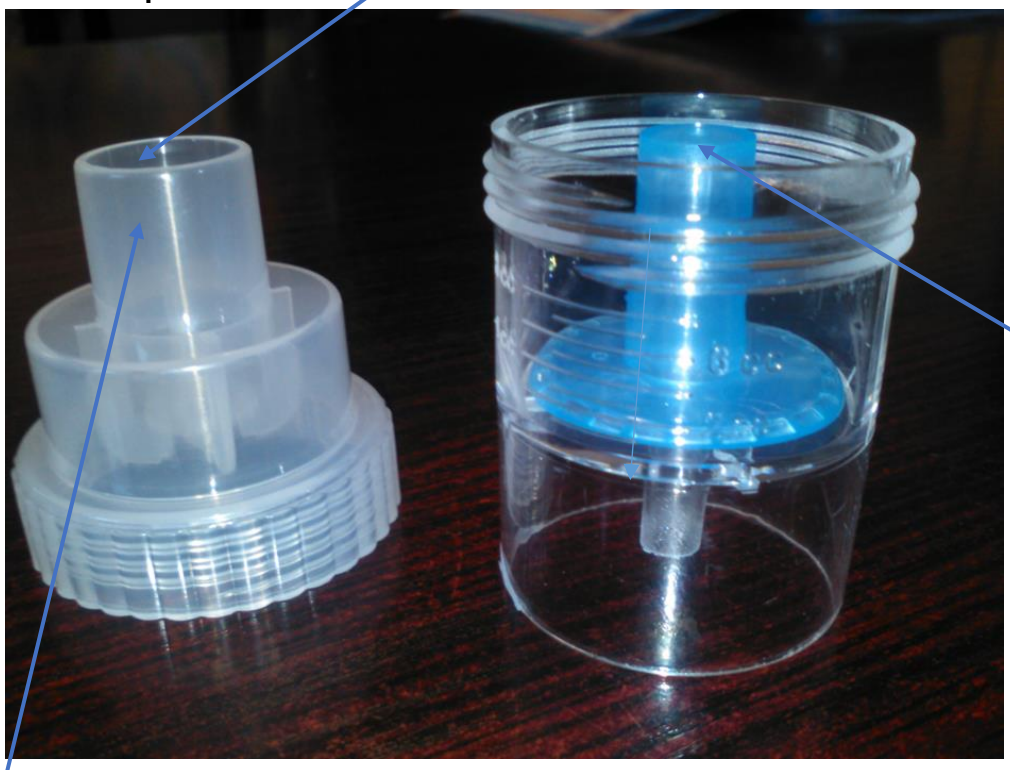
Appendix 5 Nebuliser jar parts

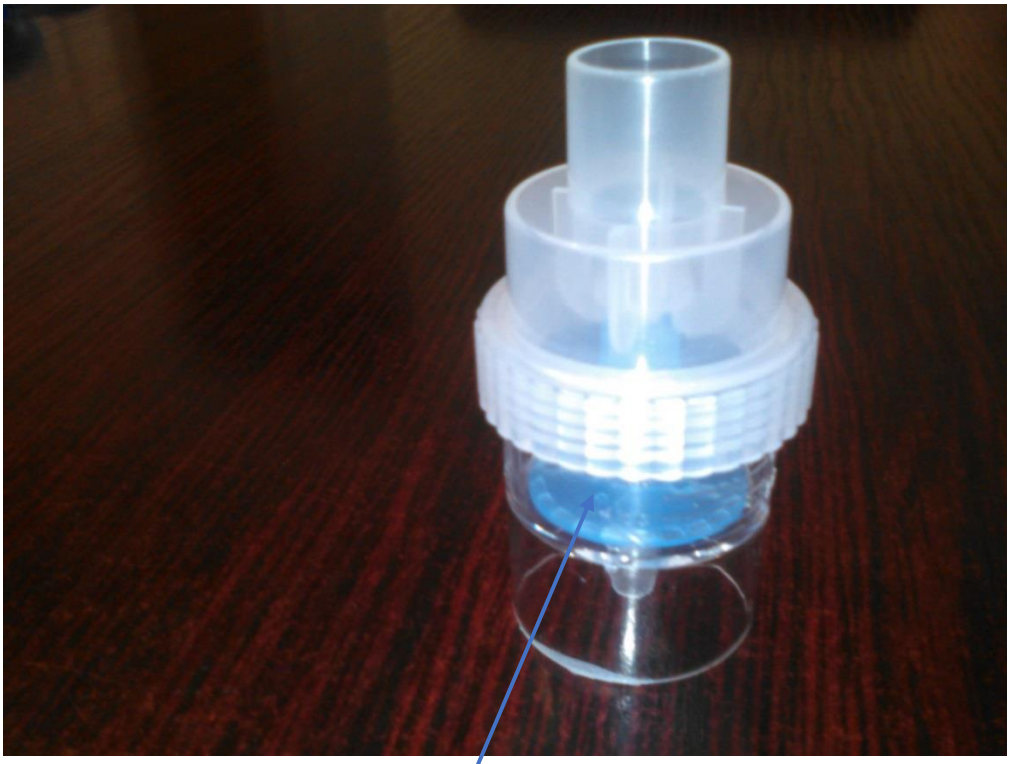
5. A. Nebulizer jar dismantled



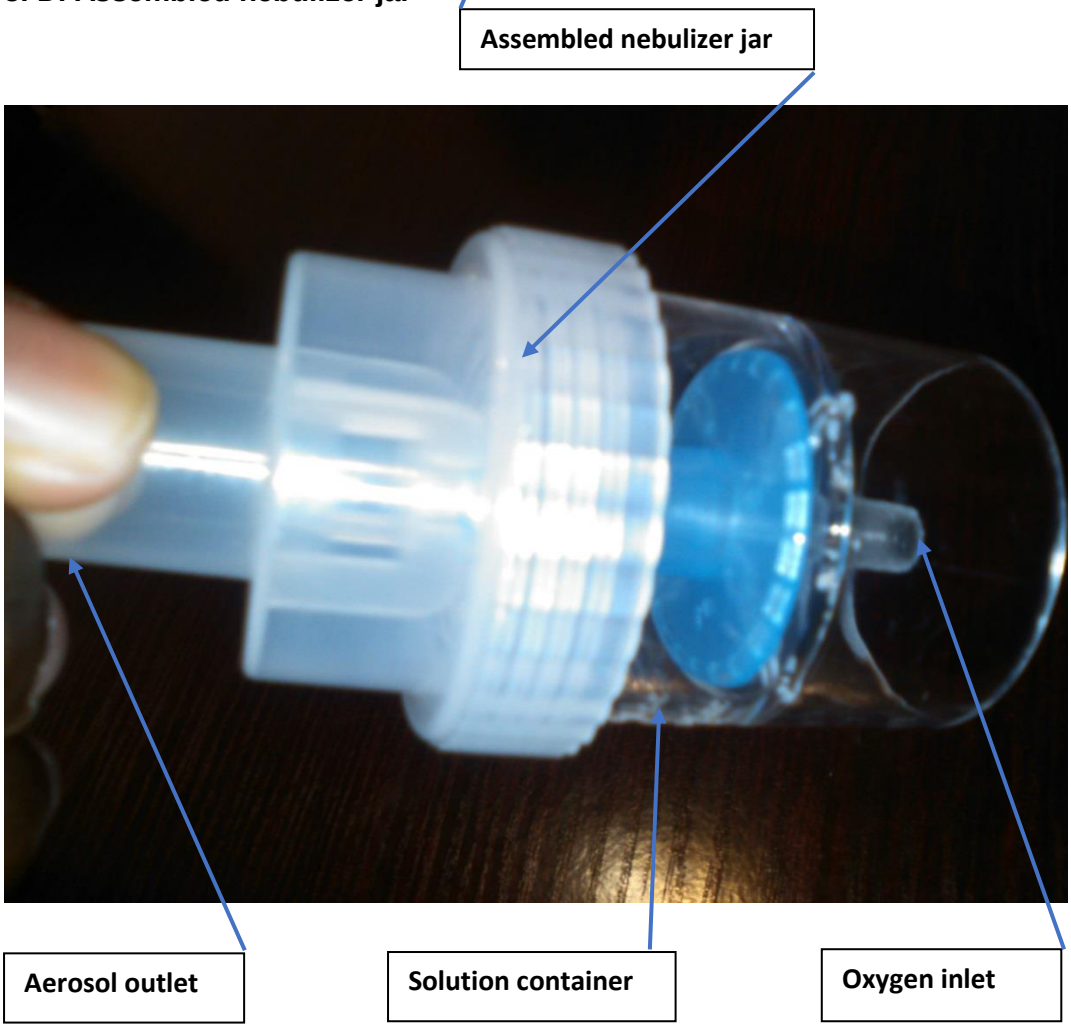
5. B. Assembling of jar parts

5. C. Mouthpiece



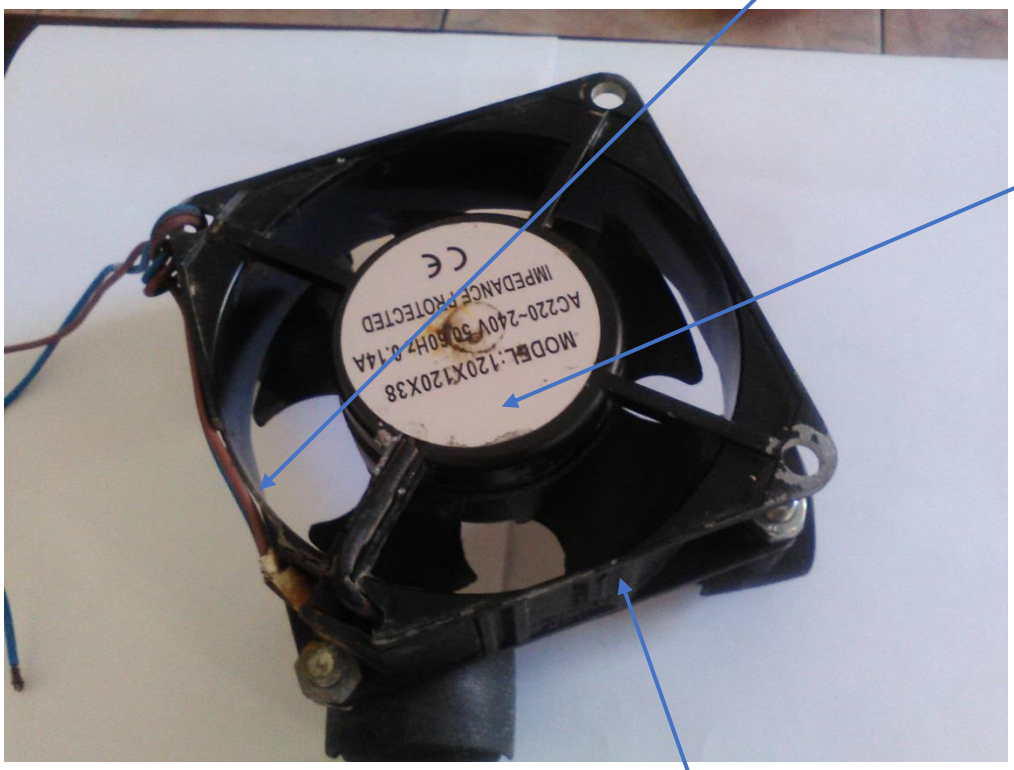


5. D. Assembled nebulizer jar

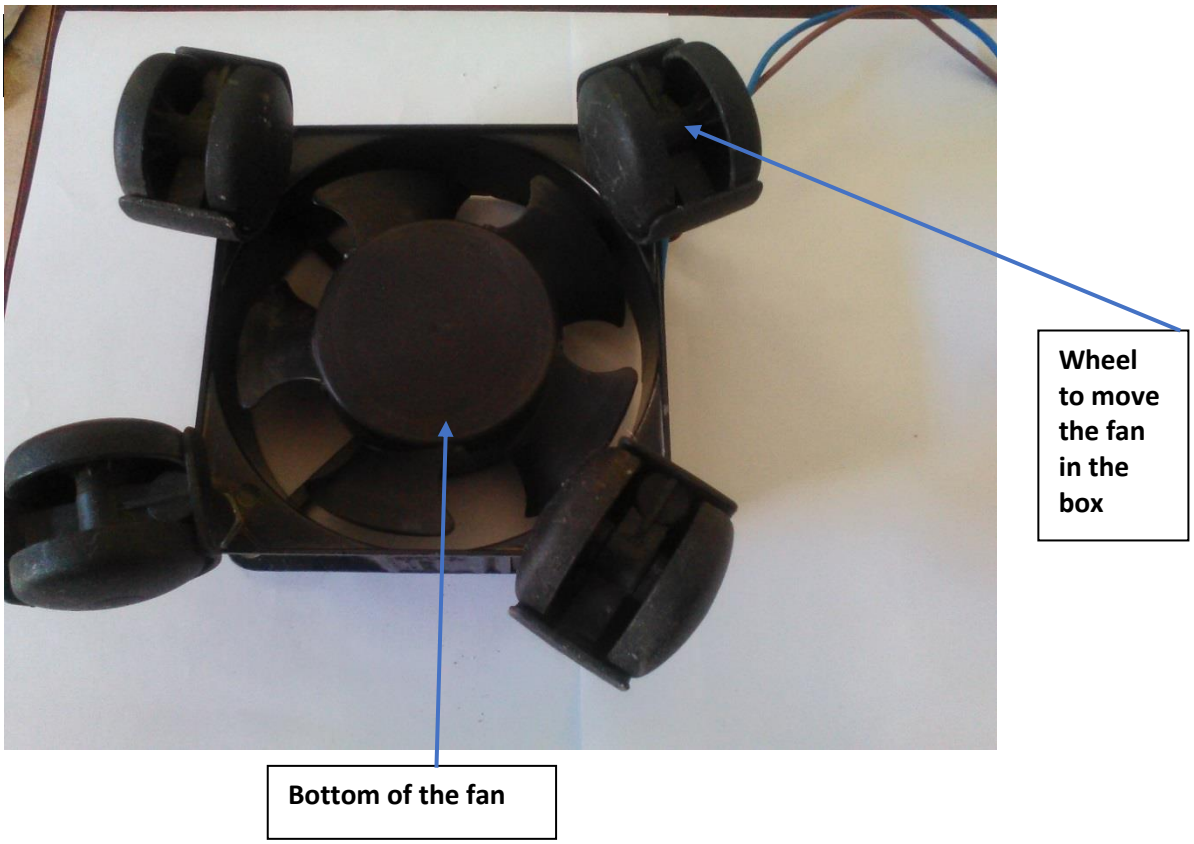


Appendix 6 Fan used to lift up the dust

6. A. Top of one of the small fans used for lifting up the dust



Appendix 6.B. Bottom one of the small fans used for lifting the dust



Appendix 7. Results of the analysis of the dust used for the study

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IMAGING UNIT

31 July 2014

Report
Analysis: SEM Unit, CAF, Stellenbosch University

Method

BSE-EDX analysis and elemental mapping was accomplished using a Zeiss EVO® MA15 Scanning Electron Microscope. Prior to analysis the samples was coated with a thin layer of gold coating in order to establish conductivity. The different elements were analyzed by means of quantitative ED analysis with a Zeiss EVO MA 15 Scanning Electron Microscope (SEM). Phase compositions were quantified via EDX analysis using an Oxford Instruments® X-Max 20mm^2 detector and Oxford INCA software. Energy dispersive spectroscopy is only suitable for determining major elements of minerals at concentrations over 0.1 wt% for heavy elements, and over 0.01 wt% for light elements. Beam conditions during the quantitative analyses were 20 KV and approximately 1.0 A, with a working distance of 8.5mm and a specimen beam current of -20.00 nA. For mineral analyses counting time was 10 seconds live-time. Natural mineral standards were used for standardization and verification of the analyses. The system is designed to perform high-resolution imaging concurrently with quantitative analysis, with errors ranging from ± 0.6 to 0.01wt% on the major elements using EDS.

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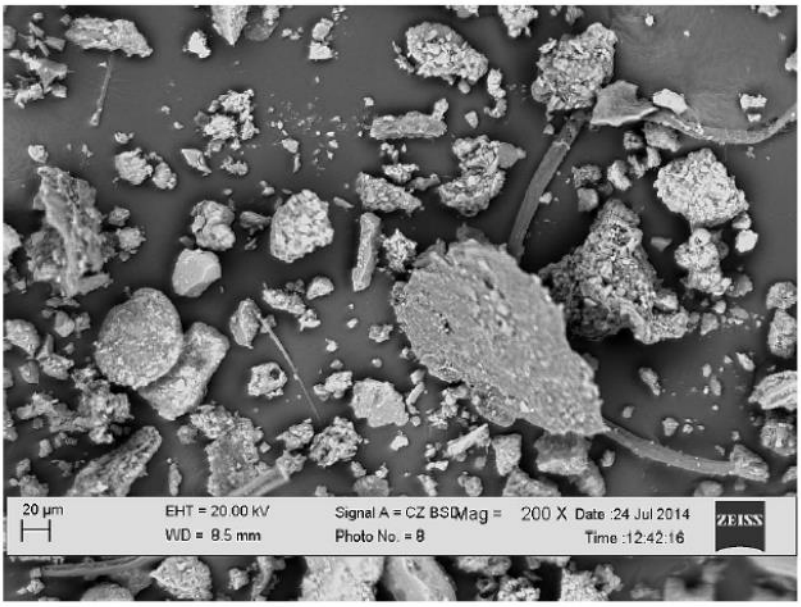
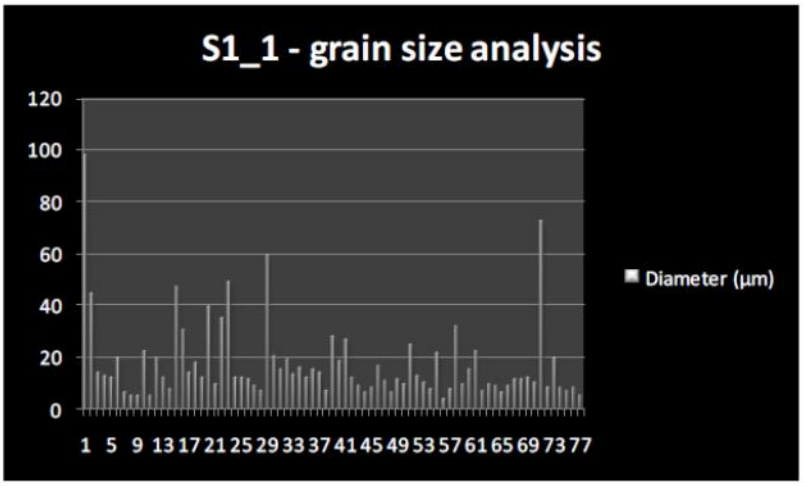
Results
BSE images and grain size analysis – Sample S1_1





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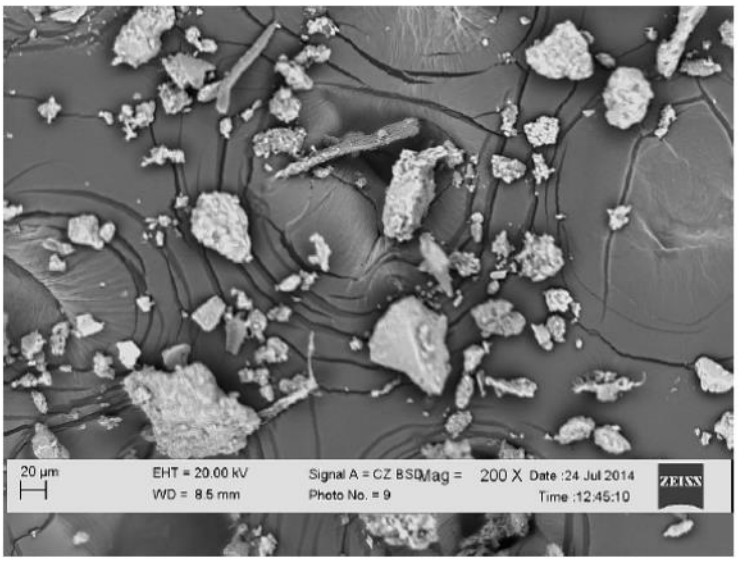
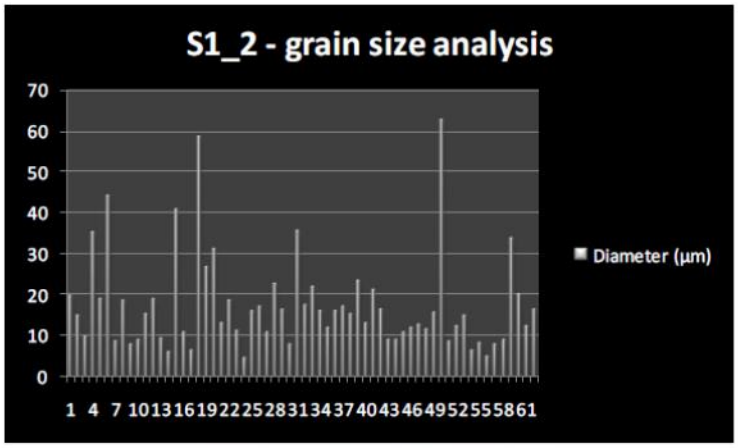
General statistical parameters for Sample S1_1 grain size (µm)	
AVE	17.05
MIN	3.93
MAX	97.70

BSE images and grain size analysis – Sample S1_2





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The S1_1 and S1_2 are two different mapping angles of sample1 as S2_1 and S2_2 are for sample2. The grains of these samples could be seen in several different angles. The

technicians could have done more mapping to get an average that could be very close to the exact size of the particles.

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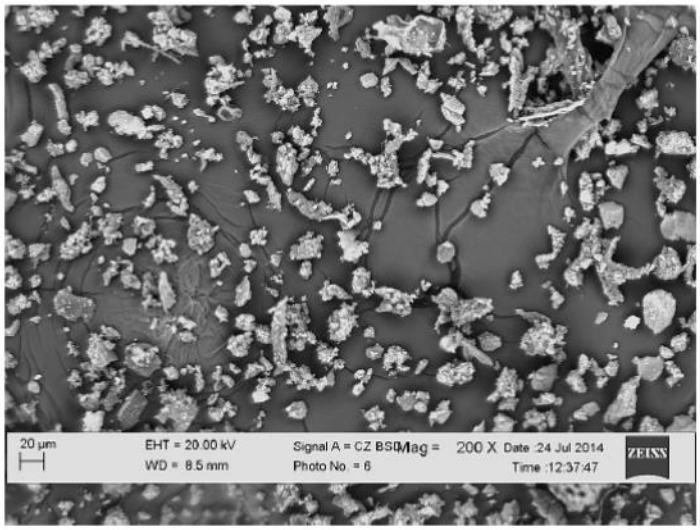
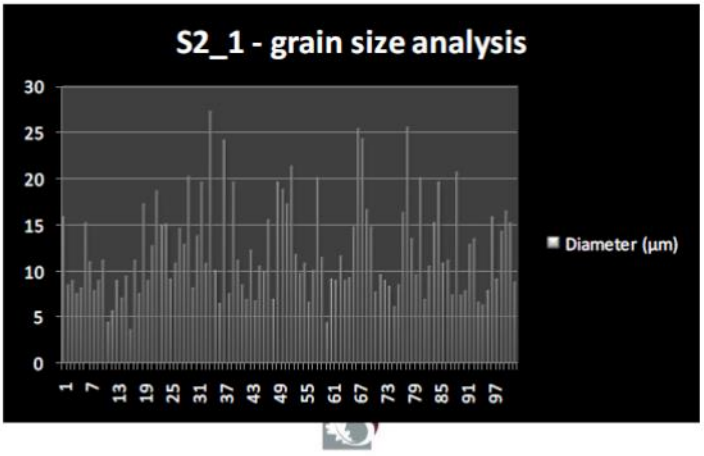
General statistical parameters for Sample S1_2 grain size (µm)	
AVE	17.14
MIN	4.44
MAX	62.62

BSE images and grain size analysis – Sample S2_1





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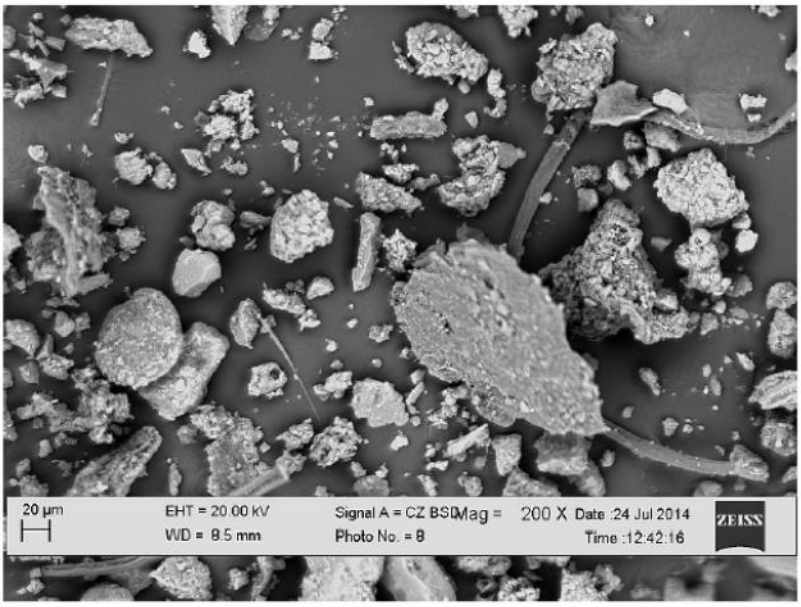
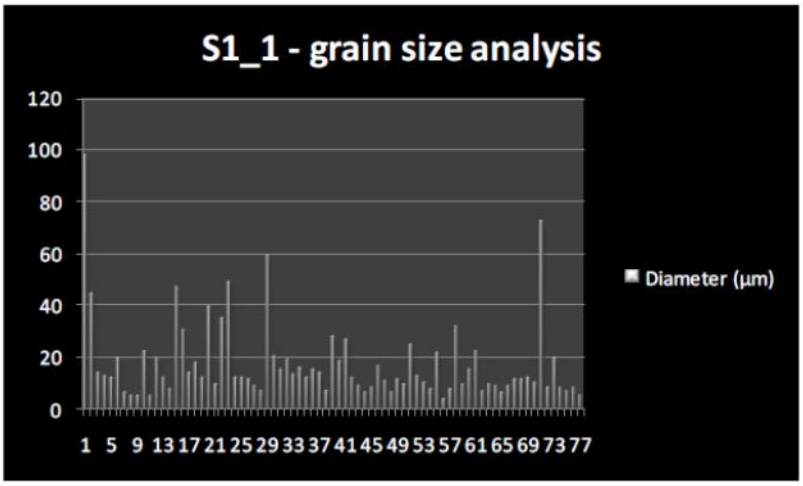
Results
BSE images and grain size analysis – Sample S1_1





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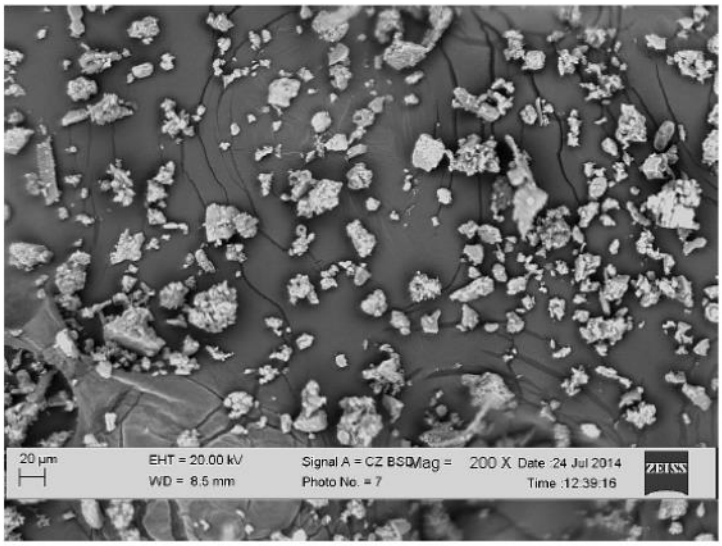
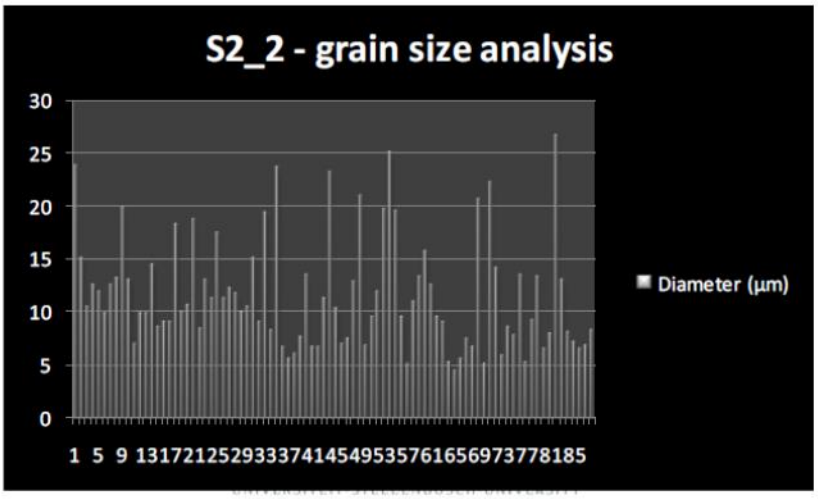
General statistical parameters for Sample S2_1 grain size (µm)	
AVE	12.02
MIN	3.56
MAX	27.24

BSE images and grain size analysis – Sample S2_2





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General statistical parameters for Sample S2_2 grain size	
AVE	11.55
MIN	4.34
MAX	26.67

EDX analysis

Sample S1

Elemental analysis normalised to 100 wt%												
Element	Na	Mg	Al	Si	S	Cl	K	Ca	Fe	Cu	O	Total
AVE	1.98	1.28	5.66	23.59	1.24	2.30	1.29	12.09	7.06	0.87	42.63	100.00

Sample S2

Elemental analysis normalised to 100 wt%													
Element	Na	Mg	Al	Si	S	Cl	K	Ca	Ti	Fe	Cu	O	Total
AVE	1.39	1.25	4.68	22.38	2.25	1.90	1.15	14.18	0.73	6.07	1.23	42.78	100.00

Elemental phase mapping

Elemental phase mapping primarily highlights the spatial distribution of each element, in this case Al, Ca, Mg, Fe, Si, K and Na across the surface of each sample. In total, two elemental maps were completed for each sample (Refer to attached files, namely S1_map1, S1_map2, S2_map1, and S2_map2). Eight images were produced for each sample, which included a backscattered electron (BSE) image and seven elemental maps. The phase mineral identification with the aid of BSE imaging and elemental maps in both samples included a variety of mineral grains which were interpreted as quartz, K-feldspar, biotite, calcite, garnet, sphene, and staurolite.

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Conclusion

In short, the particle sizes of sample S1 are generally larger than sample S2. The grain size of sample S1 is particular variable. The modal abundance of elements and mineralogical composition for both samples is strikingly similar.

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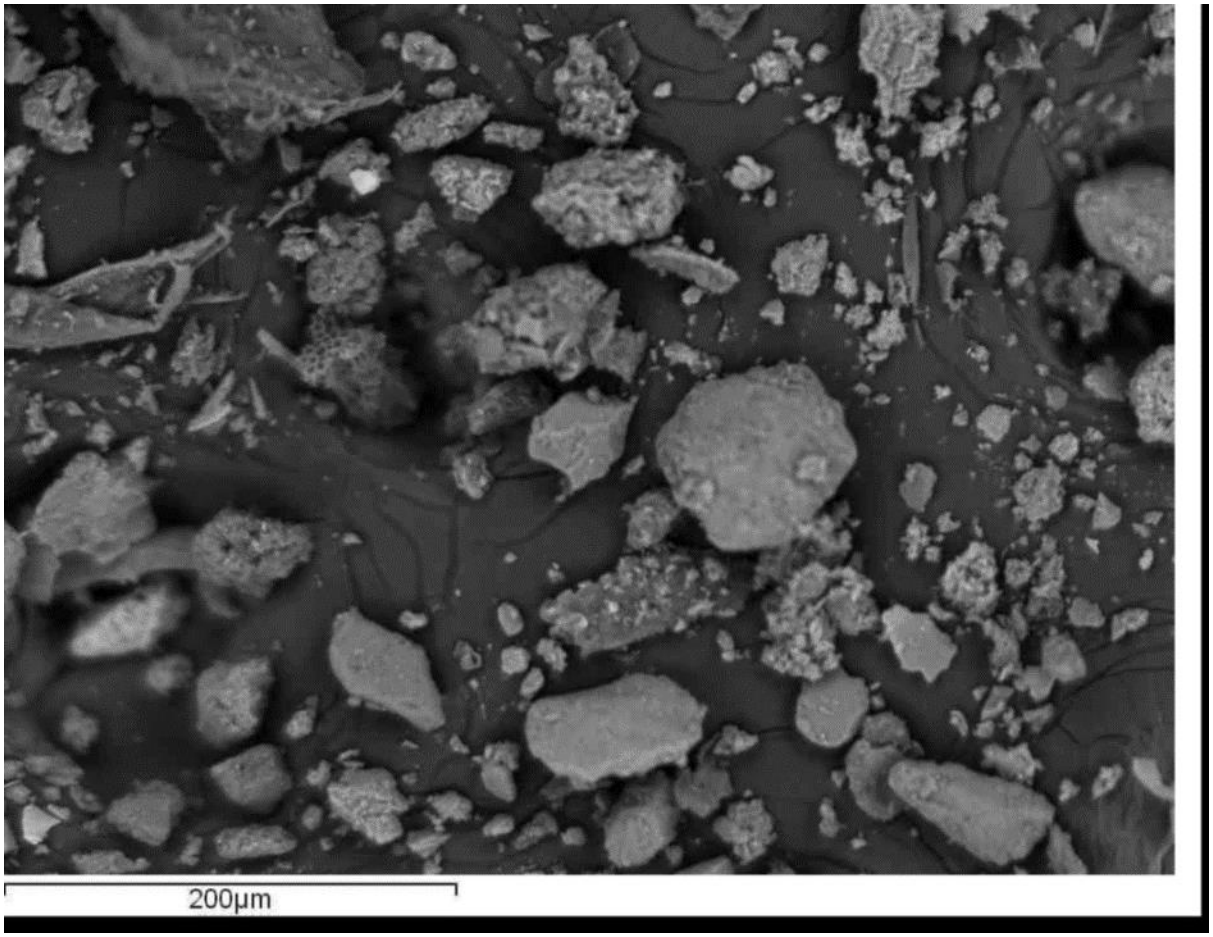
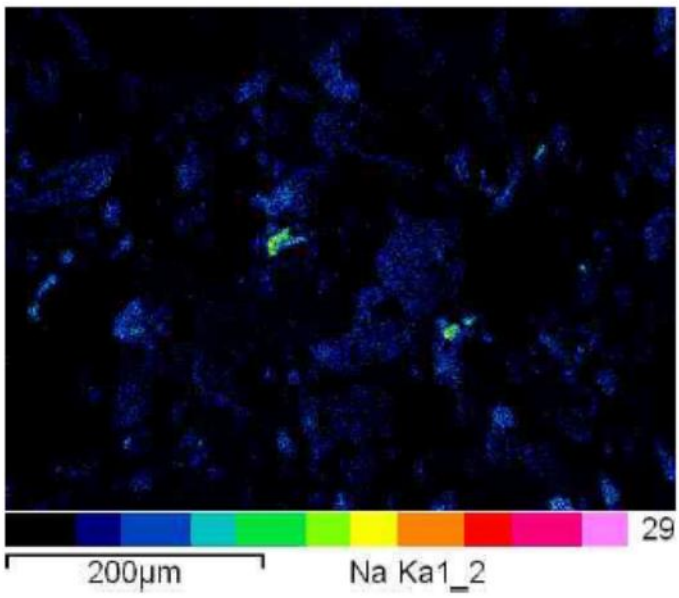
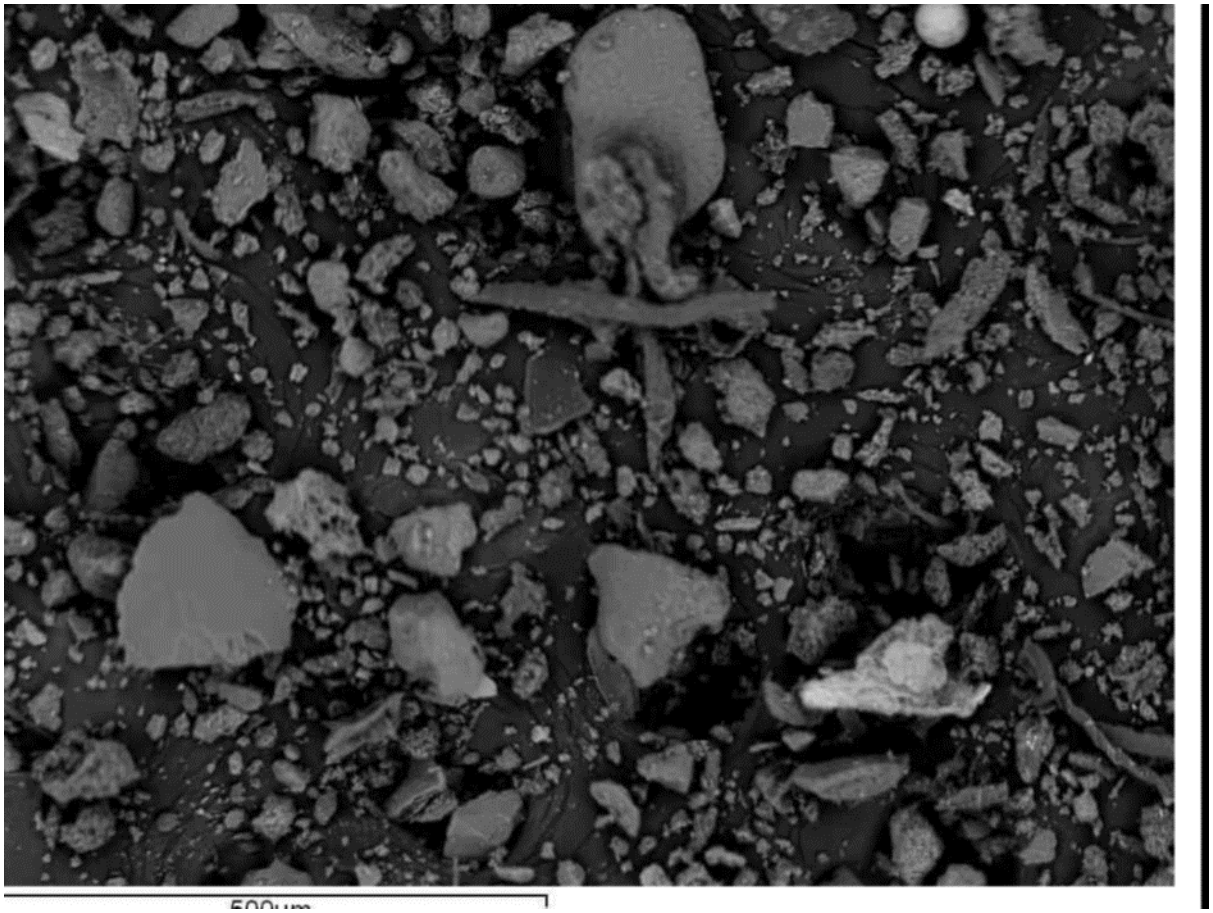
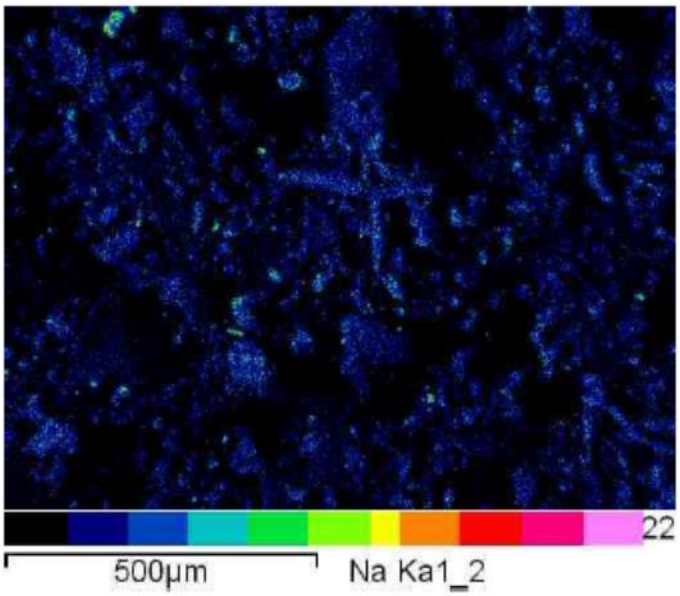




Sample 2: map 1





Sample 1: map1

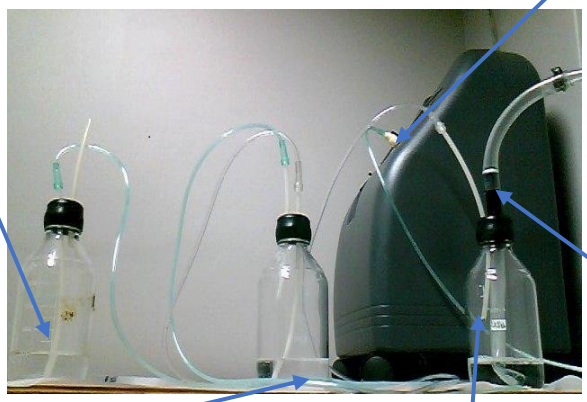
Appendix 8. Technical features of the nebulizer used for the study

V5 oxygen concentrator technical parameter

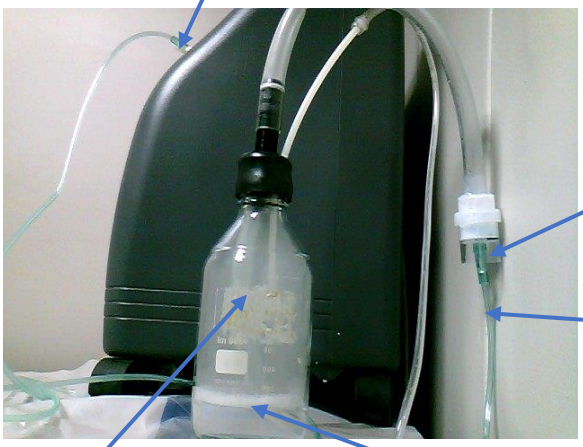
Model	V5 series
Power consumption (w)	350
Working voltage (V/Hz)	220/110V±10% 50/60 HZ±1
Flow Rate (L/min)	1-5
Concentration (%)	93±3%
Outlet pressure (Mpa)	0.05-0.08
Sound level	42
Electrical category	Class II Type B
Product Category	Class II a
Net weight (Kg)	21
Dimension (mm)	390x337x620
Atomization particle	≤ 5μ reaches 90%
Oxygen percent alarm	When oxygen purity is less than 82%, the alarm Starts up

(Shenyang Canta Medical Tech. Co. Ltd., p. 6)

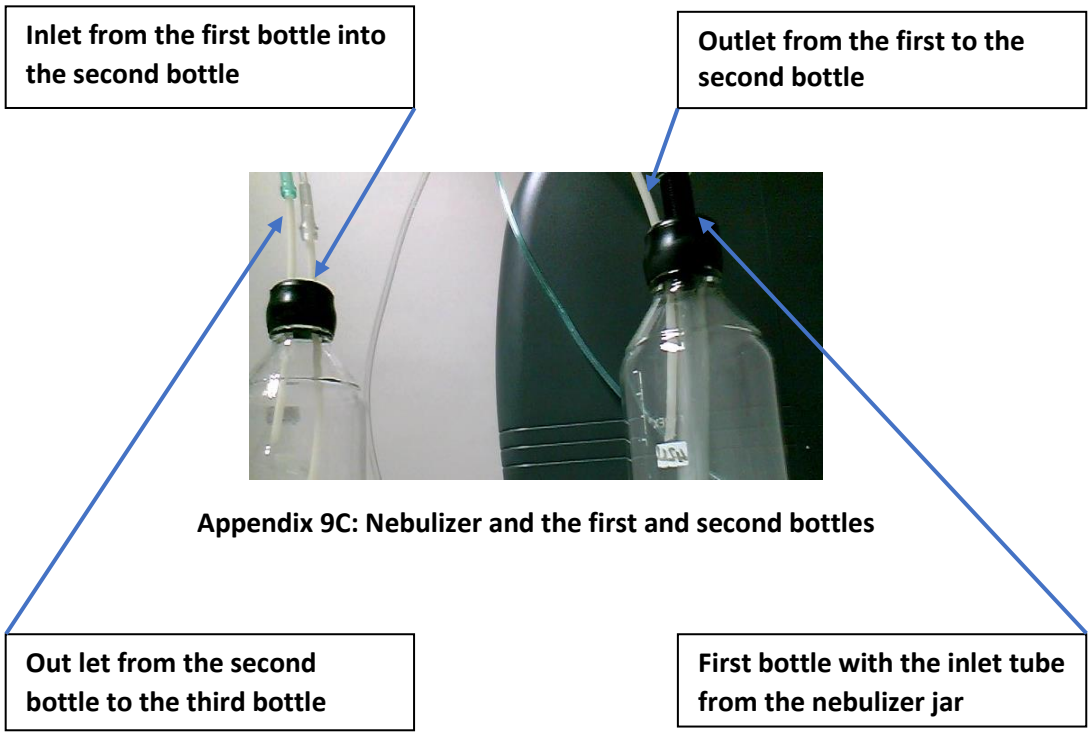
Appendix 9. Assessment of the Nebulizer



Appendix 9A: Nebulizer and three bottles



Appendix 9B: Nebulizer, the jar and the first bottle



Appendix 9C: Nebulizer and the first and second bottles

When we were submitting our proposal for the current study, we were offered The BANG or BioAerosol Nebulizing Generator as the nebulizer to be used for our research. This apparatus works on the principle of the Collison nebulizer. It was reported in its user manual that it was a unique nebulizer for the generation of aqueous aerosols at a low air flow rate and that its design would minimize the foaming of protein solutions while maximizing aerosol output. The quotation for this nebulizer, if bought in the United States of America (USA), excluding shipping and taxes costs amounted to $27,515 (US Dollars) which is equivalent to approximatively R382,152 South African Rand. This amount could not be funded for this research project in a low and middle-income country. Our target, therefore, was to find a nebulizer that would filter the air used to generate the bioaerosol as much as possible, without flawing the results, and that could produce to a certain extent respirable particles. We decided to go for a certain type of oxygen concentrator with an atomization or nebulization option used for the administration of medication or oxygen to patients. The total cost of this type of nebulizer was R10,488 South African Rand, this was nearly 1/36 times less than or approximately 3% of the cost of the BANG. We have no data on any previous use of this tool for generating bioaerosol. The question was could this instrument generate bioaerosol with enhanced viability of microorganisms for aerobiology research?

For the assessment of the nebulizer we simulated the technique of collecting bioaerosol by means of liquid impediment. The choice of this method was motivated by the fact that it was cost effective in counting the output number of microorganisms compared to the input. The preparation of the solution to be nebulized was done as described in the study. The liquid impeder used was PBS X 1 instead of PBS-T80 as the detergent Tween 80 produced foamy solutions that came out from the exhaust of the third bottle, with loss of bacteria. We initially used two bottles containing 100 ml of PBS X 1 (Griffin, *et al.*, 2010) to collect nebulized bacteria and plated a sample from two bottles, and found that there was growth of mycobacteria. We added a third bottle with the same quantity of the liquid impeder, and there was still growth from the samples taken after nebulization on 20 ml of the nebulized solution. Our aim in increasing the number of bottles was to minimize the number of escaping microbes from the third bottle. Instead of multiplying the number of bottles we decided to increase the level of the liquid impeder up to 200ml to sink deeper the inlet tube of each bottle and plated samples from the three bottles. Now, there was no growth of microorganisms after plating samples from 200 ml of the solution from the third bottle; meanwhile we notice some growth from the first two bottles. These results consolidated the use of three bottles and 200 ml of the liquid impeder in the assessment of the nebulizer.

CFU/ml= (average CFU/plate) x (dilution factor)/volume plated

CFU/ml= (1.25x100 per 100µl) x10 =1.25 x 100=1.25.10^3=1.25.10^3CFU/ml

The 20 ml were nebulized in 600 ml making a total approximate volume of 620 ml, the total CFU contained in 620ml came from 20 ml, this number of CFU is nearly 1.25.10^3 x 620=7.75.10^5CFU. The CFU number in 20 ml before nebulization was 20.10^6. From this nebulized number 7.75.10^5 were recovered using liquid impediment procedure (Appendix 9D, this represented nearly 1/25 of the initial number of CFU put in the nebulizer jar. This was probably due to the stress due to nebulization and the impaction of the mycobacteria in the liquid as well as to the escaping process of microbes from the bottles. The above results showed that this nebulizer is less performant than the Collison nebulizer in the study conducted by Melvin and his collaborators on the nebulizer characteristics for certification of biosafety cabinets with bacteria and simulants. These researchers found that with a starting suspension of 5-8.10^8. The Collison Nebulizer consistently delivered 1-4. 10^8 spores of Bacillus subtilis during 5 min of continuous operation. The Collison nebulizer is the standard in comparison to other nebulizers (Melvin, *et al.*, 1998)

From these results assessment of this new tool, we cannot make any segregation between different causes of losses during and after nebulization of mycobacteria request a further re-assessment of this tool for better accuracy.

Appendix 9D: Number of colony Forming Units from the plating 100µl of post-nebulization samples

	undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	231	7	1	0	0	0	0	0
2	221	5	1	0	0	0	0	0
3	225	6	1	0	0	0	0	0
4	221	11	1	0	0	0	0	0
5	229	13	1	0	0	0	0	0
6	225	10	1	0	0	0	0	0
7	203	19	1	0	0	0	0	0
8	228	17	1	0	0	0	0	0
9	223	18	1	0	0	0	0	0
10	219	18	1	0	0	0	0	0
11	218	16	1	0	0	0	0	0
12	219	14	1	0	0	0	0	0
13	208	17	1	0	0	0	0	0
14	227	13	1	0	0	0	0	0
15	206	13	1	0	0	0	0	0
16	221	14	1	0	0	0	0	0
17	217	12	1	0	0	0	0	0
18	199	13	1	0	0	0	0	0
19	219	12	1	0	0	0	0	0
20	229	20	2	0	0	0	0	0
21	218	20	1	0	0	0	0	0
22	202	16	1	0	0	0	0	0
23	213	17	1	0	0	0	0	0
24	228	21	2	0	0	0	0	0
25	219	20	2	0	0	0	0	0
26	222	20	2	0	0	0	0	0
27	200	19	1	0	0	0	0	0
28	226	22	2	0	0	0	0	0
29	229	21	2	0	0	0	0	0
30	220	18	2	0	0	0	0	0
31	217	18	1	0	0	0	0	0
32	202	22	2	0	0	0	0	0
Average	218.25	15.6875	1.25	0	0	0	0	0

The CFU grown from the plating of 100 µl of the mixed solution of the three bottles.

Appendix 10. An overview of the results of the pilot study

When conducting the pilot study, we sampled as follows: the ACI collected samples of the aerosols on a culture medium for incubation at T_0 = 2 min from the end of the initial aerosolization (Nebulization or re-aerosolization) of the bacterium and after each lift up of the dust (10 minute run of two small fans) in the aerosol chamber, T_1 (one hour following the end of the initial aerosolization or re-aerosolization of the dust), T_2 (4 hours from T_1), T_3 (6 hours from T_1), T_4 (24 hours from T_1 or the day after the initial aerosolization), thus T_4 time sampling preceded the agitation of dust (the latter to be considered as occurring at T_0 for this day). This allowed us to confirm that there was no microbe in the air 24 hours after nebulization or re-aerosolization. Re-aerosolization from Day 1 to Day 20 was done by agitating the dust using two small fans, 24 hours after the initial generation of aerosols (Nebulization) with the nebulizer (Appendix 2). In the current study the driving force of the dust for one small fan was at 2 Kpa and the velocity of the airflow was at average 2.035 m/s with a maximum of 2.47 m/s for the upwards airflow fan and a minimum of 1.60 m/s for the downwards airflow fan. After each sampling operation plates were removed from the sampler and the aerosol chamber and labelled for incubation. Fresh plates were reloaded in the ACI after the cleaning of the latter with ethanol 70%, and settle plates in the aerosol chamber were renewed for the next sampling operation. Plates collected from the ACI and from the experimental box were incubated for three to ten days and examined daily for assessment of the survival of the mycobacterium through colonies growth.

Below are presented some results from the pilot study:

Appendix 10A: Sampling the air with no dust spread and no microbes aerosolized

Plates and their levels	Average number of CFU/plate
SP	0
A1	0
A2	0
A3	0
A4	0
A5	0
A6	0

The above results showed that the air was not contaminated especially with *M. smegmatis* after running the ACI for 5 minutes.

Appendix 10B: Sampling of the air after dust spreading and no microbes aerosolized

Plates and their levels	Average number of CFU/plate
SP	0
A1	0
A2	0
A3	0
A4	0
A5	0
A6	0

The above results showed that the dust was not contaminated especially with *M. smegmatis* after running the ACI for 5 minutes and settle plates were kept in the chamber for about 8 minutes.

Appendix 10C: Sampling of the air after dust spreading and 5 min after the start of aerosolization of 20 ml of 10^8 CFU/ml

Plates and their levels	Average number of CFU/plate
SP	confluent
A1	confluent
A2	confluent
A3	confluent
A4	confluent
A5	confluent
A6	confluent

20 ml of a solution of 10^8 CFU/ml were nebulized at least 15 minutes after the dust was spread in the aerosol chamber. The ACI was run for 5 minutes after 5 minutes after the end of the aerosolization of solution. Colonies were confluent at all levels with no possibility of enumeration

Appendix 10D: Sampling of the air after dust spreading and 5 min from the end of aerosolization of 20 ml of 10^8 CFU/ml

Plates and their levels	Average number of CFU/plate
SP	confluent
A1	confluent
A2	confluent
A3	confluent
A4	confluent
A5	confluent
A6	400

Colonies were confluent at all levels except on the plate from the sixth level where they were matching the pattern of the 400 holes of the 6th slit of the ACI. This confluency was less than in the sampling done during the nebulization. This suggest sampling later after the end of the aerosolization process. As colonies were still confluent at most levels, the option of reducing the concentration of the nebulized solution to 10^6 CFU/ml was taken.

Appendix 10E: Sampling of the air 24 hours after dust spreading and microbes 10^8 CFU/ml aerosolized in presence of a giant fan

Plates and their levels	Average number of CFU/plate
SP	confluent
A1	0
A2	1
A3	0
A4	0
A5	14
A6	1

Sampling from the flat results suggested that may be settle plates microbes came from the giant fan or the outlet the aerosol chamber to the ACI. Thus, there is a real risk of confounding with true re-aerosolized bacteria.

Appendix 10F: Number of CFU grown on plates after lifting of the dust

	Average of CFU according to the plate level						
Sampling time after nebulization/Lift of dust	SP	A1	A2	A3	A4	A5	A6
2 minutes	3	0	1	1	4	33	3
1 hour	1	0	0	0	0	6	3
4 hours	0	0	0	0	0	1	0
6 hours	0	0	0	0	0	0	0
24 hours	0	0	0	0	0	0	0
2 minutes after lifting of the dust	0	0	0	0	0	0	0
1 hour after nebulization	0	0	0	0	0	0	0
4 hours after nebulization	0	0	0	0	0	0	0
6 hours after nebulization	0	0	0	0	0	0	0
24 hours after nebulization	0	0	0	0	0	0	0

The number of bacteria collected with settle plates or the ACI decrease as the aerosol settle as times goes after dust lifting.

Appendix 10G: Number of CFU grown from sampling 2 minutes after lifting of the dust

	Average of CFU per the plate level						
Sampling 2 min after dust lifting day after day	SP	A1	A2	A3	A4	A5	A6
1	5	0	1	0	2	18	3
2	5	0	0	0	7	1	4
3	2	0	0	0	0	2	0
4	5	0	0	1	1	0	0
5	1	0	0	0	0	0	0
6	1	0	0	0	0	0	0

The number of bacteria collected with settle plates or the ACI decrease as the aerosol settle as times goes after dust lifting.

From the above outcomes, the following changes were made: Reduction of the concentration of the solution to nebulize, use of small fans to be introduced the day following nebulization, no use of the giant fan, reduction of the sampling time to 2 min, disinfection of the outlet from the aerosol chamber to the ACI and replacement of the connecting tubes after each sampling procedure. We purchased appropriate plates for to the ACI. Actually, on the second day of incubation we had the growth becoming visibly noticeable. Ten days were used as an extension from seven days to fully cover for any late growth during incubation as some growth appeared on plates for probably late recovering microbes from stress or microbes to come late in touch with the culturing media (dust particles probably preventing early contact).

Appendix 11. Wind speed table for conversion of knots, Beaufort, m/s and km/h (Windfinder 2015)

Knots	Beaufort	m/s	km/h	mph	Label	Effect on sea	Effects on land
< 1	0	0 - 0.2	<1	<1	Calm	Sea like a mirror	Calm. Smoke rises vertically.
1-3	1	0.3-1.5	1-5	1-3	Light Air	Ripples with the appearance of scales are formed, but without foam crests	Wind motion visible in smoke.
4-6	2	1.6-3.3	6-11	4-7	Light Breeze	Small wavelets, still short, but more pronounced. Crests have a glassy appearance and do not break	Wind felt on exposed skin. Leaves rustle.
7-10	3	3.4-5.4	12-19	8-12	Gentle Breeze	Large wavelets. Crests begin to break. Foam of glassy appearance. Perhaps scattered white horses	Leaves and smaller twigs in constant motion.
11-15	4	5.5-7.9	20-28	13-17	Moderate Breeze	Small waves, becoming larger; fairly frequent white horses	Dust and loose paper raised. Small branches begin to move.
16-21	5	8.0-10.7	29-38	18-24	Fresh Breeze	Moderate waves, taking a more pronounced long form; many white horses are formed. Chance of some spray	Branches of a moderate size move. Small trees begin to sway.
22-27	6	10.8-13.8	39-49	25-30	strong Breeze	Large waves begin to form; the white foam crests are more extensive everywhere. Probably some spray	Large branches in motion. Whistling heard in overhead wires. Umbrella use becomes difficult. Empty plastic garbage cans tip over.

28-33	7	13.9-17.1	50-61	31-38	Near Gale	Sea heaps up and white foam from breaking waves begins to be blown in streaks along the direction of the wind	Whole trees in motion. Effort needed to walk against the wind. Swaying of skyscrapers may be felt, especially by people on upper floors.
34-40	8	17.2-20.7	62-74	39-46	Gale	Moderately high waves of greater length; edges of crests begin to break into spindrift. The foam is blown in well-marked streaks along the direction of the wind	Twigs broken from trees. Cars veer on road.

Knots	Beaufort	m/s	km/h	mph	Label	Effect on sea	Effects on land
41-47	9	20.8-24.4	75-88	47-54	Severe Gale	High waves. Dense streaks of foam along the direction of the wind. Crests of waves begin to topple, tumble and roll over. Spray may affect visibility	Larger branches break off trees, and some small trees blow over. Construction/temporary signs and barricades blow over. Damage to circus tents and canopies.
48-55	10	24.5-28.4	89-102	55-63	Storm	Very high waves with long over-hanging crests. The resulting foam, in great patches, is blown in dense white streaks along the direction of the wind. On the whole the surface of the sea takes on a white appearance. The 'tumbling' of the sea becomes heavy and shock-like. Visibility affected	Trees are broken off or uprooted, saplings bent and deformed, poorly attached asphalt shingles and shingles in poor condition peel off roofs.
56-63	11	28.5-32.6	103-117	64-73	Violent Storm	Exceptionally high waves (small and medium-size ships might disappear behind the waves). The sea is completely covered with long white patches of foam flying along the direction of the wind. Everywhere the edges of the wave crests are blown into froth. Visibility affected	Widespread vegetation damage. More damage to most roofing surfaces, asphalt tiles that have curled up and/or fractured due to age may break away completely.
64-71	12	>32.7	>118	>74	Hurricane	The air is filled with foam and spray. Sea	Considerable and widespread damage to vegetation, a few windows

completely white with driving spray; visibility very seriously affected	broken, structural damage to mobile homes and poorly constructed sheds and barns. Debris may be hurled about.