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Investigating microbial ecology at solid-air interfaces

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INVESTIGATING MICROBIAL ECOLOGY AT SOLID-AIR INTERFACES

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ABSTRACT

Despite the ubiquity of biofilms in many environments, most microbiological study has focussed on their existence under aqueous conditions. The ecology of bacteria at solid-air interfaces is a significant concern in clinical settings, where prevention of nosocomial infection is a priority. Although multi-species communities are predominant in the environment, most laboratory research regarding bacterial survival at solid-air interfaces has focussed on pure culture survival. Therefore, the focus of this thesis was to determine whether community interactions affect the survival of bacteria after drying on exposed surfaces. It was determined that exogenous pathogens can exhibit enhanced survival after drying in the presence of members of the indoor bacterial flora. The enhanced survival of desiccation-sensitive species in communities is likely dependent on the density of cells on the surface and is thought to involve direct or close contact between community members, allowing poor-survivors to benefit from the desiccation-tolerance mechanisms of others. This project represents an exploratory study into bacterial ecology at solid-air interfaces within indoor environments.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CFU/m^3	Colony forming units per cubic metre
CFU/mL	Colony forming units per millilitre
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substance
gfp	Green fluorescent protein
MID	Minimum infectious dose
MRSA	Methicillin-resistant *Staphylococcus aureus*
OD_{600}	Optical density at 600 nm
PCR	Polymerase chain reaction
PVDF	Polyvinyl difluoride
RAI	Room air isolate
RH	Relative humidity
RNA	Ribonucleic acid
TAE	Tris-acetate-EDTA
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet
VBNC	Viable but nonculturable

CHAPTER 1: INTRODUCTION

1.1 Background

Planktonic and especially biofilm-encapsulated bacterial cells are ubiquitous on earth and have the unique capability of proliferating in a wide range of challenging environments. Some bacterial species are able to survive for extended periods in dry environments with very little moisture. The diversity and significance of bacteria in dry, outdoor environments such as in air, arid soils, and on the surfaces of rocks, statues, monuments, and other structures has been the focus of recent research (Alakomi et al., 2006; Gorbushina and Broughton, 2009; Gorbushina, 2007). However, there is a significant lack of knowledge with regards to microbial ecology at solid-air interfaces within indoor environments. Since the average person is estimated to spend nearly 90% of their lifetime indoors (Klepeis et al., 2001), it is important that we fully understand the behaviour of bacteria in indoor environments, and accurately assess the associated risks to human health.

The prevalence of bacterial cells in indoor air and on exposed surfaces is a significant concern in clinical environments, which are often occupied by individuals in a state of weakened immunity. Nosocomial infections, also known as hospital-acquired infections, develop as a result of a person's visit to a hospital or similar healthcare environment (World Health Organization, 2002). While immune-compromised patients are most susceptible to infection, hospital staff and visitors are also at risk. These infections are a major cause of morbidity and mortality among hospitalized patients, and continue to be a major socio-economic problem in healthcare systems around the world. In Canada alone, it is estimated that one out of every ten patients admitted to a hospital will develop an infection during their stay or shortly after discharge (Office of the Auditor General of Ontario, 2008), resulting in greater than 8000 deaths each year (Zoutman et al., 2003). In addition, the increased length of stay in hospital due to

infection is a financial burden on already cash-strapped healthcare systems, and requires money which would no doubt be better spent to hiring new staff, expanding hospital facilities, and investing in new technologies.

Despite evidence that some pathogens can remain viable for extended periods after drying on surfaces (Hota, 2004; Kramer et al., 2006), the role of the inanimate environment in the transmission of infection remains controversial. While transmission via direct contact between an infected individual and a susceptible host is widely accepted as a major route of transmission, the significance of indirect contact, involving contamination of intermediate non-critical surfaces or objects, is unclear. No universal standard is enforced for cleaning hospital rooms, and practices often vary from one hospital to another (Dancer, 2009). Typically, hospitals are required to clean rooms according to a fixed schedule to keep surfaces "clean and dust-free" (Office of the Auditor General of Ontario, 2008). It must be acknowledged however, that visual inspection of the inanimate hospital environment is likely not sufficient to detect microbial contamination at solid-air interfaces, and that surfaces appearing "clean" can still harbour high numbers of bacterial cells and other microbial agents (Dancer, 2009; Office of the Auditor General of Ontario, 2008). Thus, cells can remain on surfaces even after cleaning if disinfectants or wash-clothes become contaminated during cleaning, or if insufficient contact times are used for disinfectants. As well, microscopic cracks and crevices on hospital surfaces likely provide protective environments where cells can remain viable for extended periods **(**Office of the Auditor General of Ontario, 2008). It is likely then, that the presence of bacterial cells at solid-air interfaces poses a significant risk for cross-contamination and transmission of infection.

Until recently, it was thought that infection was an unavoidable risk of hospitalization

(McCaughey, 2008). It is now clear that the majority of nosocomial infections may be preventable with a better understanding of the fate of pathogens in the hospital environment. If it is accepted that pathogenic cells can contaminate inanimate surfaces and remain viable for extended periods, despite regular cleaning and disinfection, the ecology of microorganisms at these solid-air interfaces deserves a great deal more attention in future studies. Specifically, it is necessary to identify the extrinsic and intrinsic factors that affect bacterial survival in these dry indoor environments.

1.2 Hypothesis and Objectives

In order to control and prevent contamination of solid-air interfaces in high-risk environments such as hospitals and other medical centres, it is important to first understand the factors that influence bacterial survival in these dry environments. Parameters such as the relative humidity in a room and the nutrients available on a surface are known to affect the survival of bacterial cells during dehydration and prolonged desiccation. Some species also possess natural desiccation-tolerance mechanisms which help them remain viable during extended periods on dry surfaces. It was hypothesized that interactions between members of a mixed bacterial community, particularly between desiccation-sensitive and -tolerant species, would affect bacterial survival during dehydration and desiccation. While microbial interactions within aqueous biofilms have been studied extensively, interactions affecting bacterial survival in a dried state at solid-air interfaces have not been adequately addressed by the scientific community. Thus, the overall goal of this study was to investigate bacterial survival on indoor surfaces to determine whether members of the indoor microbial flora can influence the survival of exogenous pathogenic species. This objective was accomplished by achieving the following goals: i) To isolate bacterial species from indoor air which can be used to represent the desiccation-tolerant fraction of the indoor bacterial flora, ii) To develop methods to simulate bacterial deposition onto inanimate surfaces in order to study survival during prolonged periods of desiccation, and iii) To perform experiments investigating the survival of the opportunistic pathogen *Pseudomonas aeruginosa* in the presence of various members of the indoor bacterial flora, in order to determine whether desiccation-sensitive species can benefit from the presence of desiccation-tolerant species in microbial communities at solid-air interfaces.

CHAPTER 2: LITERATURE REVIEW

2.1 Bacteria at solid-air interfaces in the outdoor environment

The Earth's lithosphere is covered by a thin layer of microbial growth. Microorganisms at this boundary exist primarily in communities known as sub-aerial biofilms (Gorbushina and Broughton, 2009). Typical members of sub-aerial biofilms include phototrophs, heterotrophs, and lithotrophs, all of which must be extremely well adapted to harsh terrestrial stresses such as desiccation, temperature fluctuations, nutrient scarcity, and solar radiation (Gorbushina and Broughton, 2009). There is a great deal of research underway regarding the fouling of statues, monuments, and other structures, that results from biofilm formation at the air-solid interface (Alakomi et al., 2006).

Atmospheric activities such as wind and storm events contribute to the ubiquitous nature of sub-aerial biofilms. Winds are capable of eroding rock surfaces with attached microbes, as well as lifting large quantities of arid soil, which can contain as many as a million bacterial cells per gram (Kellogg and Griffin, 2006). These airborne microorganisms can be carried thousands of kilometres from their source at altitudes greater than five kilometres (Kellogg and Griffin, 2006). Inevitably, all airborne particles will be deposited on some air-exposed surface (Gage et al., 1999; Gorbushina and Broughton, 2009). When these airborne microorganisms include pathogens, there can be serious economic, agricultural, and public health concerns, since long-distance dispersal of pathogens is thought to play a significant role in the transmission of both plant and animal diseases (Kellogg and Griffin, 2006). There is a need to investigate how these same principles of bacterial dispersal, deposition, and survival, apply to the indoor environment.

2.2 Bacteria in indoor air

2.2.1 The nature of bacteria in indoor air

Microorganisms mostly exist within indoor as bioaerosol particles. The term 'aerosol' refers to a solid or liquid particle suspended in a gas, and a 'bioaerosol' is an aerosol particle that contains some biological component. If the biological component in a bioaerosol is considered pathogenic, then the whole airborne particle is referred to as an infectious bioaerosol. Bacterial bioaerosols can include cells associated with dust or skin squamae, or cells contained within a liquid droplet (Tang et al., 2006). Droplet bioaerosols can vary greatly in size, from less than 0.001 μm in diameter to well over 100 μm, and are loosely classified based on size (Tang et al., 2006). Particles with a diameter greater than 60 μm are considered 'large droplets', while 'small droplets' have a diameter less than 60 μm. A third class of airborne particles, referred to as 'droplet nuclei', are typically less than 10 μm in diameter. Droplet nuclei are generated when airborne droplets evaporate rapidly upon aerosolization, resulting in a dried aerosol residue that can behave similarly to a smoke particle in indoor air (Tang et al., 2006).

2.2.2 Laboratory study of airborne bacteria

Bacteria in the airborne state are typically studied through the use of aerosol chambers. Aerosol chambers can vary greatly in size and mode of operation, but usually employ a nebulizer to aerosolize or atomize a bacterial suspension, which is injected into the chamber. The chamber air may be sampled using impingers, which draw specific volumes of air into liquid broth, permitting further investigation of sampled particles. Aerosol chambers may also employ some sort of sterilization technique, such as an installed UV lamp (Heidelberg, 1997; Levin, 1996).

Wilkinson (1966) investigated the survival ability of several bacterial strains on metal surfaces deposited via aerosolization. In this study, it was determined that aerosol deposition

could result in an appreciable and replicable number of bacteria on a surface, such that population survival could be assessed over extended periods (Wilkinson, 1966). More recently, Robine et al. (1998) constructed an aerobiocontamination system to simulate aerocontamination of surfaces, and has subsequently used it to study the survival of several bacterial aerosols after deposition onto inert surfaces (Robine et al., 2000).

Until a study by Goldberg et al. (1958) most of the research regarding bacterial aerosols focussed on transient, short-lived aerosols due to the difficulty in overcoming the physical loss of cells due to gravity. In order to overcome this problem, a 'dynamic aerosol toroid' was designed, which holds aerosols in a large rotating drum, maintaining cells in the airborne state for up to 2 days (Goldberg et al., 1958).

2.2.3 Generation of bioaerosols in clinical environments

With the exception of fungal spores, infectious bioaerosols are usually generated within hospitals by patients, staff, and visitors (Beggs, 2003). They are typically dispersed through activities such as sneezing, coughing, and even talking, in addition to routine procedures involving infected patients. The average sneeze has been shown to expel as many as 40,000 aerosol particles ranging from 10-100 μm in diameter, of which a significant number may be infectious bioaerosols, particularly if the individual is suffering from a respiratory infection (Tang et al., 2006). These particles, which can rapidly evaporate to droplet nuclei depending on their initial size and the indoor relative humidity, can be ejected several metres from the source at speeds greater than 100 metres per second (Fig. 2.1) (Tang et al., 2006; Beggs, 2003). Likewise, a cough can generate as many as 3,000 droplet nuclei, as would five minutes of normal speech (Tang et al., 2006). Biofilms within sink drains are also thought to contribute potentially infectious bioaerosols into the air, which may subsequently infect nearby patients (Hota et al.,

2009).

The exact mechanisms involved in airborne transmission of infection remain poorly understood. This is due in part to the many technical challenges involved in establishing quantitative estimates of the aerosolization process itself, such as the number of particles dispersed during an aerosolization event (Hermann et al., 2009). Additionally, difficulty arises in trying to account for the many factors involved in the dissemination of the aerosol particles, such as droplet size and the influence of air currents on the movement of these particles. Even if the aforementioned parameters are determined, their significance may be underestimated unless the probability of infection as a function of exposure dose can be ascertained (Hermann et al., 2009).

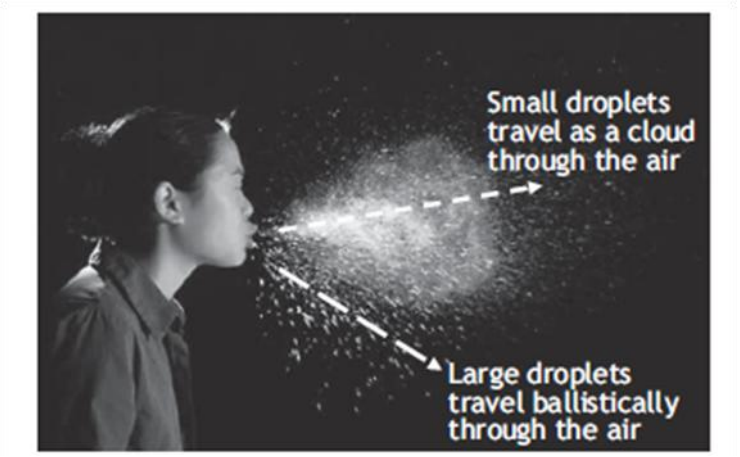


Fig. 2.1 Bioaerosol generation associated with sneezing. A typical sneeze produces roughly 40 000 particles, ejected at speeds in excess of 100 m/s. Airborne particles may land several metres from the source, contaminating the immediate environment, or they may evaporate rapidly to droplet nuclei and remain airborne indefinitely (Tang et al., 2006).

2.2.4 Microbial load of hospital air

According to Holton and Ridgeway (1993), the airborne microbial load in an empty operating room should be less than 35 bacteria-carrying particles per cubic metre of air. These particles include skin cells, hair, dust, and droplet-nuclei. When an operating room is occupied and in use, there should be no more than 180 bacterial colony forming units per cubic metre (CFU/m^3) (Holton and Ridgeway, 1993).

Despite several decades of study, there are only a small number of comprehensive and complete air sample surveys for hospital air (Beggs, 2003). The most thorough reviews date

back to the 1960s, although these studies rarely acknowledge the nonculturable fraction of cells in the air. In one of these studies, the microbial load of various hospital rooms and corridors was investigated. The average bacterial load within hospital air was determined to range between 350 CFU/m^3 and 700 CFU/m^3 with the highest counts occurring in laundry rooms and the lowest counts occurring in the operating and delivery rooms (Greene et al., 1960). In a subsequent study by Greene et al. (1962), a total of 9,938 microbial isolates were obtained from hospital air and identified based on colonial and microscopic morphology as well as various physiological tests. Approximately 40% of isolates were identified as Gram-positive cocci, almost 20 percent were Gram-positive rods and only 14% were Gram-negative rods (Table 2.1). Interestingly, the community distribution of airborne microorganisms in hospital air was not affected by seasonal changes, as is usually the case in outdoor air (Greene et al., 1962).

In a recent review article, Beggs outlined a number of pathogens that likely contribute to nosocomial infection through airborne transmission (Beggs, 2003). Gram-positive pathogens such as *Staphylococcus aureus* and specifically methicillin-resistant *S. aureus* (MRSA) are now believed to contaminate the air surrounding infected patients (Boyce et al., 1997). As well, coagulase-negative *Staphylococci* such as *S. epidermidis*, *S. hominis*, *S. simulans*, and *S. haemolyticus* are suspected to remain viable in the air

Table 2.1: Types of microbes isolated from air at two hospital environments (Greene et al., 1962).

Organisms	Hospital A	Hospital B	Total
Total no. of isolates	6,587	3,081	9,938
Gram-positive cocci (hemolytic)	14.7%	11.9%	13.8%
Gram-positive cocci (non-hemolytic)	27.6%	31.3%	28.8%
Gram-positive rods	17.0%	23.8%	19.2%
Gram-negative rods	15.5%	10.4%	14.0%
Coccobacilli, diptheroids, and others	3.6%	4.4%	3.8%
Penicillin-resistant bacteria	16.6%	10.6%	15.4%
Moulds	18.0%	15.1%	17.1%
Yeasts	1.1%	1.5%	1.2%
Actinomycetes	2.5%	1.7%	2.2%

and cause infection after inhalation (Lidwell et al., 1982). *Mycobacterium tuberculosis* is a Gram-positive pathogen responsible for the spread of tuberculosis. Its transmission through the air is well-known and occurs after inhalation of droplet nuclei containing *M. tuberculosis* cells (Beggs, 2003).

Although many Gram-negative species are poor survivors in the airborne state, some have shown the ability to remain viable in the air for extended periods. Specifically, *Acinetobacter baumannii* can lead to infections of the respiratory and circulatory systems due to its presence in the air (Bernards et al., 1998). The pathogenic bacteria *Acinetobacter anitratus* can also be isolated from indoor air (Allen and Green, 1987). *Legionella pneumophila*, the pathogen responsible for causing Legionnaire's disease, usually dwells within water systems. In hospitals however, these pathogens can often be found in the air as a result of dispersal from drain biofilms (Beggs, 2003). It is unclear whether *Pseudomonas aeruginosa* infections can be transmitted through the air, but *P. aeruginosa* has been isolated from air near infected patients (Beggs, 2003). The persistence of *P. aeruginosa* in the airborne state likely requires cells to be maintained within liquid droplets, since this bacterium is generally thought to be susceptible to air drying.

2.2.5 Viable but nonculturable bacteria in indoor air

An increasing number of studies have identified the possibility for bacterial cells to enter a metabolically inactive state during periods of environmental stress, while maintaining some degree of viability (Kell et al., 1998; Oliver, 2005; Oliver, 2010). This phenomenon is referred to as the viable but nonculturable state (VBNC) of bacteria. Typically, enumeration of airborne bacteria is reliant on cultivation-based methods, so it is believed that the overall bacterial concentration of indoor air is likely underestimated. Heidelberg et al. (1997) investigated the

effect of aerosolization on the viability and culturability of several clinically relevant bacterial species and concluded that less than 10 percent of aerosolized bacteria were capable of forming visible colonies on appropriate media. More recent publications have estimated the proportion of culturable airborne bacteria to be less than one percent of the total microbial load in the air (Beggs, 2003). Therefore, it is recommend that any reliable air quality assessment should employ viable counting methods using a combination of live/dead staining and microscopy, rather than relying strictly on culture-based techniques (Heidelberg et al., 1997).

Shahamat et al. (1997) evaluated different media formulations in an effort to maximize culturability of bacteria isolated from the airborne state. Twelve different bacterial species were tested on a total of 120 media combinations. The authors concluded that only a small portion of the total airborne bacteria can be cultivated, and they identified brain heart infusion agar (with horse serum), Mueller Hinton agar, and tryptic soy agar (TSA) as the most effective media for the recovery of bacteria from the airborne state (Shahamat et al., 1997).

2.3 Bacteria at solid-air interfaces in the indoor environment

2.3.1 Deposition of cells from indoor air onto exposed surfaces

The movement of airborne particles within indoor air depends almost entirely on the size of the particle itself and the airflow patterns within the room (Tang et al., 2006). The large surface area with respect to the small mass of most airborne droplets causes them to lose water rapidly due to evaporation (Table 2.2) (Beggs, 2003). Since the size of an airborne droplet can change with time, prediction of its behaviour in the airborne state is very difficult, although it is accepted that much of the bacterial burden in the air will eventually deposit onto exposed surfaces during periods of reduced airflow (Gage et al., 1999; Gorbushina and Broughton, 2009).

Table 2.2: Time required for aerosol droplets to evaporate and become droplet nuclei (Beggs, n.d.).

Droplet Diameter (µm)	Evaporation Time (seconds)	Distance droplet will fall before evaporation (m)
200	5.2	6.51
100	1.3	0.42
50	0.31	0.0255
25	0.08	1.59×10^{-3}
12	0.02	8.4×10^{-5}

In a study by Knight (1980), the settling time (time required to land from a particular height) of particles of various sizes was observed. It was determined that large particles of 100 µm in diameter take only ten seconds to fall three metres, while 20 µm particles take up to four minutes to settle from the same height. Particles 10 µm in diameter can remain airborne for up to seventeen minutes, while small particles less than 3 µm may remain airborne for an indefinite length of time before settling (Knight, 1980).

Short-range transport and subsequent deposition of microorganisms depends heavily on airflow patterns, temperature, and relative humidity. With the exception of wind entering through open windows or doors, indoor airflow patterns are usually not caused by atmospheric activity. Rather, ventilation systems, opening and closing doors, and even the movement of people through a room, can generate unique airflows capable of transporting microorganisms through the indoor environment, before depositing them in new locations (Fig. 2.2) (Tang et al., 2006). In an earlier study, Tang et al. (2005) determined that even in a negative pressure room, the opening of a door can cause a significant volume of air to be transported out through the doorway due to a temporary inversion of the negative pressure gradient. Thus, in the time it takes an infectious bioaerosol particle to settle onto an exposed surface, it may be carried throughout the indoor environment and colonize new surfaces.

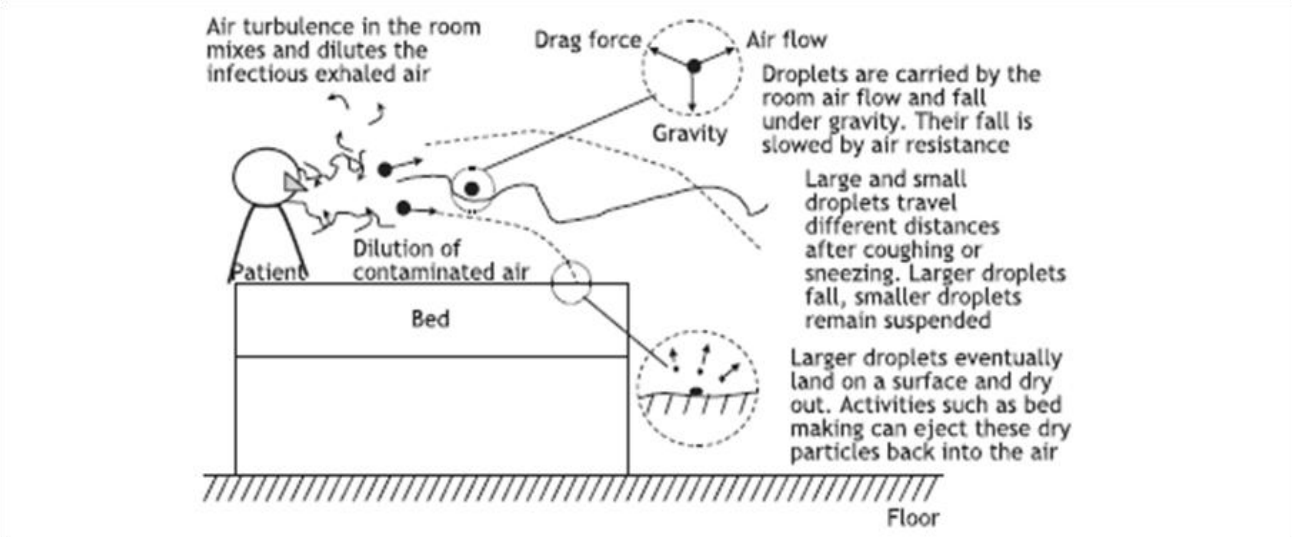


Fig. 2.2: Mechanics of aerosol droplet dispersal from an infected patient. There is likely a dynamic exchange of cells between air and exposed surfaces (Tang et al., 2006).

2.3.2 Surfaces and cleaning strategies in the clinical environment

Surfaces in the clinical environment are generally grouped into one of three categories (critical, semi-critical, and non-critical) based on the perceived likelihood of acquiring an infection via contact with each surface (Rutala and Weber, 2001). This classification system was first proposed by Dr. E.H. Spaulding in the 1970s and is still considered an important tool for dictating appropriate surface disinfection or sterilization techniques. Surfaces that regularly come into contact with normally sterile areas of the body are considered a high risk for transmitting infection, and are therefore classified as 'critical surfaces'. Due to this perceived risk, critical surfaces such as some medical instruments (endoscopes, scalpels, etc.) receive high level disinfection (Rutala and Weber, 2001). 'Semi-critical surfaces' are thought to pose a moderate risk for infection transmission, since they only come into contact with mucous membranes of the body. For this reason, they may not receive the same degree of disinfection as critical surfaces, but still must be deemed sterile prior to use. Examples of semi-critical surfaces include medical instruments such as laryngoscope blades, breast pumps, and endotracheal tubes (Rutala and Weber, 2001). 'Non-critical surfaces' such as floors, walls, furniture, and various

medical equipment, only come into contact with intact skin and have therefore traditionally been thought to pose minor risk for transmitting infection. As a result, most hospital room cleaning measures only involve low-level disinfection or soap-and-water treatment of non-critical surfaces (Rutala and Weber, 2001). Several recent publications however, have highlighted the ability for non-critical surfaces to act as long-term pathogen reservoirs (Hota, 2004; Kramer et al., 2006).

Simple 'cleaning' in the clinical context, is the process of removing foreign material from objects and surfaces. A non-critical surface is generally considered 'clean' if it is visibly clear of dust, dirt, and other organic material (Office of the Auditor General of Ontario, 2008). However, there is a lack of scientific standards to assess the effectiveness of various cleaners and disinfectants, and to accurately measure surface cleanliness (Dancer, 2009). Visual inspection of the inanimate hospital environment is likely not sufficient to detect microbial contamination, and surfaces that appear 'clean' can still harbour high numbers of bacterial cells and other microbial agents (Dancer, 2009; Office of the Auditor General of Ontario, 2008). Bacterial cells may remain on surfaces even after disinfection if the cleaning solution or wash cloth becomes contaminated or if insufficient contact times are used for particular disinfectants (Office of the Auditor General of Ontario, 2008; Rutala and Weber, 2001). Microscopic cracks and crevices in non-critical surfaces likely also provide environments where cells are protected from the disinfectants or shielded from mechanical scrubbing (Office of the Auditor General of Ontario, 2008). Also noteworthy is the fact that elevated surfaces which are out of reach of cleaning staff may not receive any cleaning at all.

2.3.3 Clinical surfaces as reservoirs for nosocomial pathogens

The role of inanimate surfaces in the transmission of nosocomial infection is controversial. In a systematic review of the literature, Kramer et al. (2006) highlighted the fact

that many known pathogens can remain viable for extended periods on inanimate surfaces (Table 2.3). It is thought that pathogens persisting on surfaces in the clinical environment may provide a continuous source of infection through direct transmission from surfaces to susceptible patients or via the hands of healthcare workers and other personnel within hospitals (Fig. 2.3). However, as Hota (2004) explains, it is difficult to determine whether the bacterial florae on clinical surfaces are "innocent bystanders" or potential sources of infection.

Aside from objects in direct contact with patients such as blood pressure cuffs, bed linen, and patient gowns, other surfaces within the hospital environment are at high risk for contamination. These can include floors, walls, washbasins, furniture, and overhead tables (Ayliffe et al., 1967; Boyce et al., 1997). It is important to note however, that the presence of a pathogen in the inanimate hospital environment is not necessarily indicative of a causal role in disease transmission (Beggs, 2003).

Table 2.3: Experimentally observed persistence of clinically relevant bacteria on inanimate surfaces (Kramer et al., 2006).

Bacterium	Duration of persistence (range)	Bacterium	Duration of persistence (range)
Acinetobacter spp.	3 days to 5 months	*Mycobacterium bovis*	> 2 months
Bordetella pertussis	3 – 5 days	*Mycobacterium tuberculosis*	1 day – 4 months
Campylobacter jejuni	up to 6 days	*Neisseria gonorrhoeae*	1 – 3 days
Clostridium difficile (spores	5 months	*Proteus vulgaris*	1 – 2 days
Chlamydia pneumoniae, C. Trachomatis	≤ 30 hours	*Pseudomonas aeruginosa*	6 hours – 16 months; on dry floor: 5 weeks
Chlamydia psittaci	15 days	*Salmonella typhi*	6 hours – 4 weeks
Corynebacterium diphtheriae	7 days – 6 months	*Salmonella typhimurium*	10 days – 4.2 years
Corynebacterium pseudotuberculosis	1–8 days	*Salmonella* spp.	1 day
Escherichia coli	1.5 hours – 16 months	*Serratia marcescens*	3 days – 2 months; on dry floor: 5 weeks
Enterococcus spp. including VRE and VSE	5 days – 4 months	*Shigella* spp.	2 days – 5 months
Haemophilus influenzae	12 days	*Staphylococcus aureus, including MRSA*	7 days – 7 months
Helicobacter pylori	≤ 90 minutes	*Streptococcus pneumoniae*	1 – 20 days
Klebsiella spp.	2 hours to > 30 months	*Streptococcus pyogenes*	3 days – 6.5 months
Listeria spp.	1 day – months	*Vibrio cholerae*	1 – 7 days

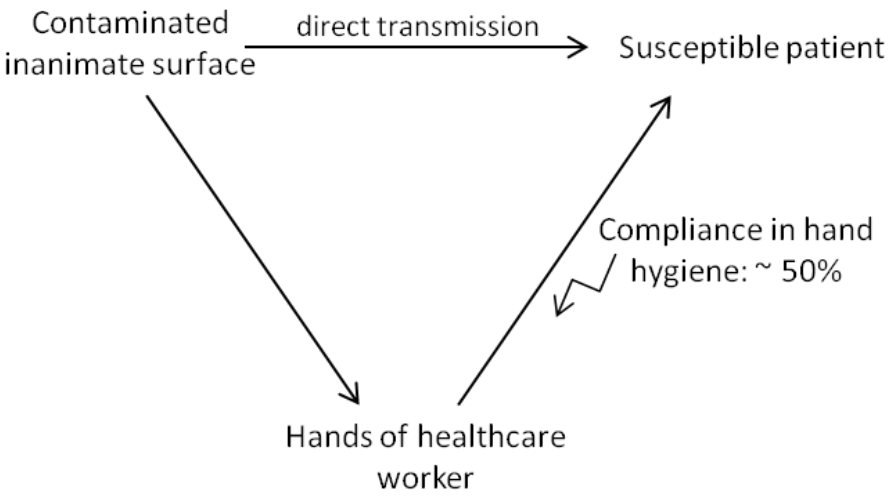


Figure 2.3: Possible modes of infection transmission from inanimate surfaces to patients (Kramer et al., 2006).

Some studies suggest that non-critical surfaces such as floors, maintain a baseline in regards to microbial load. Ayliffe et al. (1967) suggested that the removal of microorganisms from floors via regular cleaning and disinfection is balanced by the deposition of microbes from the air, as well as recontamination via shoes and trolley wheels. In this study, the authors concluded that daily disinfection of floors actually contributed little to the bacteriological cleanliness of the floor (Ayliffe et al., 1967).

Boyce et al. (1997) investigated the possible role of contaminated environmental surfaces as reservoirs for MRSA, and found that surface contamination of MRSA occurred in 73 percent of the rooms of MRSA-infected patients, and 69 percent of the rooms of MRSA-colonised patients. Interestingly, the uniforms and gloves of a significant number of hospital staff who had no direct contact with infected patients were contaminated. It was concluded that this cross-contamination likely occurred when staff members came into contact with contaminated surfaces within patient rooms (Boyce et al., 1997).

C. difficile is a common nosocomial pathogen that has been shown to be resistant to

standard cleaning and can remain viable on hospital surfaces such as commodes, bedpans, blood pressure cuffs, walls, floors, washbasins, and furniture, for up to five months (Hota, 2004). Although most Gram-negative bacilli are generally thought to lose viability after drying on surfaces, *Acinetobacter baumannii* has been isolated from inanimate reservoirs in hospital environments surrounding infected patients (Kramer et al., 2006). Some strains of *S. aureus*, including MRSA are able to remain viable on dry inanimate surfaces for up to nine weeks (Hota, 2004).

2.4 Survival of bacteria after drying on inanimate surfaces

The vast majority of living organisms are killed or irreversibly damaged when dried to equilibrium with air (Alpert, 2005). However, a small percentage of animals, plants, and bacteria are able to remain viable despite sustained exposure to extremely dry conditions. The availability of water is considered one of the most important evolutionary pressures, since a tolerance to desiccation is an obvious evolutionary advantage (Alpert, 2005). Research is underway investigating the mechanisms involved in desiccation tolerance, and the ability of desiccation-tolerant microorganisms to trigger nosocomial outbreaks by surviving in dry indoor environments. There is also ongoing work studying the possibility of inducing desiccation tolerance among traditionally sensitive species.

2.4.1 Desiccation-tolerance in bacteria

The ability of some bacterial species to withstand the drying process and remain viable at solid-air interfaces is dependent on a variety of factors. Extrinsic factors such as the availability of nutrients in the form of blood, sugars, serum, and complex media affect the viability of cells during and after dehydration (Potts, 1994). The nature (material, charge, etc.) of the substrate upon which cells are dried also affects survival, as very different survival times may be seen for

the same species on two different materials. The mode of drying (rapid vs. slow, active vs. passive), the inoculum size, and growth phase of cells during dehydration and desiccation are also considered important factors dictating bacterial survival (Billi and Potts, 2002). Equally important is the amount of moisture available to cells during periods of dehydration. Cells dried onto surfaces may receive moisture via condensation, the occasional spill, regular cleaning with aqueous solutions, or in the form of humidity in the air.

Relative humidity (RH) is the amount of water vapour in the air and is expressed as the ratio of actual partial vapour pressure of water to the saturation vapour pressure at the temperature in question (Bauermeister et al., 2011). Several studies have been carried out in an effort to assess the influence of RH on bacterial survival in the dried state (Bauermeister et al., 2011; Turner and Salmonsen, 1973; Walters et al., 2005). While it seems that the effect of RH on bacterial survival is very much dependent on the species in question, a general trend of good survival at low and mid RH, and poor survival at high RH was observed for several stress-tolerant species.

Some bacterial species possess intrinsic tolerance mechanisms that allow cells to maintain viability during prolonged periods of desiccation. The average bacterial cell has an internal water content of approximately 70% or 0.7 g water/g dry weight (Potts, 1994). Desiccation-sensitive cells typically die when their cellular water content reaches 0.3 g water/g dry weight, however, a small number of bacterial species considered tolerant to desiccation can survive when their internal water content reaches below 0.1 g water/g dry weight (Billi and Potts, 2002). Through experimental analysis, it has been determined that drying cells to equilibrium with air at 40% relative humidity results in an internal water content of roughly 0.1 g water/g dry weight, whereas drying cells in air with 30% relative humidity causes cells to reach an internal

water content of approximately 0.03 g water/g dry weight (Potts, 1994).

The mechanisms involved in desiccation-tolerance among bacteria remain somewhat unclear, though they are thought to involve both cellular protection and damage repair systems (Billi and Potts, 2002). It is difficult to study desiccation-induced mechanisms, since desiccation is often superimposed on a host of other stressors such as UV radiation, temperature fluctuations, osmotic shock, and nutrient scarcities (Bauermeister et al., 2011). One of the major theories with respect to desiccation-tolerance is the water replacement hypothesis. It has been determined experimentally that desiccated cells can accumulate a great deal of trehalose and sucrose. In some instances, these disaccharides can comprise more than 20% of the dry weight during desiccation (Potts, 1994). The water replacement hypothesis suggests that trehalose and other polyhydroxyl compounds are able to replace the layer of water around macromolecules, thus preventing desiccation-induced damage. As a result, it is believed that cells respond to dehydration by directing a large portion of energy and nutrients towards polysaccharide production (Potts, 1994). The presence of extracellular polymeric substance (EPS), which is composed of many polysaccharides, including trehalose, is also thought to protect cells from desiccation by providing a matrix for various water-stress proteins and polysaccharides (Billi and Potts, 2002). While some bacterial species have the ability to form endospores which can withstand partial desiccation, these structures are not considered truly anhydrobiotic, since the internal water content of spores is usually 21-58% (Billi and Potts, 2002).

2.4.2 Survival of *Arthrobacter* spp.

Arthrobacter spp. are a major member of most soil communities (Mongodin, 2006), but have also been isolated from other dry environments such as indoor air (Krysinska-Traczyk et al., 2002; Li et al., 2004). Although these species are not thought to be pathogenic, isolation of

Arthrobacter spp. from human specimens has occurred (Funke et al., 1996), and they have been implicated in various skin and eye infections (Bodaghi et al., 1998; Imirizalioglu et al., 2010). These species are taxonomically clustered with Micrococcaceae as they are all high-GC Gram positive bacteria (Mongodin, 2006). The relative ubiquity of *Arthrobacter* spp. in both the natural and artificial environment is likely due to its ability to remain viable during long periods of stress, which may include desiccation, nutrient starvation, temperature fluctuations, and ionizing radiation (Cacciari and Billi, 1987; Mongodin, 2006). Interestingly, most *Arthrobacter* spp. can switch between two distinct cell shapes during their life cycle (Boylen, 1973). Cells from younger cultures usually appear rod-shaped, while older cells appear as small cocci (Mongodin, 2006). The cocci form is thought to be the more stable of the two, as it is more frequently isolated from dry environments (Boylen, 1973). The exact mechanisms used by *Arthrobacter* spp. to tolerate desiccation are not fully understood, but are thought to involve EPS accumulation and trehalose production, since many species of the *Arthrobacter* genus are known to possess trehalose synthesis pathways (Higo et al., 2006; Maruta et al., 1995; Mongodin et al., 2006; Narvaez-Reinaldo et al., 2010). In addition, trehalose production has been observed experimentally when *Arthrobacter* spp. was exposed to osmotic stress and nutrient starvation.

2.4.3 Desiccation-induced cell damage in non-tolerant species

There are a variety of reasons why most cells are sensitive to desiccation. Water is a critical component of all cells, comprising roughly 70% of the cell mass (Potts, 1994). It is the major component of the cytosol, periplasm, and capsule, and confers structural order on cells (Potts, 1994). Water is also critical for the proper function of reaction mechanisms and contributes to the stability of proteins, DNA, and lipids (Potts, 1994). In a truly desiccated environment, there is not even enough moisture to form a monolayer around macromolecules

(Billi and Potts, 2002). As a result, proteins undergo conformational changes causing them to malfunction, triggering an accumulation of free radicals, which in turn can cause further protein denaturation, lipid peroxidation, and DNA mutation (Billi and Potts, 2002). Phenotypic changes of the bacterial community can also occur as a result of air drying. Changes such as an increase in surface area, colony colour, texture, and size, may occur after prolonged desiccation (Potts et al., 2005). Individual cells may also experience shrinkage of their capsular layers, concentration of intracellular salt, crowding of macromolecules, changes in surface tension, and increased viscosity (Potts, 1994) (Table 2.4).

Table 2.4: Responses to cellular dehydration (Potts, 1994).

Level of Effect	Response
Community	change (usually increase) in surface area
	shrinkage
	salt precipitation
	change in texture
	change in shape
	change in colour (oxidation of pigments)
Cell	shrinkage of capsular layers
	increase in intracellular salt levels
	crowding of macromolecules
	changes in volumes of cell compartments
	changes in biophysical properties (eg. surface tension)
	reduced fluidity (increased viscosity)
	damage to external layers (eg. pili, membranes)
	change in physiological processes (eg. growth arrest)

2.4.4 Survival of *P. aeruginosa*

P. aeruginosa is a common nosocomial pathogen that is one of the leading causes of morbidity and mortality among patients with cystic fibrosis (Cystic Fibrosis Trust, 2004; Lanini et al., 2011; Stover et al., 2000). Direct transmission of *P. aeruginosa* from one patient to another is known to occur, but it is also possible that the inanimate hospital environment represents an important source of cross-contamination. *P. aeruginosa* is relatively ubiquitous in moist reservoirs within hospitals (Panagea et al., 2005). Hota et al. (2009) identified sink drain biofilms containing *P. aeruginosa* as the major source of transmission during a large outbreak. Lanini et al. (2011) found that a contaminated triclosan soap dispenser was the main source of *P. aeruginosa* infection during an outbreak in a hematology unit. Outbreak of *P. aeruginosa* infections have also been linked to contaminated sanitary equipment and surface cleaning equipment (Englehart et al., 2002), contaminated bronchoscopes (Silva et al., 2003)**,** hand-lotion (Becks and Lorenzoni, 1995), moist mouth swabs (Iverson et al., 2007), and nebulizers used to treat patients with chronic obstructive airways disease (Cobben et al., 1996).

2.4.5 Laboratory study of *P. aeruginosa* survival during desiccation on surfaces

The ability of *P. aeruginosa* to survive on dry surfaces has been the subject of many studies, although these investigations often present conflicting reports regarding the susceptibility of *P. aeruginosa* to dehydration and desiccation. Although it is generally thought that *P. aeruginosa* is sensitive to desiccation and loses viability rapidly upon drying, the disparity among experimentally observed survival times is likely because *P. aeruginosa* is highly dependent on ideal combinations of environmental factors for long-term survival.

In an early study, Stephens (1957) tested the ability of a range of solutes to keep *P. aeruginosa* viable after drying, and found that a combination of casein, sucrose, and granular

mucin resulted in the best survival after 24 hours. Skaliy and Eagon (1972) studied the survival of *P. aeruginosa* OSU 64, as well as several other *P. aeruginosa* strains isolated from patients and the hospital environment. After drying these strains on glass and maintaining them at 53% relative humidity, they found that the age of the culture prior to inoculation played a significant role in survival ability. More specifically, they observed the longest survival time (3 days) for cells taken from a seven day culture, and found no significant difference in survival between the isolated strains and the laboratory strain OSU 64. These findings do not agree with the previous conclusion of Pettit and Lowbury (1968) who found that patient-isolated strains had a lower percent survival after 2 hours of drying compared to isolates from the inanimate environment. In this study however, all strains exhibited poor survival after 24 hours on a glass substrate at ambient relative humidity (35-50%).

McDade and Hall (1964) tested survival of a clinically isolated *P. aeruginosa* strain, suspended in tryptic soy broth, and dried onto glass, ceramic, and stainless steel, at relative humidity values of 11, 53, and 85%. They found that survival was best (up to 7 days) at 11% humidity, but observed near complete die-off within 24 hours when exposed to 53 and 85% humidity. No apparent difference in survival was noted between the different substrates. Zimakoff et al. (1983) also studied the survival of clinically isolated *P. aeruginosa* strains, specifically looking at survival on a polyvinyl substrate at 60% humidity. They found that best survival (more than 5 days) occurred when cells were suspended in sputum before drying, while a rapid die-off occurred within three hours when cells were suspended in saline solution. Scott and Bloomfield (1990) looked at survival of *P. aeruginosa* NCTC 6749 on a laminate substrate at 40-45% relative humidity, and compared survival in 'clean conditions' (cells suspended in distilled water) with 'soiled conditions' (cells suspended in tryptic soy broth). They found that

while both treatments resulted in a rapid die-off, a small number of cells were still recoverable after 24 hours from the 'soiled' surfaces. Hirai (1991) found that no cells survived past seven hours when *P. aeruginosa* IID 1042 was suspended in distilled water and dried onto a glass substrate at 50% humidity. Panagea et al. (2005) studied survival of several clinical isolates as well as the lab strain *P. aeruginosa* PAO1, suspended in deionized water and dried onto a plastic substrate at 35-50% humidity. They observed a considerable reduction in cell number within one hour (2.5-4.5 log), but did find that low numbers were detectable for most of the test strains after 48 hours (Panagea et al., 2005).

It seems as though survival of *P. aeruginosa* cells is also dependent on the method with which they are dried. Fuster-Valls et al., (2008) found that slow air-drying (exposure to ambient environmental conditions for 72 hours) was less harmful to *P. aeruginosa* ATCC 15442 cells than rapid air-drying (exposure to safety-hood airflow for one hour) when inoculated and dried onto stainless-steel. The substrate on which the cells are dried was also shown by Yazgi et al. (2009) to play a role in *P. aeruginosa* survival. They looked at the survival of *P. aeruginosa* ATCC 27853 on a variety of substrates maintained at 50% humidity. Cells suspended and dried in saline solution were still detectable after 6 days on an inox sheet, after 11 days on a vinyl substrate, after 25 days on a laminate substrate, and after 30 days on a ceramic substrate (Yazgi et al., 2009).

Overall, the ability of *P. aeruginosa* to remain viable after drying to equilibrium with air seems to vary greatly depending on a variety of factors. Some of the studies regarding *P. aeruginosa* survival report a rapid loss of viability almost immediately upon drying, while others show extended survival of several days or weeks. These apparent discrepancies are likely due to the different parameters tested in each experiment, such as the strain itself (patient or

environmental isolate vs. laboratory strains), the suspending medium, the nature of the substrate upon which cells are dried, and the relative humidity. These conflicting data highlight the need for a more thorough understanding of the fate of *P. aeruginosa* in the clinical environment in order to accurately assess the role of the dry surfaces in the proliferation and transmission of *P. aeruginosa*.

2.5 Minimum infectious doses of nosocomial pathogens

Minimum infectious dose (MID) refers to the minimum number of cells required to trigger the first signs of an infection (World Health Organization, 2009). Theoretically, if a single pathogenic cell is placed in an ideal environment for growth, it is capable of replicating to reach an infective concentration (Bolashikov and Melikov, 2009). Thus, the persistence of even low cell numbers in the inanimate hospital environment may represent a significant risk for infection transmission, especially among immuno-compromised individuals.

A variety of factors influence the pathogenicity of microorganisms, and it is therefore difficult to achieve accurate estimates of MID for many pathogens. The inability to carry out controlled human experiments also makes accurate assessment extremely complicated (Bolashikov and Melikov, 2009). In addition, the state of the host's immune system is one of the major factors dictating susceptibility to infection, so a MID value determined for one individual is likely not very applicable to another. The route of infection also influences the minimum cell numbers required to trigger infection, as infection via inhalation usually requires a lower MID than infection via ingestion (Cafruny and Hovinen, 1988).

Despite the difficulties encountered in accurately determining the MID for various bacterial species, rough estimates do exist for a number of common nosocomial pathogens. The MID can vary greatly depending on the infecting pathogen, ranging from a single cell for *M.*

tuberculosis, to greater than 10^7 cells for many strains of *S. aureus* (Schaad, 1983). For some infections associated with *P. aeruginosa*, the MID is thought to be approximately 10^8-10^9 CFU for healthy individuals, although the infective dose for patients with compromised immune systems is unknown and likely much lower (Sacchetti et al., 2009). For enterohemorrhagic and enteroinvasive strains of *Escherichia coli*, the MID may be as low as 10 cells (Public Health Agency of Canada., 2011). Infections caused by *Enterobacter* spp. may require only 10^3 cells to cause infection, while infections caused by *Shigella* spp. may require only 10-100 cells (Public Health Agency of Canada., 2011). For infections with *Klebsiella, Serratia* spp., and other enterobacteriaceae, the MID is likely greater than 10^4 cells (World Health Organization, 2009). The infectious dose of *Clostridium difficile* in humans is not known, however it is likely very low, since as few as two cells were shown to cause infection in hamsters (Larson and Borriello, 1990).

2.6 Future considerations for studying bacterial ecology and epidemiology at solid-air interfaces

Nosocomial infections continue to be a significant burden on healthcare systems worldwide. Effective treatment and prevention strategies for these infections require a thorough understanding of the causes and modes of transmission during an outbreak. It is possible that non-critical surfaces in clinical environments have been underestimated in terms of their role in the transmission of infection. In order to determine how surfaces affect pathogen epidemiology, it is necessary to first gain a more thorough understanding of bacterial ecology at these solid-air interfaces. Virtually all of the researching regarding bacterial survival on inanimate surfaces has focussed on the survival of pure culture cells, despite the predominance of mixed communities in both indoor and outdoor environments. Thus, there is a need for future studies to investigate

bacterial survival in communities, and to assess whether interactions among community members can affect the survival ability of individual members.

CHAPTER 3: INVESTIGATING THE SURVIVAL OF MIXED BACTERIAL COMMUNITIES AT SOLID-AIR INTERFACES

3.1 Introduction

Despite several decades of ongoing debate, the role of the inanimate hospital environment in the transmission of nosocomial infection remains unclear (Allerberger et al., 2002; Ayliffe et al., 1967; Hota, 2004; Rutala and Weber, 2001). Nonetheless, preventing bacterial contamination and colonization on dry surfaces remains a concern not just in clinical settings, but also in high risk areas such as manufacturing clean rooms and food processing plants. In a review article, Tang et al. (2006) summarized a range of studies addressing the ability for bacterial cells and other infectious agents to remain viable for extended periods in indoor air, highlighting the fact that small bioaerosol particles can spread throughout the indoor environment due to airflows. It is generally accepted that much of the bacterial burden within air will eventually be deposited onto some air-exposed surface (Gage et al., 1999; Gorbushina and Broughton, 2009). While much of the bacterial burden in indoor air is considered non-pathogenic, there is a concern with regards to the fate of these cells after deposition. If cells remain viable for extended periods after depositing on surfaces, then they may provide an environment for enhanced survival of exogenous pathogenic species. Several recent publications have emphasized the ability of potentially pathogenic microorganisms to remain viable for extended periods on dry surfaces (Hota, 2004; Kramer et al., 2006).

Extended bacterial survival under desiccated conditions depends on a variety of factors. Many Gram-positive species, such as *Arthrobacter* spp., *Bacillus* spp., *Micrococcus* spp., and *Staphylococcus* spp., are thought to possess a natural tolerance to desiccation, as they are commonly isolated from dry indoor environments such as operating rooms, clean rooms, factories, and dental offices (Chaibenjawong and Foster, 2011; Murayama et al., 2010;

Krysinska-Traczyk et al., 2002; Wan et al., 2011; Wu and Liu, 2007). Although the exact mechanisms involved in prokaryotic desiccation tolerance are not fully understood, some publications (Alpert, 2005; Billi and Potts, 2002) have suggested that their tolerance to desiccation might involve both cellular protection and damage repair systems. Overall, these desiccation-tolerant species could potentially be used in experiments investigating interactions between community members during dehydration and periods of desiccation.

Data regarding the survival of some pathogenic species, such as *Pseudomonas aeruginosa* (a Gram-negative, rod-shaped opportunistic pathogen), after drying on surfaces seems to suggest a large variation in the ability to survive the drying process. In some instances, a complete and rapid loss of viability was observed immediately after or within hours of drying (Hirai, 1991; Scott and Bloomfield, 1990; Skaliy and Eagon, 1972), while in other cases, viability was maintained for several days or even weeks (McDade and Hall, 1964; Yazgi et al., 2009). *P. aeruginosa* is one of the major causes of morbidity and mortality among patients with compromised immune systems, especially those suffering from cystic fibrosis (Cystic Fibrosis Trust, 2004; Lanini et al., 2007; Stover et al., 2000). Studies have shown the ability for this bacterium to persist in moist reservoirs in the clinical environment, such as in sink drains (Hota et al., 2009), soap dispensers (Lanini et al., 2007), sinks, toilets, and humidifier solutions (Zimakoff et al., 1983), which can lead to infection of nearby patients. It is possible that dry surfaces may also represent an important source of cross-contamination during nosocomial outbreak of *P. aeruginosa* (Panagea, et al., 2005). The conflicting data regarding the survival of *P. aeruginosa* after drying highlighted the need for a more thorough understanding of the fate of *P. aeruginosa* in the inanimate environment.

Most of the work studying bacterial survival on dry surfaces has been carried out using

pure cultures, despite the predominance of mixed communities in natural and indoor environments. To the authors' knowledge, no studies could be found which focussed on the viability of dried bacterial communities on indoor surfaces. Therefore, the purpose of our study was to investigate the influence of community interactions on the survival of bacterial cells after drying on a glass surface. Specifically, the objectives of this study involved i) isolating bacterial species from indoor air, ii) developing methods to simulate bacterial deposition onto exposed surfaces, and iii) investigating the survival of *Pseudomonas aeruginosa* (PAO1) after drying in the presence of various members of the indoor bacterial flora, in order to determine whether desiccation-sensitive species can benefit from the presence of desiccation-tolerant species, like *Arthrobacter* spp., in microbial communities at solid-air interfaces.

Most studies investigating long-term bacterial survival do so using a large droplet inoculation method, in which large volumes (typically 0.05 to 0.2 mL) of bacterial suspensions are inoculated onto test surfaces (Jawad et al., 1996; Makison and Swan, 2006; Rose et al., 2003; Wendt et al., 1997; Yazgi et al., 2009). This method is advantageous in that it allows for rapid and reproducible inoculation of a large number of test substrates, and was therefore used for much of the present study. However, large droplet inoculation does not accurately represent the natural deposition of cells from the air onto exposed surfaces. Therefore, the survival curves obtained with this method were compared to results obtained using an additional method of bioaerosol generation and deposition (Wilkinson, 1966; Robine et al., 1998, Robine et al., 2000). We hypothesized that survival of desiccation-sensitive species such as *P. aeruginosa* is enhanced when cells are dried in the presence of desiccation-tolerant members of the indoor bacterial flora, or as part of a diverse mixed community, compared with survival as a pure culture.

3.2 Materials and Methods

Isolation of bacterial species from indoor air

Indoor air isolates were obtained from a high traffic university science laboratory room. Indoor air was sampled by impaction for twenty minute intervals using a Mold Sampler for Viable and Non-Viable Aerosols (Biochem Tech) onto tryptic soy agar (TSA) plates (full strength (30 g/L) and 10%) (EMD Chemicals Inc., Mississauga, ON, Canada). After sampling, all plates were incubated at room temperature for 14 days. Unique colonies were picked on days 3, 7, and 14 based on colour, morphology, and growth rate. The picked colonies were re-streaked to check for purity and then re-grown overnight in tryptic soy broth (10%) for cryopreservation, genomic DNA extraction, 16S rRNA PCR amplification, and sequencing identification.

DNA Extraction and PCR amplification

The genomic DNA from each isolate was extracted using the MoBio UltraClean Soil DNA Extraction Kit (MoBio Laboratories INC., Carlsbad, CA, USA) following the manufacturer's protocol. Genomic DNA was stored at -20°C until further analysis. PCR was performed on all extracted DNA using the bacteria-specific forward primer U341F (5'-CCTACGGGAGGCAGC AG-3') and reverse primer U758R (5'-CTACCAGGGTATCTAATCC-3'), which results in a 418-bp fragment corresponding to the variable V3 and V4 regions of the 16S sequence in *Escherichia coli* (Rolleke et al., 1996).

Each 50 µL PCR reaction contained 1 µL of genomic DNA, 25 pmol of both U341F and U758R primers, 6.875 µg of BSA, 200 µM of each of the four dNTPs, and 2.5 units of Taq polymerase in Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) (New England BioLabs, Pickering, ON, Canada). The samples were first denatured at 96°C for 5 min and

thermocycling at 94°C for 1 min. An initial annealing temperature of 65°C was lowered by 1°C every cycle for a total of 20 cycles, using an elongation time of 3 min at 72°C. An additional 15-20 cycles were performed with a 55°C annealing temperature (Yeung et al., 2010). The correct size of the PCR products was subsequently confirmed on a 1% agarose gel containing SYBR Safe DNA gel stain (Invitrogen, Burlington, ON, Canada). PCR products were quantified against a standard curve prepared with several dilutions of a 100-bp molecular weight ladder (MBI Fermentas, Amherst, NY, USA).

Sequencing of PCR products and phylogenetic analysis

Following quantification, PCR products were sent for sequencing at the Centre for Applied Genomics at SickKids in Toronto, using the Applied Biosystems SOLiD 3.0 System. The resulting forward and reverse nucleotide sequences were used to create a single consensus sequence using BioEdit Sequence Alignment Editor (Version 7.0.9.0; Hall, 1999), and the consensus sequence was subjected to a BLAST search using the NCBI database of 16s rRNA sequences (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*). ClustalW (*http://www.ebi.ac.uk/clustalw/*) was used to align sequences, and the neighbour-joining algorithm of MEGA v5.05 was used for evolutionary analyses and construction of the phylogenetic tree.

Preparation of inocula for survival experiments

Overnight cultures (roughly 10^8 CFU/mL) were grown in 10% TSB for all the survival experiments. Growth curves constructed for each bacterial species suggests that this incubation time corresponded to the late exponential or early stationary phase of growth. All indoor air isolates as well as the laboratory strain *Pseudomonas aeruginosa* PAO1::*gfp-2* (herein referred to as PAO1) were grown at room temperature with agitation. Cells were washed twice with sterilized tap water to remove the growth media (a possible nutrient source available to cells after

drying). This was performed by centrifuging at 7000 x g for 5 minutes, which was shown in preliminary experiments not to damage or destroy the cells. Sterilized tap water was used as the suspending medium rather than other common solutions such as physiological saline or phosphate buffered saline in order to prevent potential osmotic shock to the cells from residual salt crystals formed during the drying process.

Surface inoculation and humidity control

Autoclave-sterilized glass microscopy coverslips (1 mm x 18 mm x 18 mm, VWR International, Mississauga, ON, Canada) were used as the substrate for all survival experiments. Two methods were used to inoculate the coverslip substrates. Survival of cells was assessed using a large droplet inoculation method, in which 0.05-0.1 mL of washed culture was pipette onto the coverslips. This large droplet inoculation method is commonly used to assess bacterial viability after extended periods of desiccation on surfaces (Jawad et al., 1996; Makison and Swan, 2006; Rose et al., 2003; Wendt et al., 1997; Yazgi et al., 2009). An additional experiment was carried out studying survival of deposited bioaerosols (Wilkinson, 1966; Robine et al., 1998, Robine et al., 2000) using our specially designed aerosol chamber (Fig. 3.1). In this method, sterile air was passed through a nebulizer (MedPro, Langley, BC, Canada), a medical device commonly used for drug delivery (Fig. 3.1C), containing the same washed bacterial suspension prepared as mentioned previously. The aerosolized suspension passed through a short piece of silicon tubing before injection into an aerosol chamber (Fig. 3.1D), where bioaerosols were allowed to deposit onto glass coverslips placed inside the chamber (Fig. 3.1F). An aerosolization time of ten minutes was used for each species, followed by a five minute deposition period during which all ports were sealed and the chamber was left undisturbed. Coverslips were

arranged in a circular pattern around the bioaerosol inlet port in order to ensure uniform deposition on all samples.

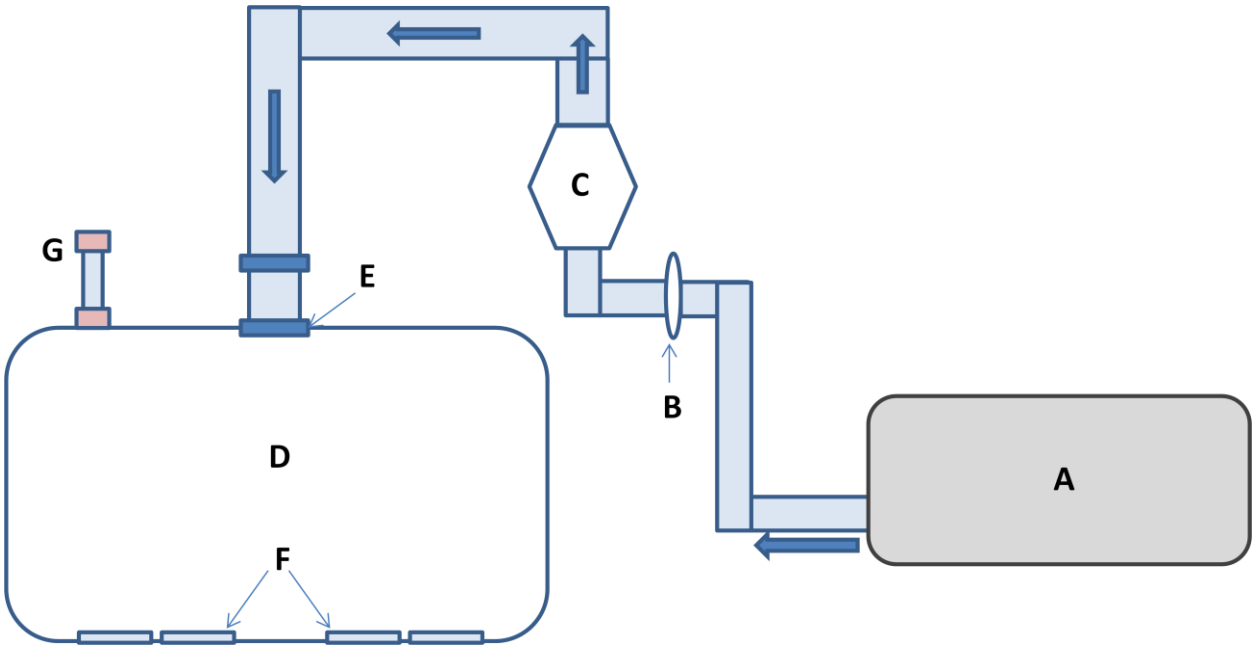


Fig. 3.1: Schematic diagram of the aerosol chamber constructed for simulating bioaerosol generation and deposition onto exposed surfaces. Air was passed from an air pump (A) through a 0.2 µm filter (B) in order to remove contaminants before aerosolizing the bacterial suspension in the nebulizer (C). The bioaerosol particles were carried with the airflow through a length of tubing and injected into an aerosol chamber (2.5 L, D) at the aerosol injection port (E). Bioaerosol particles were allowed to settle naturally onto exposed coverslips (F). Humidity within the chamber was monitored via a humidity sensor port (G).

The starting cell number on the coverslips was between 10^7-10^8 CFU for each species being tested. Similar inoculation numbers were used for both the large droplet inoculation and bioaerosol deposition methods. After inoculation, all coverslips where allowed to dry in a laminar flow ventilation hood, during which time humidity was not regulated and coverslips were subject to ambient relative humidity (60 ± 5%). Coverslips were left to dry for 3 hours when inoculated using the large droplet inoculation method, but only required 2 minutes to dry

when inoculated with deposited bioaerosols. Immediately after drying, coverslips were sacrificed in duplicate to determine the proportion of cells lost due to the inoculation/deposition and drying processes. All remaining coverslips were placed in a humidity chamber for the duration of the experiment so that the dried cells would be exposed to a constant relative humidity. Humidity chambers were adapted from GasPak chambers (Becton Dickson, Franklin Lakes, NJ, USA), and humidity was monitored throughout with an Indoor/Outdoor Hygro-Thermometer (Extech Instruments, Waltham, MA, USA).

Humidity within the chambers was controlled using a range of saturated salt solutions (Greenspan, 1977). Saturated potassium acetate, magnesium chloride, and potassium sulfate where used to maintain relative humidity values of 25 ± 1% (low), 42 ± 3% (mid), and 95 ± 2% (high), respectively. Saturated solutions were prepared as slushy mixtures in 150 mL beakers by adding crystals to distilled water at approximately two to three times the solubility limit. The saturated solutions were placed inside their respective experimental chambers at least three days prior to commencing experiments in order to allow the humidity to equilibrate. When chambers where opened to retrieve coverslips for enumeration, the relative humidity returned to the desired level within 1-2 hours.

Survival Scenarios

Several survival scenarios were tested involving dual-species and mixed community conditions. First, the desiccation tolerance of each indoor air isolate as well as PAO1 was assessed in pure culture using the large droplet inoculation method. Relative humidity during desiccation was maintained at mid humidity (i.e. 42 ± 3%). For all subsequent experiments, pure culture controls were run in parallel with community scenarios. The effect of interactions between desiccation-sensitive and -tolerant species was studied at mid humidity by inoculating

PAO1 in the presence of a room air isolate (RAI-3) which exhibited the best desiccation survival ability. In this experiment, a number of inoculation sequences were tested for the dual-species community, including co-inoculation (both species mixed prior to inoculation), and sequential inoculation (one species inoculated first and allowed to dry, before inoculation of the second species). Survival of PAO1 was subsequently tested in the presence of RAI-3 at three relative humidity levels. This involved sequential inoculation of PAO1 onto dried RAI-3 cells, followed by exposure to low, mid, and high humidity. PAO1 survival was further studied when co-inoculated as a member of an artificial mixed community, and when inoculated as a secondary droplet onto a dried layer of mixed community cells. The artificial mixed community was created with bacterial species obtained during the isolation experiment. Each of the aforementioned experiments was performed using the large droplet inoculation method. Only the PAO1 with RAI-3 experiment at mid humidity was performed using the bioaerosol deposition method. In this dual-species bioaerosol experiment, RAI-3 bioaerosols were deposited and dried first, followed by deposition of PAO1 bioaerosols.

Enumeration of viable bacteria

During the sampling, coverslips were removed from the humidity chambers in duplicate without replacement at various time points for enumeration. Each sacrificed coverslip was placed in a 50 mL polyethylene tube containing 5 mL of sterile 0.9% NaCl and vortexed at top speed with a benchtop vortex for 1 minute to remove adherent cells. Control trials showed that vortexing for 1 minute resulted in the highest recovery of viable cells, and microscopy showed that low numbers of cells remained on the coverslip after vortexing (data not shown). A serial dilution of the collection liquid was carried out and plated in duplicate onto 10% TSA plates. When low counts were expected, one millilitre of the collection liquid was also filtered through a

0.2 µm nitrocellulose membrane filter, which was then placed on a 10% TSA plate. All plates were incubated at room temperature for 2-5 days, as some of the environmental isolates exhibited slower growth rates on solid media. Each isolate was checked for autofluorescent colonies under UV light and none were observed. Thus, PAO1 colonies constitutively expressing green fluorescent protein could be differentiated from other colonies in the community using a Leica fluorescent dissection microscope (Leica Microsystems, Concord, ON, Canada).

DGGE analysis of a mixed community during desiccation

An artificial mixed community was constructed using six bacterial species obtained during the isolation experiment, as well as PAO1. Each species was washed with sterile tap water as described above, and adjusted to a concentration of roughly 10^7 to 10^8 CFU/mL prior to creation of the artificial mixed community. Glass coverslips were inoculated and dried using the large droplet inoculation method. After drying, all coverslips were transferred to a humidity chamber and exposed to mid humidity for seven days, during which duplicates of coverslips were sacrificed on days 1, 4, and 7. Sacrificed coverslips were placed in 50 mL polyethylene tubes containing 5 mL of sterile 0.9% NaCl and vortexed at top speed for 1 minute to remove adherent cells.

The collection liquid was filtered through a PVDF membrane filter (25 mm diameter, 0.45µm pore size) (Pall Corporation, Ann Arbor, MI, USA), which was subsequently added to a bead-beating tube provided in the MoBio UltraClean Soil DNA Extraction Kit. DNA was extracted from cells trapped on the filter by following the manufacturer's protocol. PCR was performed on extracted DNA targeting the 16S rRNA gene, using the same procedure and primer set described above, except the forward primer U341F had a GC clamp attached to the 5' end

(5'-GGCGGGGCGGGGGCACGGGGGGCGCGGCGGGCGGGGCGGGGG-3') (Muyzer et al., 1993). PCR products were purified using the illustra GFX PCR purification kit (GE Healthcare), at which point DNA from duplicate samples were combined in order to eliminate extraction bias. Purified PCR products were run on a DGGE gel using 8% polyacrylamide with a denaturing gradient of 30-70% (7 M urea and 40% deionized formamide were regarded as 100% denaturant). The gel was cast with a gradient former (BioRad Laboratories, Mississauga, ON, Canada) and approximately 200 ng of pure culture 16S rRNA gene product and 500 ng of the mixed community gene products were loaded into each well. The gel was run with a DCode Universal Mutation Detection System (BioRad Laboratories, Mississauga, ON, Canada), at a constant voltage of 80V for 60 hours at 60°C in 1X TAE running buffer. The resulting gel was developed in SYBR Gold (Invitrogen, Burlington, ON, Canada) for 30 minutes followed by de-staining in 1X TAE. The gel image was digitized using a Gel Logic 1500 Imaging System (Kodak, Rochester, NY, USA), and a dendrogram profile and cluster analysis were created using GelCompare II (Applied Maths, Sint-Martens-Latem, Belgium).

3.3 Results

Isolation of bacterial species from indoor air

A total of six bacterial species were isolated from indoor air in a university science laboratory. The isolates are referred to as "room air isolates" (RAI). Results from phylogenetic analysis based on 16S rRNA gene sequences suggested that all isolates are closely (>98%) related to species which have been isolated from similar dry indoor environments, including hospital air and clean room floors (Fig. 3.2). Only one of the room air isolates (RAI-1) was determined to be Gram-negative, based on sequence similarity to other Gram-negatives, while five isolates (RAI-2, 3, 4, 5, and 6) clustered closely with Gram-positive species. The genera represented by the isolates were all very closely related to members from the following genera: *Herbaspirillum* spp. (RAI-1, 98%), *Brevibacterium* spp. (RAI-2, 100%), *Arthrobacter* spp. (RAI-3, 99%), *Micrococcus* spp. (RAI-4, 100%), *Bacillus* spp. (RAI-5, 99%), and *Microbacterium* spp. (RAI-6, 99%). Based on these sequence similarities, certain physiological properties, such as natural resistance to desiccation, were deduced.

Assessing desiccation tolerance in pure culture

Desiccation tolerance and culturability after drying was assessed for these six room air isolates and PAO1 using the large droplet inoculation method to mimic drying on exposed surfaces. After inoculation and drying on coverslips, culturable cell numbers were monitored during desiccation at mid humidity. A large variation was observed in the ability of these isolates to remain culturable during prolonged desiccation (Fig. 3.3). RAI-1, RAI-4, and RAI-5 each experienced a significant reduction in culturable cell numbers, but were still detectable after four days of desiccation, indicating at least some degree of desiccation tolerance. RAI-6 and RAI-2 were undetectable after 2 and 3 days of desiccation respectively, suggesting that they are

sensitive to dehydration and desiccation. Best survival was observed for RAI-3, which maintained relatively consistent cell numbers throughout the four day experiment. Poorest survival was observed for the lab strain PAO1, which experienced a significant reduction in culturable cell numbers after just one day of desiccation, and was no longer detectable after two days.

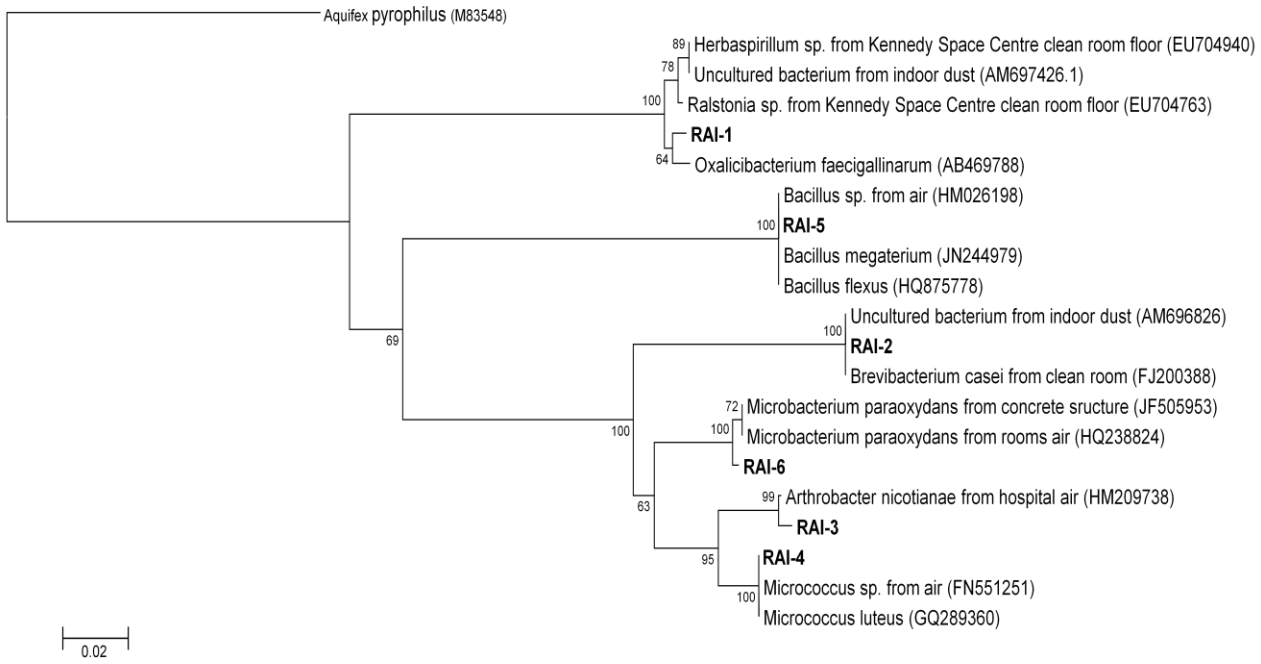


Figure 3.2: Phylogenetic position of the 6 room air isolates (RAI) and other similar species, including some which have been obtained from similar dry environments. Similarities are based on 16S rRNA gene fragment sequences. The tree was constructed using the neighbour-joining algorithm and numbers on the nodes are bootstrap values based on 1000 replicates. Aquifex pyrophilus was used as the outgroup. The scale bar indicates the estimated number of base changes per nucleotide sequence position.

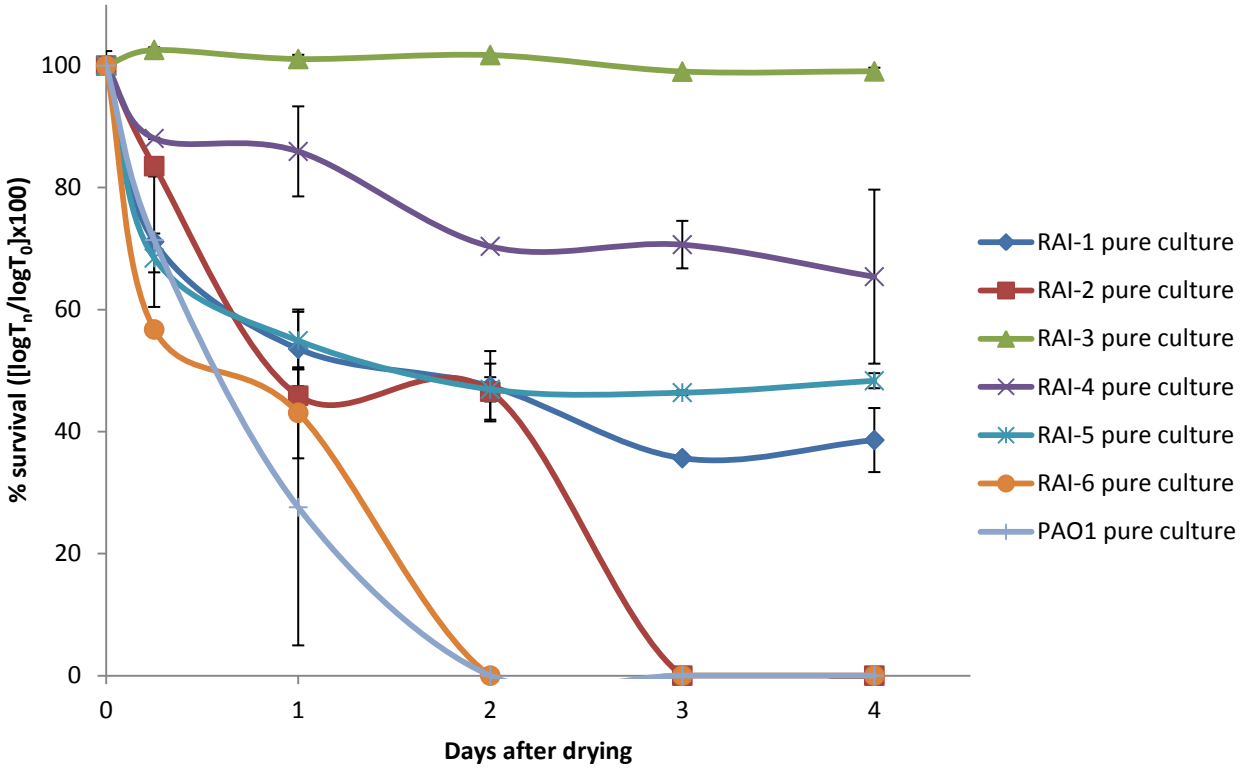


Figure 3.3: Survival curves showing percent reduction in cell numbers for 6 room air isolates (RAI), as well as the laboratory strain *P. aeruginosa* PAO1::*gfp-2*. A constant relative humidity of 42 ± 3% was maintained throughout the experiment, except during the initial three hour drying period, which was carried out at ambient RH (60 ± 5%). Values at time 0 represent the inoculum number before drying. Coverslips were sacrificed for enumeration immediately after a three hour drying period, followed by daily sampling for four days. Error bars represent range of CFU values obtained for each time point.

Investigating the effect of interactions between desiccation-sensitive and -tolerant species

In order to investigate the influence of species interaction on the fate of bacterial cells during prolonged desiccation, a species surviving well during desiccation (i.e. RAI-3) and a poor survivor (PAO1) were chosen for further study. Using the large droplet inoculation method, these two species were inoculated in pure culture as controls, and as a dual-species community using various inoculation sequences (Fig. 3.4). In each of the scenarios tested, RAI-3 maintained relatively consistent cell numbers. When PAO1 was dried as a pure culture, no culturable cells

were detected after just one day of desiccation. The same trend of decline was observed when PAO1 was dried on top of sterile tap water residue. This control was carried out to ensure that no survival advantage would be instilled by the initial presence of additional sterile tap water during dual-species survival. In each of the dual-species scenarios, PAO1 exhibited significantly better survival than as the single culture by itself and when inoculated onto tap water residue. Overall, survival of PAO1 was best when inoculated as a secondary droplet onto a primary dried droplet of RAI-3 cells (Fig. 3.4).

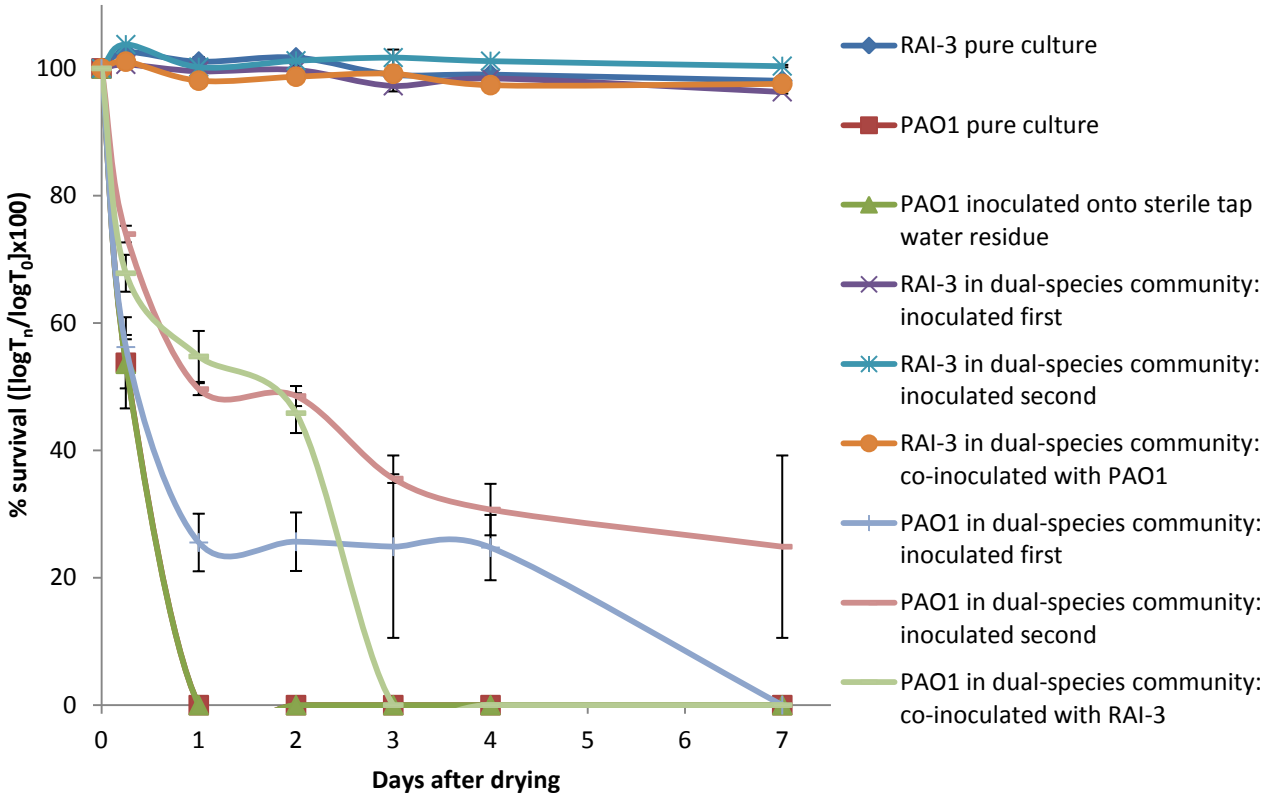


Figure 3.4: Survival curves showing percent reduction in cell numbers obtained for *P. aeruginosa* PAO1::*gfp-2* and RAI-3 in pure culture and dual-species community. The community survival scenarios included co-inoculation of the species (mixed prior to inoculation), and sequential inoculation (one species inoculated first and dried, followed by inoculation with the second species). A constant relative humidity of 42 ± 3% was maintained throughout the experiment, except during the initial three hour drying period, which was carried out at ambient RH (60 ± 5%). Values for time 0 represent the inoculum number before drying. Coverslips were sacrificed for enumeration immediately after a three hour drying period, followed by regular sampling for seven days. Error bars represent range of CFU values obtained for each time point.

Investigating the effect of relative humidity on survival in dual-species community

The same combination of desiccation-tolerant and -sensitive species was used to study survival over a range of relative humidity values. Following inoculation and drying, survival of PAO1 and RAI-3 was assessed in pure culture and dual-species community during desiccation at low, mid, and high humidity (Figs. 3.5, 3.6, and 3.7). The dual-species community involved sequential inoculation, where RAI-3 was inoculated and dried first, followed by inoculation of PAO1. At all three humidity levels, when PAO1 was inoculated as a pure culture, no culturable cells were recovered after just one day. At low and mid humidity, RAI-3 maintained relatively consistent cell numbers during seven days of desiccation in both the pure culture and community scenarios. At these humidity levels, PAO1 maintained culturability much longer when dried in a dual-species community than as a pure culture. Conversely, RAI-3 cells seemed to be more susceptible to desiccation at higher humidity, since a significant decline in cell number was observed in both pure culture and the dual-species community at high humidity. No apparent difference in survival between pure culture and dual-species community was observed for PAO1 during desiccation at high humidity.

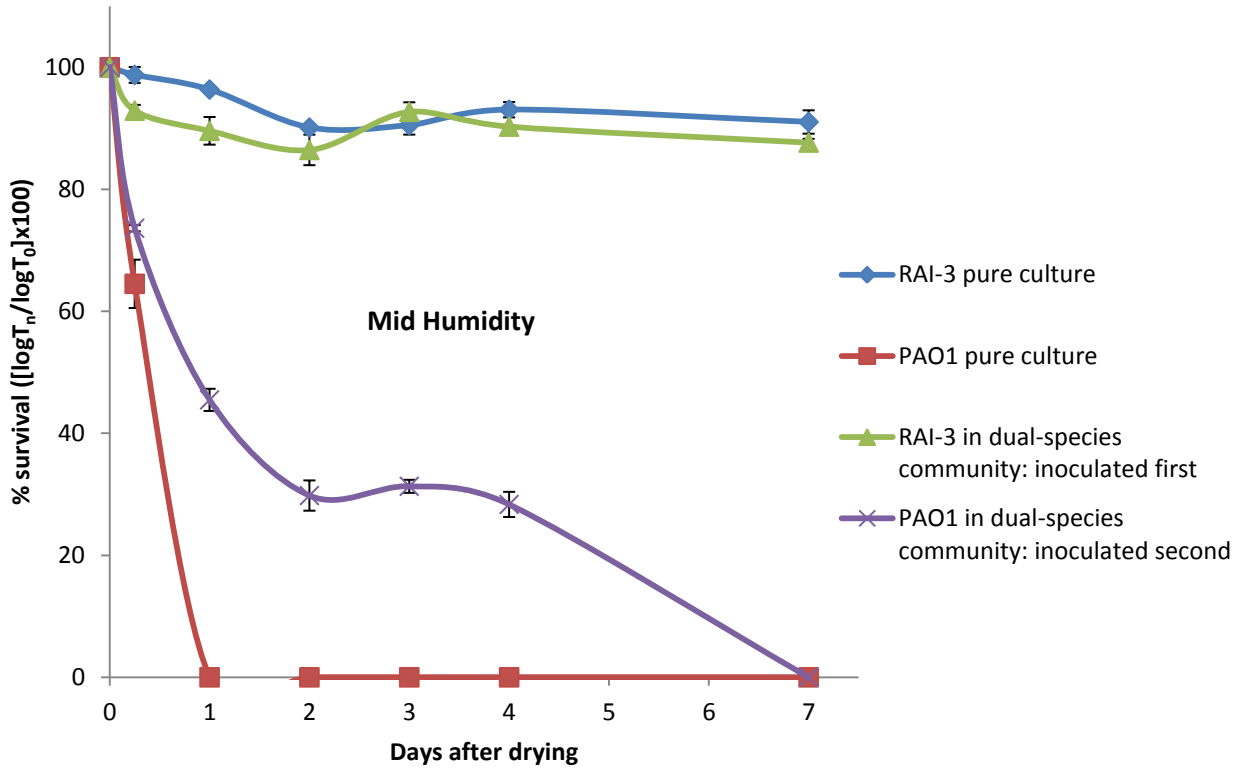


Figure 3.5: Survival curves showing percent reduction in cell numbers obtained for *P. aeruginosa* PAO1::*gfp-2* and RAI-3 in pure culture and dual-species community. The community survival scenario involved sequential inoculation, where RAI-3 was inoculated first and dried, followed by inoculation with *P. aeruginosa* PAO1::*gfp-2*. A constant relative humidity of 42 ± 3% was maintained throughout the experiment, except during the initial three hour drying period, which was carried out at ambient RH (60 ± 5%). Values for time 0 represent the inoculum number before drying. Coverslips were sacrificed for enumeration immediately after a three hour drying period, followed by regular sampling for seven days. Error bars represent range of CFU values obtained for each time point.

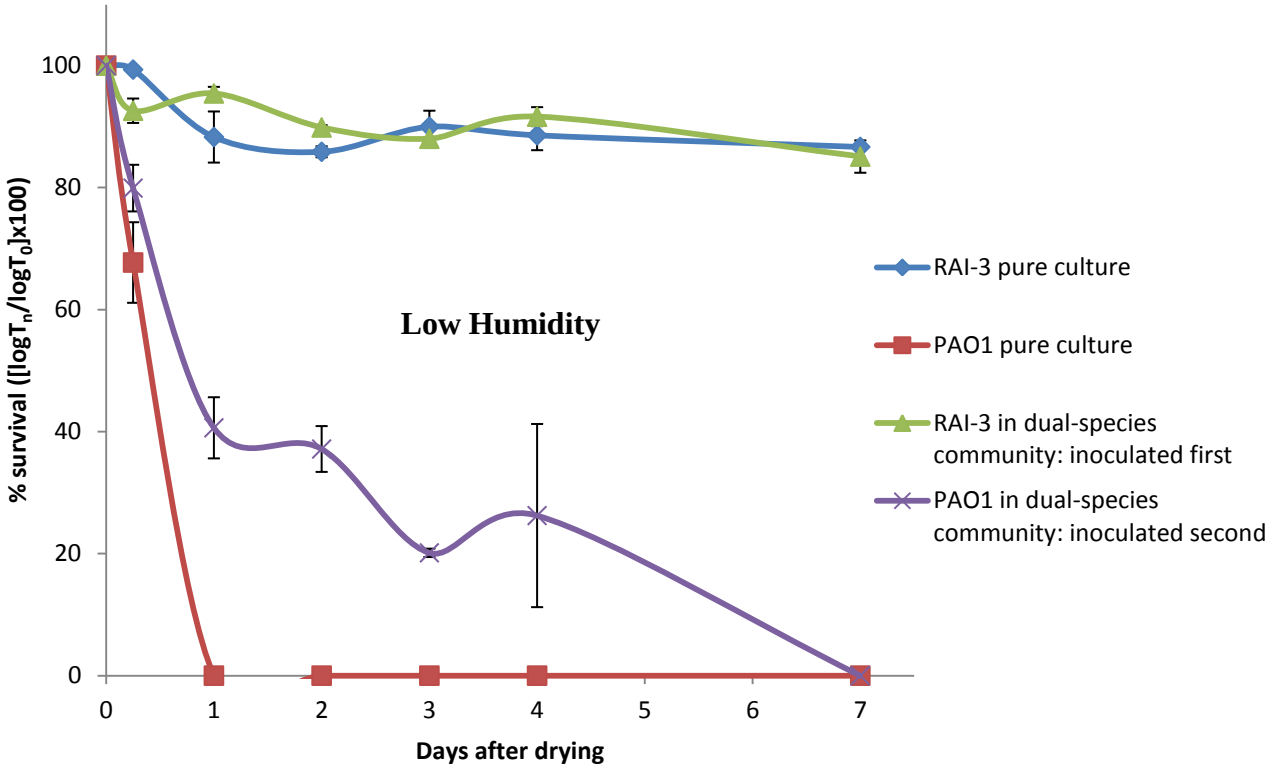


Figure 3.6: Survival curves showing percent reduction in cell numbers obtained for *P. aeruginosa* PAO1::*gfp-2* and RAI-3 in pure culture and dual-species community. The community survival scenario involved sequential inoculation, where RAI-3 was inoculated first and dried, followed by inoculation with *P. aeruginosa* PAO1::*gfp-2*. A constant relative humidity of 25 ± 1% was maintained throughout the experiment, except during the initial three hour drying period, which was carried out at ambient RH (60 ± 5%). Values for time 0 represent the inoculum number before drying. Coverslips were sacrificed for enumeration immediately after a three hour drying period, followed by regular sampling for seven days. Error bars represent range of CFU values obtained for each time point.

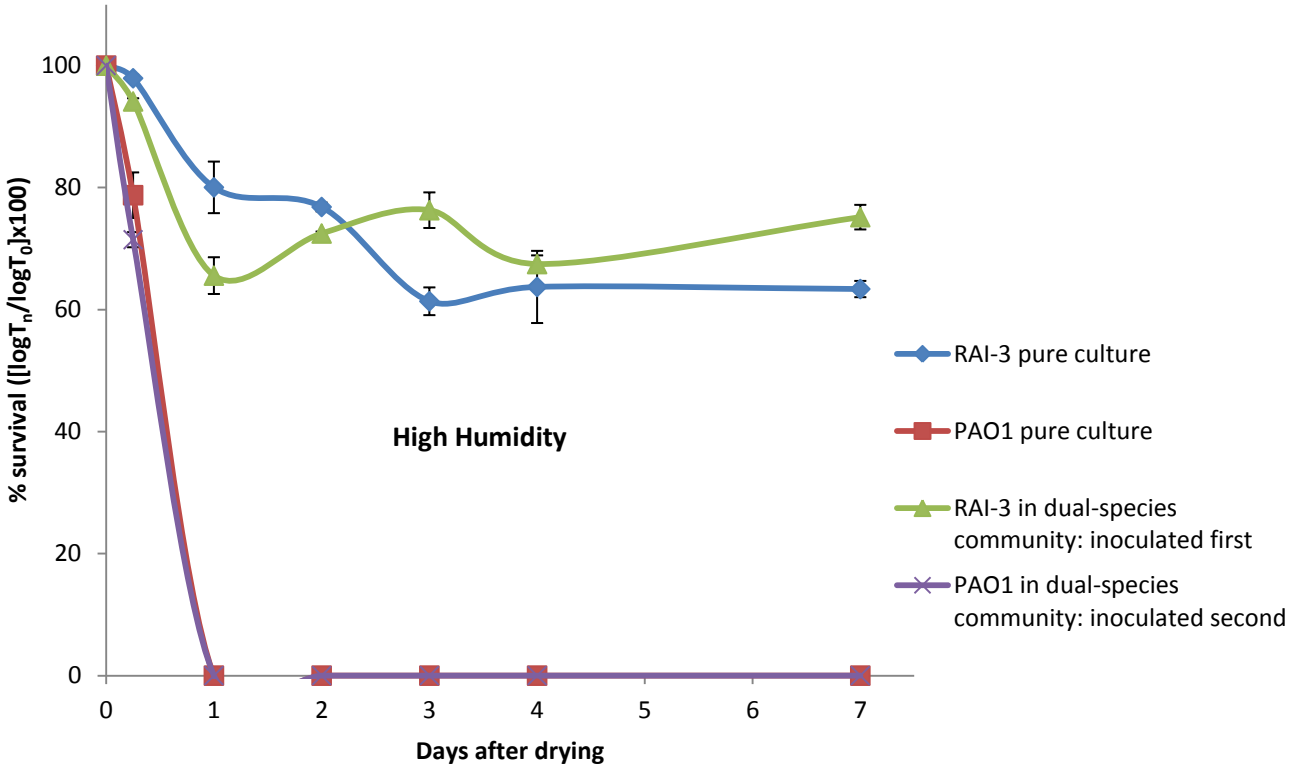


Figure 3.7: Survival curves showing percent reduction in cell numbers obtained for *P. aeruginosa* PAO1::*gfp-2* and RAI-3 in pure culture and dual-species community. The community survival scenario involved sequential inoculation, where RAI-3 was inoculated first and dried, followed by inoculation with *P. aeruginosa* PAO1::*gfp-2*. A constant relative humidity of 95 ± 2% was maintained throughout the experiment, except during the initial three hour drying period, which was carried out at ambient RH (60 ± 5%). Values for time 0 represent the inoculum number before drying. Coverslips were sacrificed for enumeration immediately after a three hour drying period, followed by regular sampling for seven days. Error bars represent range of CFU values obtained for each time point.

Investigating survival of a desiccation-sensitive species in a diverse bacterial community

Survival of PAO1 was subsequently assessed when inoculated as a member of a more diverse community. All six room air isolates were combined at roughly equal concentrations to give and artificial mixed community with diversity more representative of the indoor air microbial flora. PAO1 survival was studied when co-inoculated with this artificial mixed community (combined with community prior to inoculation), and when inoculated as a

secondary droplet onto a dried layer of community cells (Fig. 3.8). Survival of PAO1 was only slightly enhanced in the mixed community scenarios compared to pure culture survival.

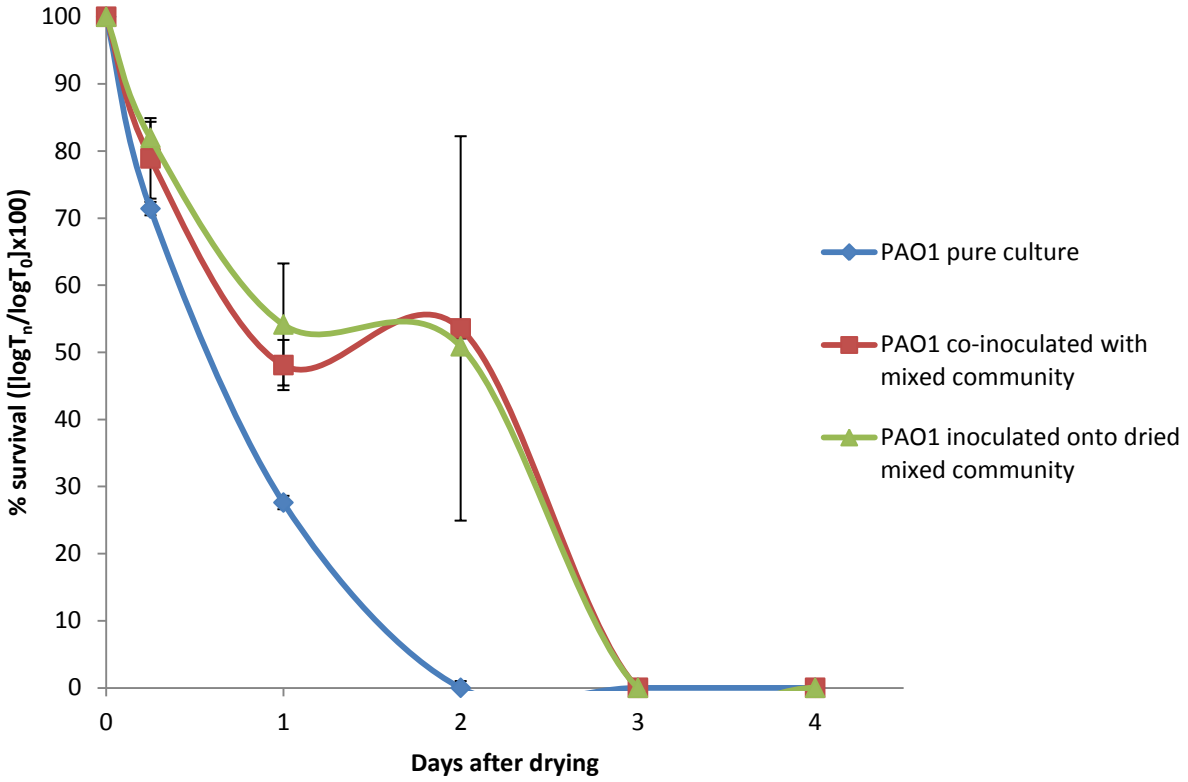


Figure 3.8: Survival curves obtained for *P. aeruginosa PAO1::gfp-2* under three survival scenarios. The mixed community contained six room air isolates, which were combined at roughly equivalent concentrations. A constant relative humidity of 42 ± 3% was maintained throughout the experiment, except during the initial three hour drying period, which was carried out at ambient RH (60 ± 5%). Values for time 0 represent the inoculum number before drying. Coverslips were sacrificed for enumeration immediately a three hour drying period, followed by regular sampling for seven days. Error bars represent range of CFU values obtained for each time point.

Molecular analysis of an artificial mixed community during desiccation

To assess the integrity of the mixed community DNA during desiccaiton, an artifiical mixed community was created with the six indoor air isolates and PAO1. After inoculation using the large droplet inoculation method, community DNA was extracted for DGGE analysis based on 16S rRNA gene fragments. Cluster analysis of DGGE banding patterns suggests no change in community profile during the first four days, and very little change during the 7 day desiccation experiment ($S_{AB} \geq 85$) (Fig. 3.9).

Assessing survival of bioaerosols deposited in a dual-species community

Survival of RAI-3 and PAO1 was tested in pure culture and as a dual-species community using a bioaerosol deposition method, rather than inoculation of large volume droplets. This was accomplished through the use of an aerosol chamber (Fig. 3.1) containing glass coverslips, upon which aerosolized bacterial suspensions were allowed to deposit naturally. Aerosolized cells were distributed uniformly over the surface of the coverslips, and dried rapidly upon deposition. Immediately after drying, all coverslips were exposed to mid humidity for 7 days. RAI-3 deposited as bioaerosol particles again maintained relatively consistent culturable cell numbers for both pure culture survival and survival in the dual-species community (Fig. 3.10). Conversely, no apparent difference was observed for PAO1 survival in pure culture and when deposited onto RAI-3 bioaerosols. In both instances, no culturable PAO1 cells were detected after just one day of desiccation.

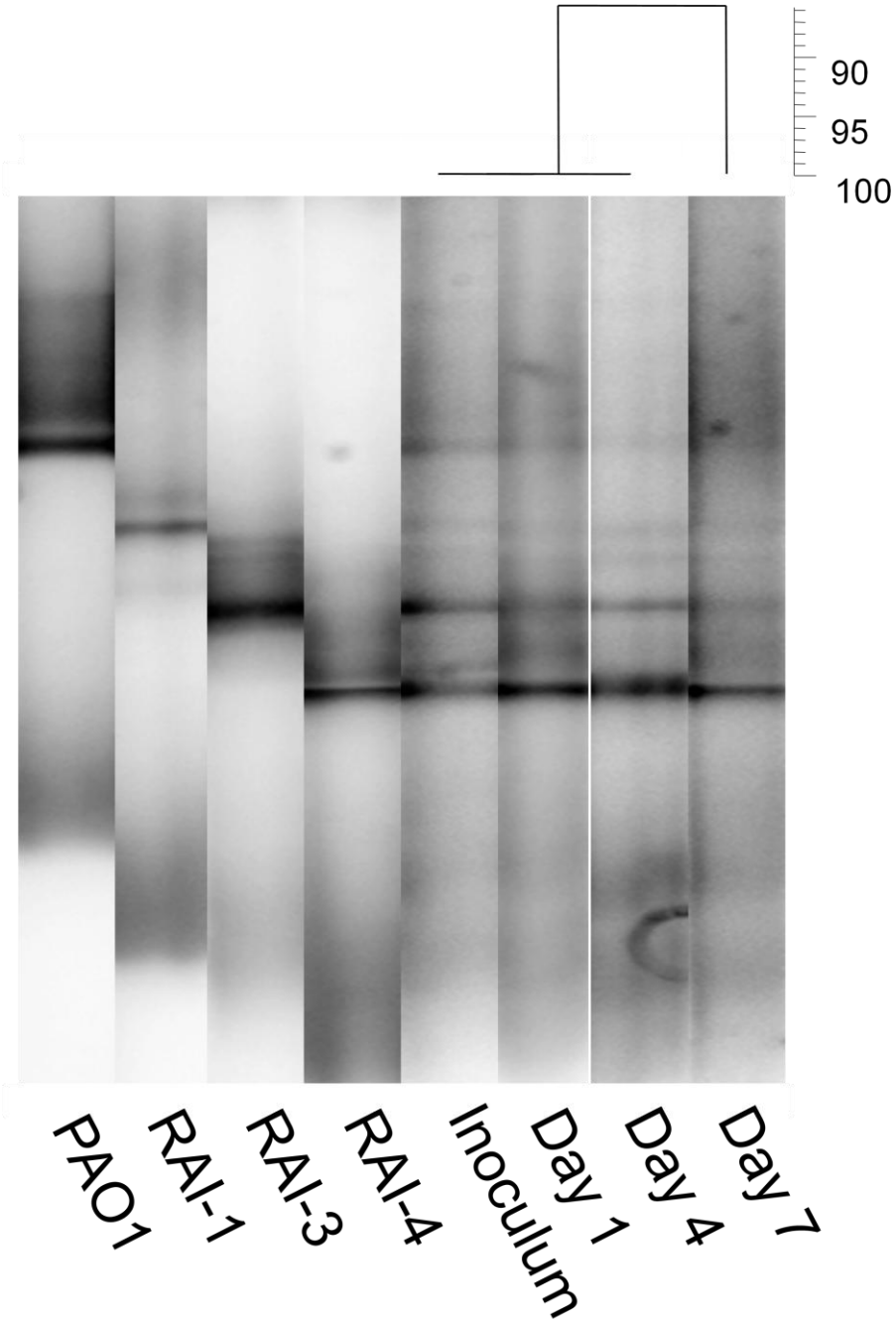


Figure 3.9: Cluster analysis of DGGE banding pattern for an artificial mixed community during prolonged desiccation. Coverslips were inoculated with the seven-member mixed community, dried, and exposed to a relative humidity of 42 ± 3% for seven days. At time points during desiccation, coverslips were sacrificed for DNA extraction, PCR, and DGGE analysis. The identity of the major bands was established by loading pure culture DNA in adjacent DGGE lanes.

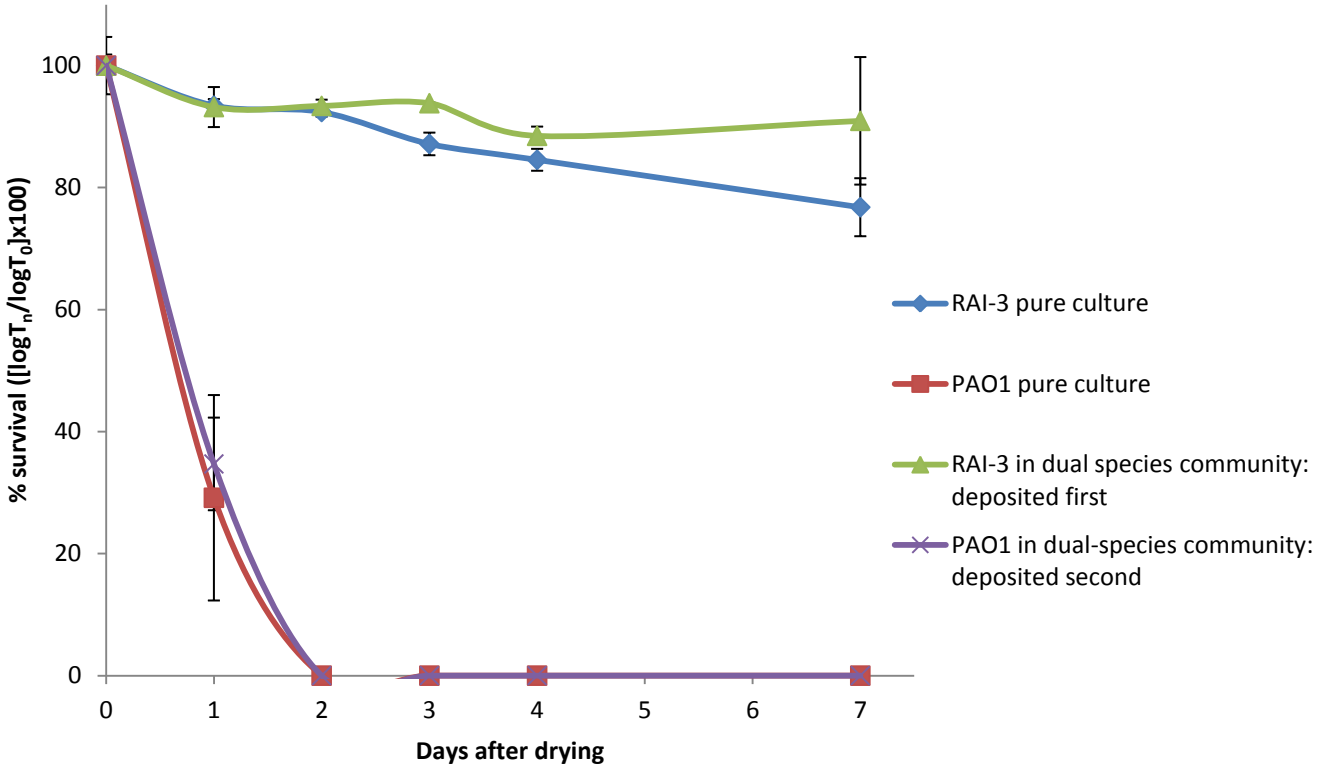


Figure 3.10: Survival curves obtained for *P. aeruginosa* PAO1::*gfp-2* and RAI-3 in pure culture and dual-species community after aerosol generation and deposition. The community survival scenario involved sequential deposition, where RAI-3 was aerosolized and allowed to deposit onto the coverslips, followed by aerosolization and deposition of *P. aeruginosa* PAO1::*gfp-2*. A constant relative humidity of 42 ± 3% was maintained throughout the experiment. Drying of the deposited bioaerosols occurred very rapidly upon removal of substrates from the aerosol chamber (1-2 minutes). Values for time 0 represent CFUs recovered immediately after deposition, before coverslips were allowed to dry. Error bars represent range of CFU values obtained for each time point.

3.4 Discussion

Isolation of bacterial species from indoor air and desiccation-tolerance

When studying infection transmission and epidemiology, it is often helpful to take a step back and consider matters in the broader ecological context. The contamination of the hospital environment by pathogenic microbes is a form of biological invasion. Bio-invasion is a three-step process involving (i) initial dispersal, during which organisms move from their normal habitat to a new environment, (ii) establishment of populations in these new habitats, and (iii) spreading to nearby habitats (Puth and Post, 2005). In the clinical environment, prevention and control of nosocomial infections can be achieved by disrupting invasion at any of these stages. This however, requires a thorough understanding of the factors that influence progression through these steps, particularly how pathogens are able to establish themselves in the inanimate environment and subsequently spread and infect susceptible individuals. This study sought to identify whether members of the indoor bacterial flora can provide an environment that would facilitate the establishment of potential pathogens in the inanimate environment.

The isolation was carried out in a high traffic university science laboratory where microbes and their vectors likely evolved from very different biota. In that respect, this location is comparable to other high traffic environments such as hospitals and other medical centers, where prevention and control of surface contamination is a high priority. Among all the room air isolates, only one Gram-negative species was isolated, and five out of six were Gram-positive (Fig. 3.2). A number of studies which have performed more thorough examinations of the bacterial burden in indoor air have found a similar predominance of Gram-positives over Gram-negatives (Augustowska and Dutkiewicz, 2006; Gilbert et al., 2010; Rintala et al., 2010). The differences in cell wall structure and composition, endospore-forming capability of some Gram-positives, and intrinsic tolerance mechanisms are thought to be primary reasons for this disparity

(Beggs, 2003).

RAI-1 was found to be closely related to species of beta proteobacteria including *Herbaspirillum* spp., *Oxalicibacterium* spp., and *Ralstonia* spp. These Gram-negative species have been isolated from both indoor and outdoor air in previous studies (Blatny et al., 2011; Norris et al., 2011). RAI-2 was closely related to *Brevibacterium* spp., which has been isolated from indoor dust samples in schools and daycares (Andersson et al., 1999). RAI-3 was identified as *Arthrobacter* spp., which can often be isolated from dry indoor environments (Krysinska-Traczyk et al., 2002; Li et al., 2004) and is known to contain many stress-tolerant species (Cacciari and Lippi, 1987). RAI-4 and RAI-5 were closely related to *Micrococcus* spp. and *Bacillus* spp., respectively, both of which are commonly found in indoor air (Bonetta et al., 2010; Gilbert et al., 2010; Gorny et al., 1999; Guilo et al., 2010). RAI-6 was highly similar to *Microbacterium* spp., a genus that has been previously identified on indoor surfaces (Lee et al., 2007). Since a review of the literature suggests the ability for each of the isolated species to persist in dry indoor environments, including high-risk areas such as hospitals and child-care facilities, the six species isolated in this study are considered a good representation of the indoor bacterial flora, and can be used to study potential interactions between the indoor flora and exogenous pathogens.

Studying pure culture survival of room air isolates and PAO1 during desiccation

A large variation was observed in the ability of the room air isolates to maintain culturability during desiccation as pure cultures, despite a high inoculation number (Fig. 3.3). RAI-3, which is closely related (>99%) to *Arthrobacter* spp., was found to maintain consistent cell numbers during prolonged desiccation (only two log reductions were observed during one month of desiccation at mid humidity (Chapter 4, Fig. 4.7). Members of the *Arthrobacter* genus

commonly exhibit considerable ability to survive as vegetative cells during prolonged periods of desiccation (Boylen, 1972; Cacciari and Billi, 1987). *Arthrobacter* spp. are also particularly resistant to a range of other stresses such as nutrient starvation and the presence of oxygen radicals or toxic chemicals. This resistance is likely due to the presence of two plasmids coding for a large number of stress-response proteins (Mongodin et al., 2006). For this reason, *Arthrobacter* spp. are often the most numerous species isolated from dry soils (Mongodin et al., 2006).

The molecular mechanisms for desiccation tolerance in prokaryotes remain poorly understood (Higo et al., 2006), but likely involve the replacement of water by the non-reducing disaccharide trehalose, as proposed by the water replacement hypothesis (Golovina et al., 2009). It has been determined experimentally that desiccation-tolerant cells accumulate large amounts of trehalose and other sugars, which are thought to replace the layer of water surrounding macromolecules and prevent desiccation-induced damage (Potts, 1994). The secretion of large amounts of extracellular polymeric substance (EPS) by many desiccation-tolerant species probably also provides a protective matrix in which water stress proteins can be maintained (Billi and Potts, 2002). The tolerance mechanisms employed by *Arthrobacter* cells almost certainly involve EPS accumulation and trehalose production, since species of the *Arthrobacter* genus are known to possess trehalose synthesis pathways (Maruta et al., 1995; Mongodin et al., 2006), and have been shown to produce large amounts of trehalose during osmotic stress and nutrient starvation (Zevenhuizen, 1992).

Pseudomonas spp., like PAO1, are far more susceptible to drying (Skaliy and Eagon, 1972), and rely on an ideal combination of various factors, such as relative humidity (Makison and Swan, 2006), surface material (Wendt et al., 1997; Yazgi et al., 2009), and nutrient

availability in the form of proteins, sputum, serum, blood, and dust (Hirai, 1991; Jawad et al., 1996; Kramer et al., 2006; Rose et al., 2003), to survive on dry indoor surfaces. In this experiment, PAO1 experienced a roughly 25% reduction in cell number due to just the drying process itself, followed by a rapid decline to become undetectable after 2 days of desiccation.

Dehydration can cause a range of deleterious effects on desiccation-sensitive species. This is because water is usually critical to confer structural order on cells, ensuring the proper function of reaction mechanisms and contributing to the stability of macromolecules (Potts, 1994). In a truly desiccated cell (i.e. less than 0.3 g water/g dry weight), there is not enough water to form a monolayer around macromolecules, causing proteins to undergo conformational changes and malfunction. This can lead to accumulation of free radicals which cause further protein damage as well as lipid peroxidation and DNA mutation (Billi and Potts, 2002).

Studying survival of bacterial communities during desiccation

Several researchers have investigated whether desiccation tolerance can be conferred upon traditionally desiccation-sensitive species through genetic engineering and exogenous manipulation (Billi et al., 2000; Welsh and Herbert, 1999). No studies could be found which focus on the natural ability of desiccation-sensitive species to exhibit increased tolerance when present in a mixed community containing desiccation-tolerant species. As such, experiments were performed to identify whether PAO1 displayed different survival characteristics when dried in the presence of the desiccation-tolerant isolate RAI-3, versus pure culture survival (Fig. 3.4). In these tests, PAO1 as a pure culture rapidly lost culturability and was no longer detectable after one day. It was also determined that the sterile tap water used as the suspending medium did not provide any added benefits that would alter survival characteristics. The tolerance to desiccation by RAI-3 was once again demonstrated in that consistent culturable cell numbers

were observed for both pure culture and dual-species community survival. PAO1 in each of the dual-species scenarios was enhanced compared with pure culture survival. The best survival was seen when PAO1 was inoculated as a secondary droplet onto a dried primary droplet containing RAI-3 cells.

A potential explanation for this phenomenon is that the survival of PAO1 in the dual-species community is enhanced by close contact between the two species, and that the PAO1 cells are benefitting from some desiccation-tolerance mechanisms possessed by the RAI-3 cells, such trehalose production or EPS secretion. A number of studies have shown that transfection of desiccation-sensitive cells with genes permitting trehalose or sucrose synthesis, induction of endogenous trehalose synthase genes, or exogenous addition of trehalose to cells prior to drying, can confer desiccation-tolerance (Billi et al., 2000; Garcia et al., 2000; Welsh and Herbert, 1999). It is therefore likely that desiccation-sensitive cells in close contact with cells that possess natural tolerance mechanisms would benefit from the extracellular secretion of trehalose and other sugars which typically comprise the EPS.

Relative humidity is one of the most important factors determining bacterial viability during extended periods of desiccation. Results from the humidity survival experiments suggested that survival of PAO1 was enhanced in the presence of RAI-3 at both low and mid relative humidity levels. Interestingly however, PAO1 did not seem to survive when the dual-species community was subject to desiccation at high humidity (Figs. 3.5, 3.6, and 3.7). RAI-3 cells maintained relatively consistent numbers in both pure culture and dual-species scenarios when exposed to low and mid-range humidity. Conversely, RAI-3 was much more susceptible to desiccation at high humidity, and experienced a significant reduction in culturable cell counts. Studies investigating the effect of relative humidity on survival of other stress-tolerant species

such as *Deinococcus radiodurans* (Bauermeister et al., 2011), *Serratia marcescens* (Walters et al., 2005), and *Klebsiella* spp. (Turner and Salmonsen, 1973) have found similar trends, where survival was impeded by high humidity. It is possible that the high humidity inhibits the desiccation tolerance mechanisms of these species, resulting in poor survival during dehydration and desiccation. If tolerance mechanisms of RAI-3 are in fact inhibited by high humidity, then it is logical that PAO1 in a dual-species community with RAI-3 would exhibit similar survival kinetics as in pure culture, since PAO1 would no longer be able to benefit from the natural tolerance mechanisms of RAI-3.

Enhanced survival of PAO1 cells was also seen when co-inoculated and dried as part of a more diverse mixed community containing the six room air isolates. However, the differences between pure culture and mixed community survival were much less pronounced than for the dual-species community of PAO1 and RAI-3 (Fig. 3.8). As seen in Fig. 3.2, not all of the isolated species were able to maintain high cell numbers during desiccation, and so it is likely that not all the community members have the tolerance mechanisms possessed by RAI-3. Therefore, the likelihood of PAO1 cells coming into close contact with desiccation-tolerant members of the mixed community is less in this more diverse community than in the dual-species scenario.

Molecular analysis of a mixed community during desiccation

The ability of bacterial cells to enter a viable but nonculturable state during times of environmental stress has been emphasized by a number of recent publications (Kell et al., 1998; Oliver, 2005; Oliver, 2010). In this state, bacteria do not grow in the media in which they would normally grow, but remain viable and able to resume metabolic activity upon resuscitation (Oliver, 2005). Despite the apparent loss of culturability of some members of the artificial

mixed community during desiccation, DGGE cluster analysis for the mixed community of room air isolates and PAO1 suggests the community profile remains relatively steady, indicating that DNA from all the members within the community remained intact during exposure to mid-range humidity (Fig. 3.9). Entry of *P. aeruginosa* cells into the viabile but non-cultrable state has been documented in other stressful environments (Kell et al., 1998), however it is unclear whether desiccation on indoor surfaces also triggers this phenomenon. Therefore, further study is required in order to assess the viability of bacterial species during desiccation, particularly for opportunistic pathogens such as *P. aeruginosa*. If pathogenic cells do in fact maintain viability during desiccation, then they likely also maintain some degree of virulence. A lack of culturability may cause these cells to go undetected on surfaces, and therefore the potential danger associated with their presence may be overlooked. This phenomenon suggests that future analysis of surface cleanliness should employ culture-independent methods (ie. molecular methods) in addition to culture-dependent methods.

Bacterial survival in deposited bioaerosols versus large volume droplets

Large droplet inoculation is a method commonly used to test bacterial survival during dehydration and desiccation under varying environmental conditions, as it allows for the rapid and reproducible inoculation of a large number of substrates. This method however, does not accurately simulate the natural deposition of cells from the airborne state onto exposed surfaces. More realistic methods should be employed to assess bacterial survival after deposition onto indoor surfaces. In this study, an aerosol chamber was constructed to study bacterial survival in deposited bioaerosols (Fig. 3.1). The scenarios tested with this method included pure culture controls of PAO1 and RAI-3, as well as sequential deposition of RAI-3 followed by PAO1 (our best survival scenario). The results from this test were contrary to what was observed using the

large droplet inoculation method, even though similar starting inoculation numbers were achieved with both methods (Fig. 3.10). Preliminary experiments showed that culturability of PAO1 was not affected by the aerosolization process itself, since deposition onto a moist, nutrient-rich medium such as tryptic soy agar resulted in a high degree of bacterial growth (data not shown).

Two major differences exist between the large droplet inoculation and bioaerosol deposition methods used in this study. Firstly, with bioaerosol deposition, cells were more uniformly distributed over the entire surface of the coverslip, whereas in the large droplet inoculation, cell density likely decreased from the center of the coverslip outward towards the edges. It is possible that since the density of cells deposited as bioaerosol particles is lower, the probability of close contact between the two species is reduced, and therefore PAO1 cells are not able to benefit from the extracellular production of desiccation-tolerance compounds by RAI-3 cells. Secondly, while large droplets took roughly three hours to dry after inoculation, deposited bioaerosols dried almost instantaneously upon removal of the coverslips from the aerosol chamber (approximately 1-2 minutes). It is possible that rapid drying of the deposited bioaerosol particles did not provide enough time for the RAI-3 cells to appropriately prepare for prolonged desiccation by producing adequate amounts of trehalose and other desiccation-tolerance compounds which comprise the EPS.

Conclusions

It is likely that other nosocomial pathogens besides *P. aeruginosa* also exhibit different survival characteristics in a mixed community versus survival as a pure culture. It is known that individual species within mixed community aqueous biofilms can exhibit phenotypes such as

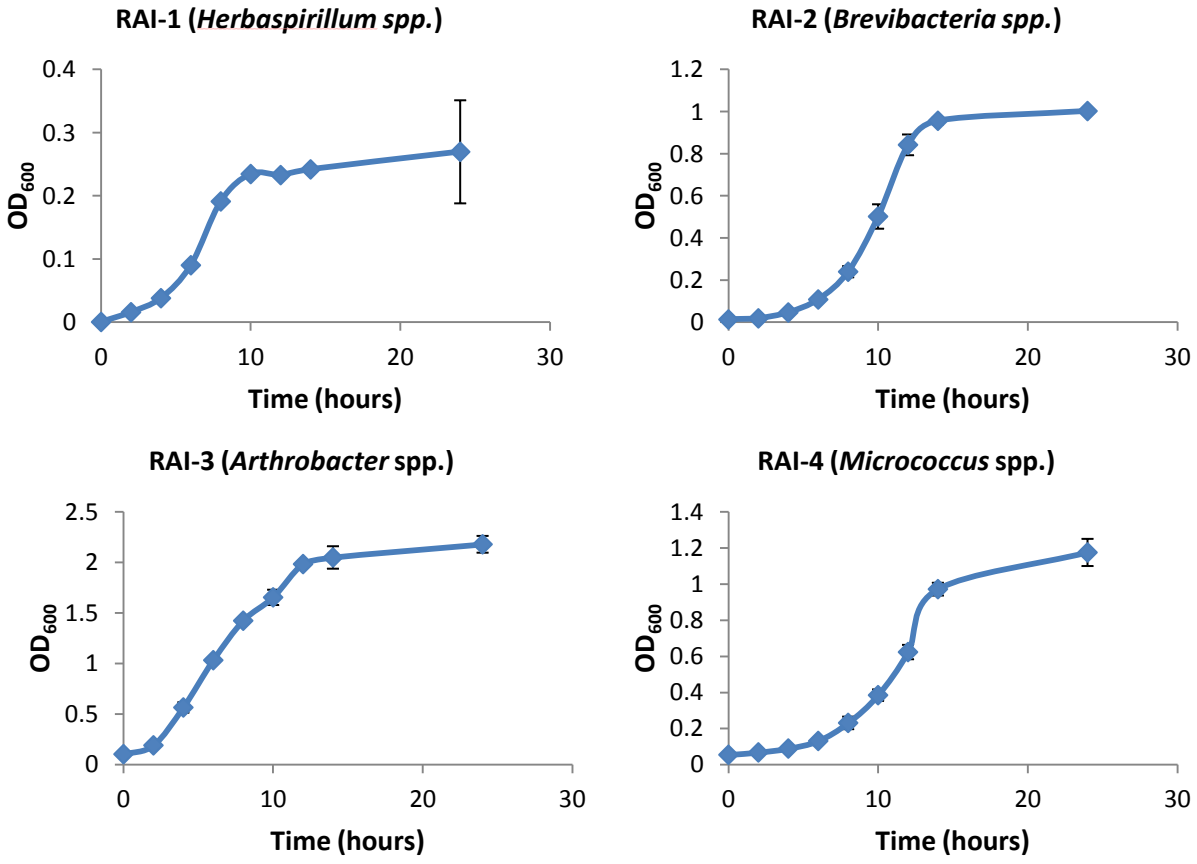
enhanced antibiotic resistance or increased virulence, which are not be present among planktonic pure culture cells. Thus, it is possible that similar interactions can occur between community members during dehydration on exposed surfaces, resulting in improved survival of desiccation-sensitive species. This is significant, since bacterial cells are rarely found as pure cultures in both the natural and indoor environment. Most of the studies investigating bacterial survival during desiccation on indoor surfaces have focussed on pure culture survival with varying parameters such as relative humidity, suspending media, and nutrient availability. While these are all important variables that dictate the length of time cells can remain viable on dry surfaces, the results of this study suggested that interactions among community members during dehydration and desiccation is also an important factor influencing bacterial survival.

It is accepted that bacteria within air can be carried throughout the indoor environment, and are continuously deposited onto exposed surfaces. In this study, it was found that *P. aeruginosa* PAO1::*gfp-2* can remain culturable longer and maintain higher cell numbers when dried in the presence of desiccation-tolerant members of the indoor bacterial flora. It is proposed that desiccation-sensitive species can benefit from the tolerance mechanisms of other species, especially if these mechanisms involve extracellular secretion of sugars and other desiccation-response molecules. This enhanced survival is probably dependent on close contact between the desiccation-sensitive and -tolerant species, which is less likely to occur when cells are deposited from the air as bioaerosol particles rather than inoculation of large-volume droplets. Despite this, the phenomenon described in this study should be considered when assessing bacterial survival in the indoor inanimate environment, as it may permit certain species to maintain viability longer than expected after drying, and maintain cell numbers high enough to trigger an infection in susceptible individuals.

CHAPTER 4: SUPPLEMENTAL MATERIAL

4.1 Determining growth curves and maximum growth rate for room air isolates and PAO1

Growth curves were constructed for the room air isolates, as well as the lab strain *P. aeruginosa* PAO1::*gfp-2* (Fig. 4.1). Three colonies were picked for each isolate from the appropriate TSA stock plate and inoculated into 30 mL of tryptic soy broth. Duplicate culture tubes were inoculated for each isolate. All tubes were grown at room temperature with agitation. One millilitre samples were removed from each culture tube at two hour intervals during incubation and the optical density (OD_{600}) was measured using an Eppendorf Biophotometer (Eppendorf AG, Hamburg, Germany). At every sampling point, three OD_{600} readings were averaged for each culture tube, and OD_{600} values obtained for duplicate culture tubes were subsequently averaged. These data were used to calculate the maximum specific growth rate for each species (Table 4.1).



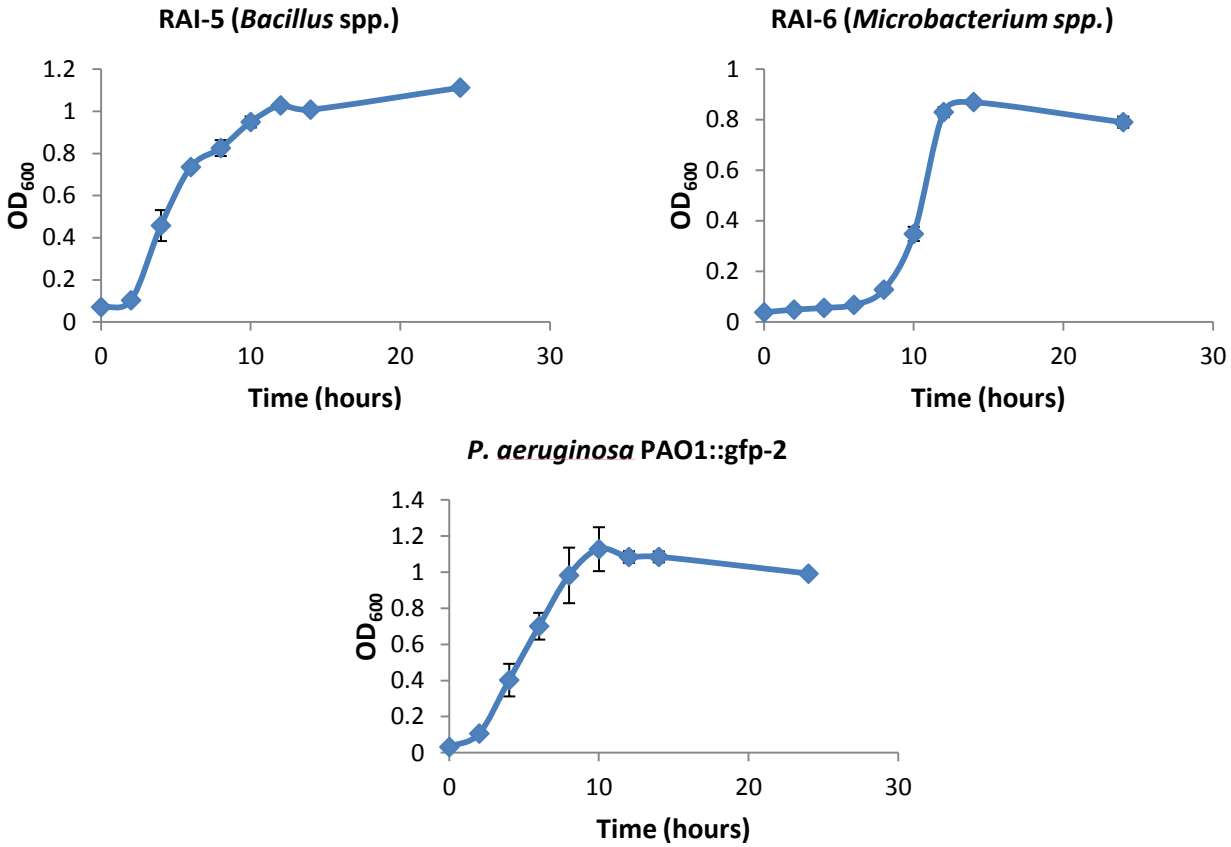


Fig. 4.1: Growth curves obtained for six room air isolates (RAI) and the laboratory strain *P. aeruginosa* PAO1::*gfp-2*. Error bars represent range of OD_{600} values obtained for each time point.

Table 4.1: Maximum specific growth rate of room air isolates and *P. aeruginosa* PAO1::*gfp-2*.

Species	Maximum Specific Growth Rate (μ_{max})
RAI-1	0.43 h^{-1}
RAI-2	0.44 h^{-1}
RAI-3	0.54 h^{-1}
RAI-4	0.26 h^{-1}
RAI-5	0.75 h^{-1}
RAI-6	0.50 h^{-1}
P. aeruginosa PAO1::*gfp-2*	0.66 h^{-1}

4.2 Validation of vortex method for removal of dried cells from coverslips

Sterile glass coverslips were inoculated with washed RAI-3 cells suspended in sterile tap water using the large droplet inoculation method described in Chapter 2. A staring inoculum number of approximately 6 x 10^7 CFU was used in this experiment. Coverslips were dried at ambient relative humidity (60 ± 5%) in a laminar flow ventilation hood for roughly three hours. When coverslips were visibly dry, they were placed in a 50 mL polyethylene tube containing 5 mL of sterile 0.9% NaCl and the number of CFUs removed for various vortex times was determined using plate counts (Fig. 4.2).

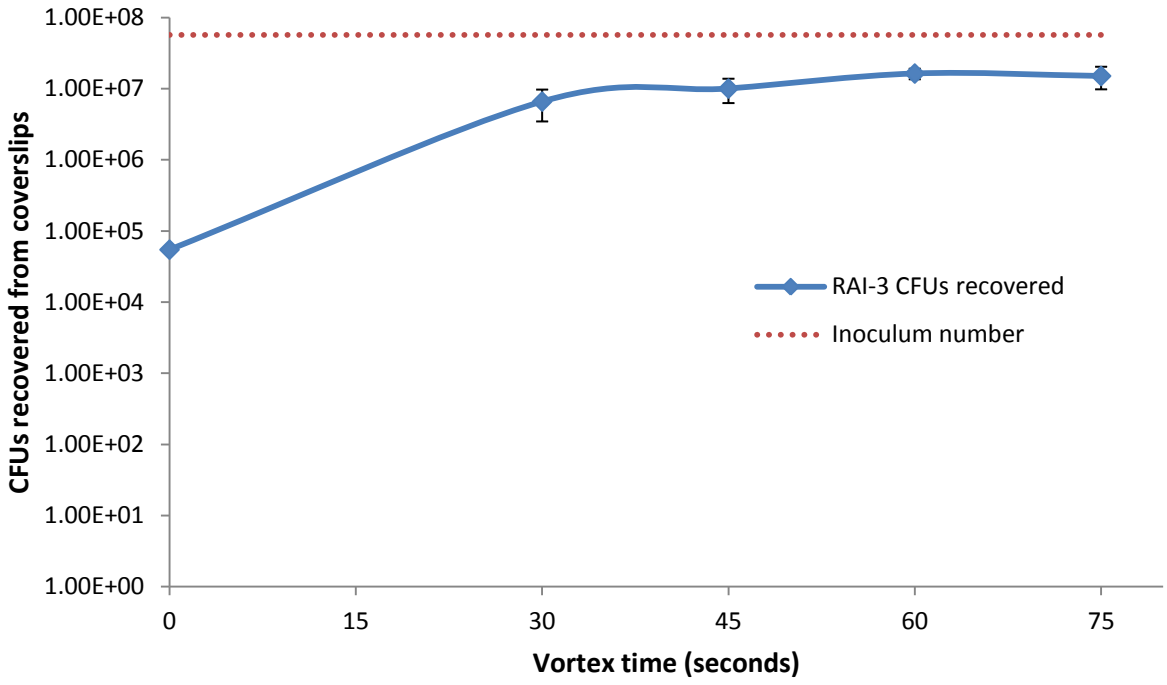


Fig. 4.2: Validation of the vortexing method for removal of dried cells from coverslips. Coverslips were inoculated with approximately 6 x 10^7 cells of RAI-3 sacrificed for enumeration immediately after coverslips were visibly dry (roughly 3 hours in a laminar flow ventilation hood). A vortex time of 60 seconds was used in subsequent experiments, as it gave the highest recovery of viable cells.

4.3 Preliminary survival experiments testing large droplet inoculation method

Several preliminary experiments were carried out using the large droplet inoculation method. In these experiments, sterile glass coverslips were inoculated with RAI-3 cells, PAO1 cells, or a combination of both species. Inoculated coverslips were not maintained in humidity chambers, so desiccated cells were exposed to ambient RH (not measured) for the duration of the experiments. In the first test (Fig. 4.3), a dual-species community scenario was tested in which PAO1 cells were inoculated onto dried RAI-3 cells. Enhanced survival of PAO1 was observed in the dual-species community compared with pure culture survival. A lack of data points between day two and day six meant that the actual survival time of PAO1 in this scenario could not be determined. Thus, in two subsequent preliminary experiments, coverslips were sacrificed daily for the first four days after drying (Figs. 4.4 and 4.5). In these two experiments, which were in effect replicates of each other, multiple inoculation sequences were tested for the RAI-3 and PAO1 dual-species community. Enhanced survival of PAO1 was observed when PAO1 was inoculated onto dried RAI-3 cells, and when co-inoculated and dried with RAI-3. When PAO1 was inoculated as the primary droplet, followed by inoculation of RAI-3, conflicting results were observed between the two replicate experiments. As well, overall survival of RAI-3 seemed to vary between the two trials. Fluctuating environmental conditions such as temperature and relative humidity within each experiment and between the two replicate experiments were most likely the source of these variations. For this reason, all subsequent survival experiments were carried out in humidity chambers with controlled relative humidity.

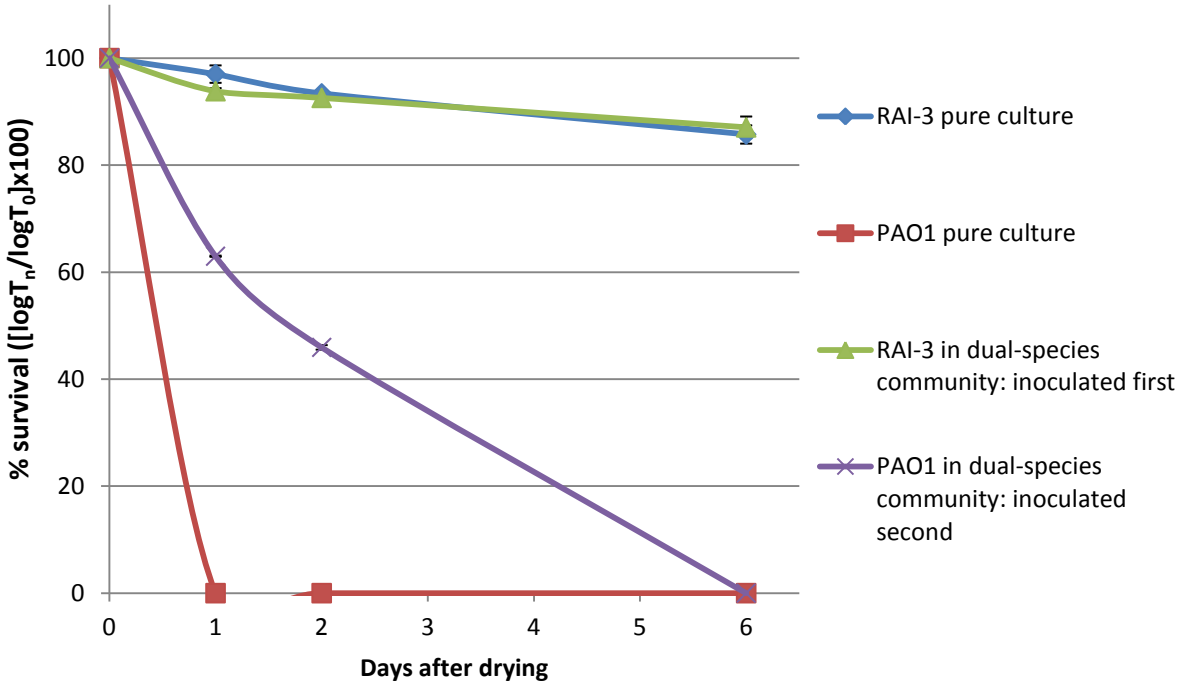


Fig. 4.3: Preliminary survival experiment #1 testing the large droplet inoculation method. Survival curves were obtained for *P. aeruginosa* PAO1::*gfp-2* and RAI-3 in pure culture and dual-species community. The community survival scenario involved sequential inoculation, where RAI-3 was inoculated first and dried, followed by inoculation with *P. aeruginosa* PAO1::*gfp-2*. Relative humidity was not controlled during this experiment, so the dried cells were exposed to natural fluctuations in ambient relative humidity. Values for time 0 represent the inoculum number before drying. Error bars represent range of CFU values obtained for each time point.

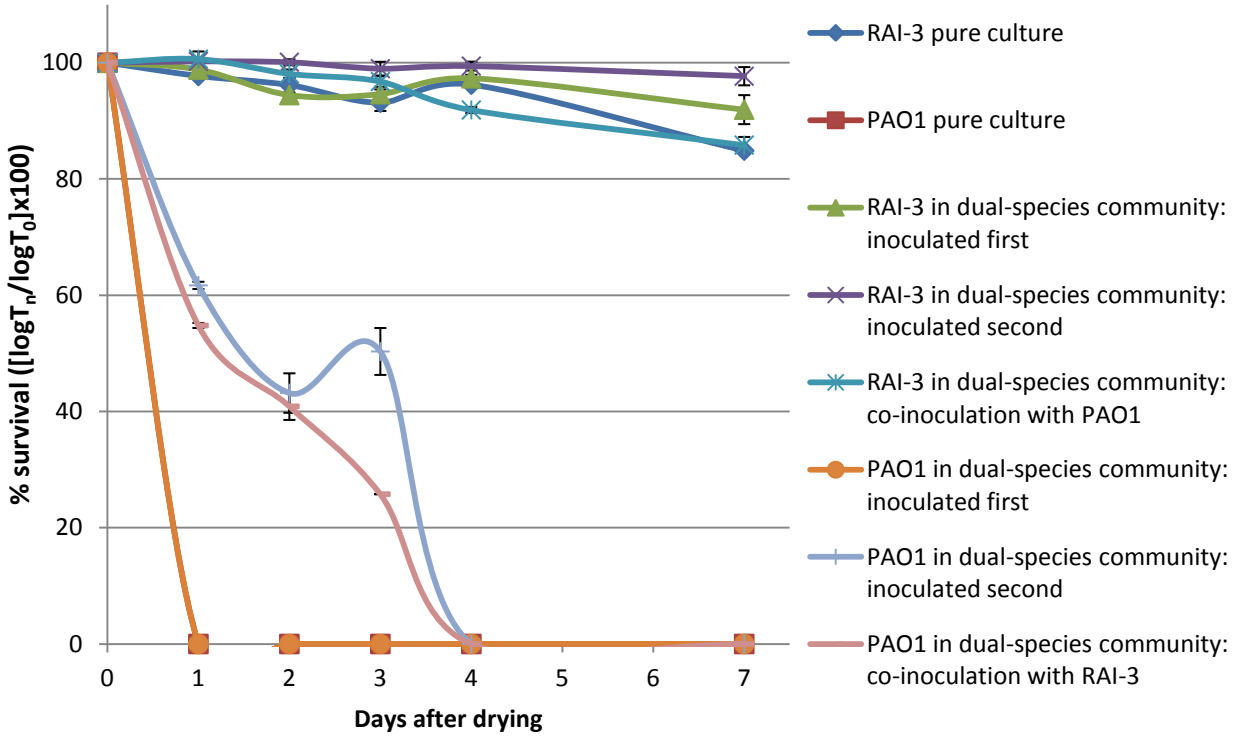


Fig. 4.4: Preliminary survival experiment #2 testing the large droplet inoculation method. Survival curves were obtained for *P. aeruginosa* PAO1::*gfp-2* and RAI-3 in pure culture and dual-species community. The community survival scenarios included co-inoculation of the species (mixed prior to inoculation), and sequential inoculation (one species inoculated first and dried, followed by inoculation with the second species). Relative humidity was not controlled during this experiment, so the dried cells were exposed to natural fluctuations in ambient relative humidity. Values for time 0 represent the inoculum number before drying. Error bars represent range of CFU values obtained for each time point.

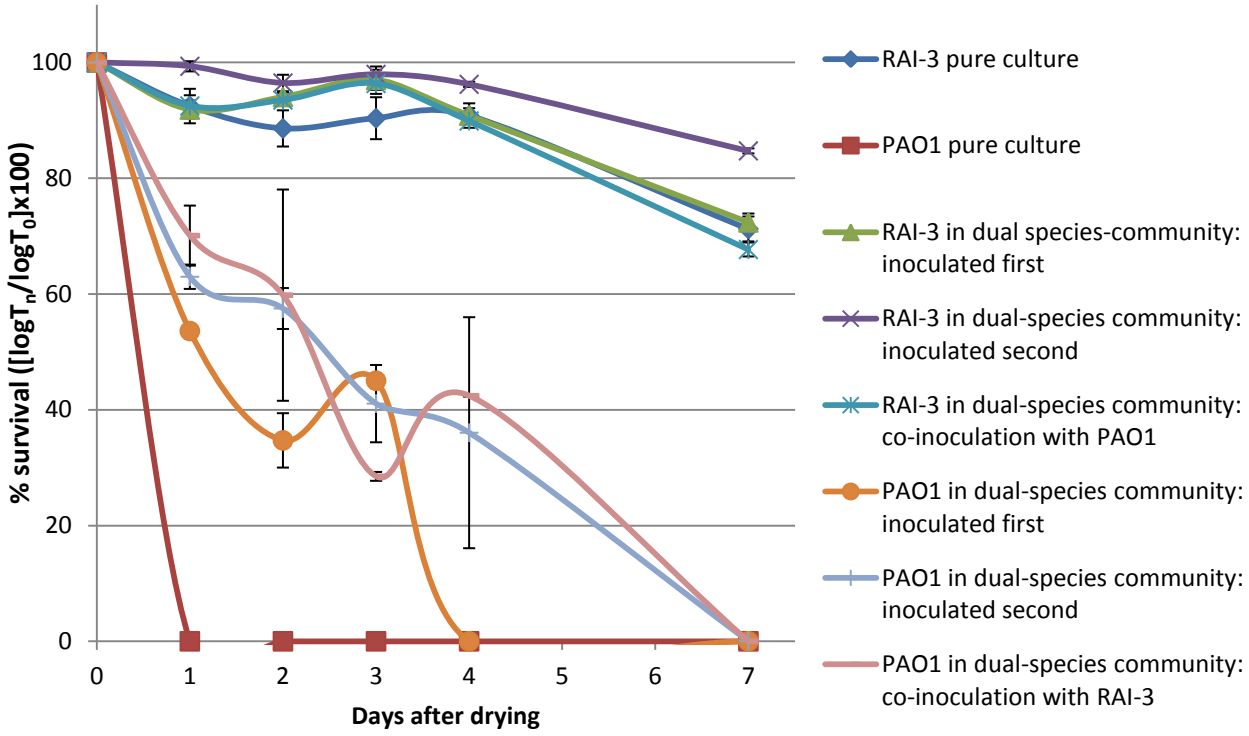


Fig. 4.5: Preliminary survival experiment #3 testing the large droplet inoculation method. Survival curves were obtained for *P. aeruginosa* PAO1::*gfp-2* and RAI-3 in pure culture and dual-species community. The community survival scenarios included co-inoculation of the species (mixed prior to inoculation), and sequential inoculation (one species inoculated first and dried, followed by inoculation with the second species). Relative humidity was not controlled during this experiment, so the dried cells were exposed to natural fluctuations in ambient relative humidity. Values for time 0 represent the inoculum number before drying. Error bars represent range of CFU values obtained for each time point.

4.4 Testing humidity control using saturated salt solutions

The use of saturated salt solutions is a very simple and convenient means of controlling relative humidity during any type of experimental analysis. By providing excess solute, the solution remains saturated even in the presence of modest moisture sinks or sources (Greenspan, 1977). However, relative humidity and the buffering capacity of saturated salt solutions are affected by temperature. Thus, experimentally observed humidity values may differ slightly from the theoretical value. In this thesis, three saturated salt solutions were used to maintain

low, mid, and high humidity conditions within experimental chambers. The observed humidity values differed slightly from the expected values (Table 4.2), but constant humidity levels were maintained nonetheless (Fig. 4.6).

Table 4.2: Saturated salt solutions used to maintain constant relative humidity

Saturated Salt Solution	Observed RH During 7 Day Survival Experiment	Expected RH at 25°C	Reference
magnesium chloride	42 ± 3%	32.78 ± 0.16%	Greenspan (1977)
potassium acetate	25 ± 1%	22.51 ± 0.32%	Greenspan (1977)
potassium sulfate	95 ± 2%	97.30 ± 0.45%	Greenspan (1977)

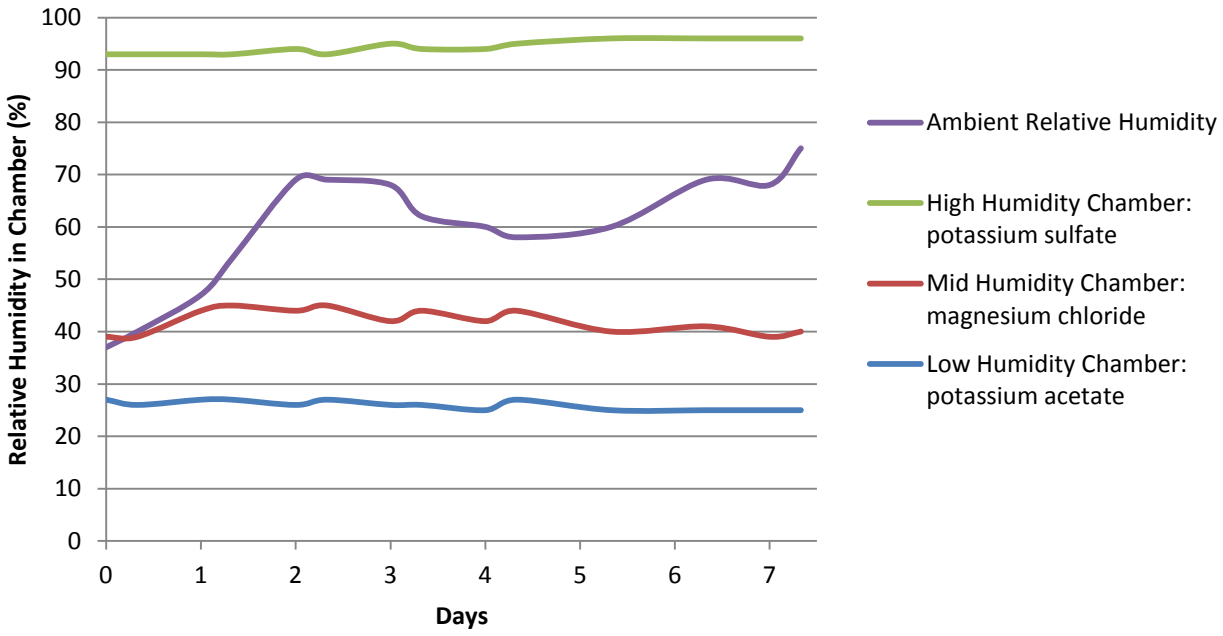


Fig. 4.6: Constant humidity levels were maintained within humidity chambers using various saturated salt solutions. The above graph shows humidity values obtained over the course of a seven day experiment. The humidity in each chamber was monitored using an Indoor/Outdoor Hygro-Thermometer (Extech Instruments, Waltham, MA, USA).

4.5 Determining long-term survival capability of RAI-3

Sterile glass coverslips were inoculated with washed RAI-3 (*Arthrobacter* spp., 99%) cells suspended in sterile tap water and inoculated onto sterile glass coverslips using the large droplet inoculation method described in Chapter 2. A starting inoculum number of approximately 5 x 10^7 CFU was used in this experiment. Coverslips were dried at ambient relative humidity (60 ± 5%) in a laminar flow ventilation hood for roughly three hours. When coverslips were visibly dry, they were transferred to a humidity chamber in which a constant humidity of 42 ± 3% was maintained using a saturated magnesium chloride solution. Coverslips were sacrificed in duplicate for enumeration at time points during prolonged exposure to desiccation (Fig. 4.7).

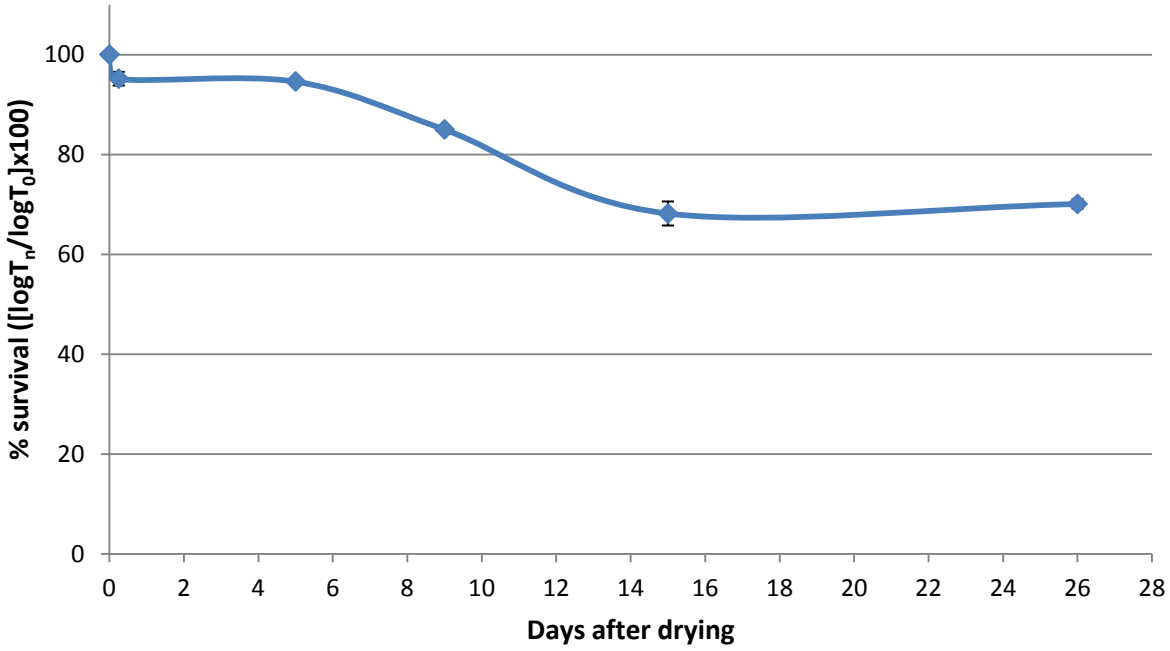


Fig. 4.7: Survival curve obtained for RAI-3 in pure culture during prolonged exposure to desiccation at 42 ± 3% relative humidity. Values for time 0 represent the inoculum number. Coverslips were sacrificed for enumeration immediately after drying, followed by regular sampling for 26 days. Error bars represent range of CFU values obtained for each time point.

4.6 Agar imprint method for qualitative assessment of survival

For many of the survival experiments discussed in Chapter 3, an additional agar imprint method was used to confirm the trend of enhanced PAO1 survival in the presence of RAI-3. Sterile glass coverslips were inoculated using the large droplet inoculation method. At various time points after drying, coverslips were sacrificed and placed face down on 10%TSA plates (cells towards the agar surface). A contact time of two hours proved sufficient for the transfer of cells to the growth medium, and plates were then incubated at room temperature for two days (Fig. 4.8). While this method does not permit enumeration, it is a simple and rapid means of assessing relative survival in different scenarios.

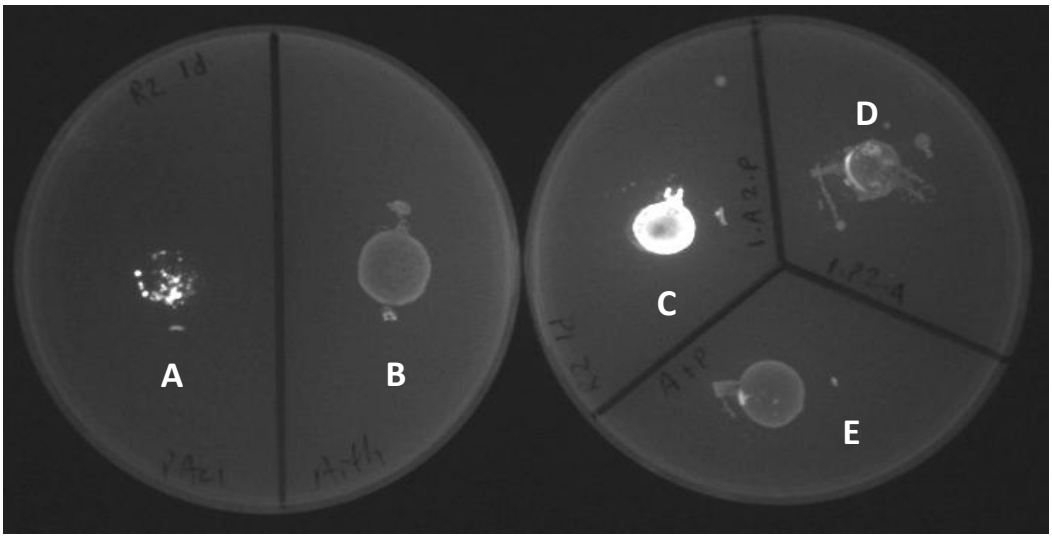


Fig. 4.8: Agar imprint method used for qualitative assessment of relative survival in different scenarios. Coverslips were inoculated and allowed to dry. The above image shows coverslip imprints 1 day after drying. Several survival scenarios involved PAO1 and RAI-3 in pure culture and dual-species community. A: PAO1 pure culture, B: RAI-3 pure culture, C: sequential inoculation of RAI-3 followed by PAO1, D: sequential inoculation of PAO1 followed by RAI-3, E: co-inoculation of PAO1 and RAI-3. The plates were imaged under UV light using a Gel Logic 1500 Imaging System (Kodak, Rochester, NY, USA), so that fluorescent PAO1 colonies could be differentiated from non-fluorescent RAI-3 colonies. In this experiment, best PAO1 survival occurred when PAO1 cells were inoculated onto a dried layer of RAI-3 cells (C).

4.7 In-situ culturing method to investigate microbial load on non-critical surfaces

A new method of in-situ surface culturing was developed to assess the microbial load on exposed surfaces. The advantage of this method compared to traditional methods, such as swabbing and contact plates, is that it does not rely on the removal of cells from the surface. While both of these traditional methods have their own advantages, they likely provide inaccurate estimations of microbial load when dried cells are strongly adhered to surfaces. Furthermore, swabbing also relies on the additional step of removing cells from the swab before inoculation onto growth media, which may represent a further source of error (Moore and Griffith, 2007).

For this in-situ surface culturing method, sterile molten growth media was cooled to slightly above the point of solidification. It was then poured liberally onto the surface of interest, and the bottom of an empty Petri dish was immediately placed face down on the surface to prevent the media from spreading too thin over the surface, and to avoid airborne contamination during incubation on the surface. The solidified media provided a nutrient rich environment for culturable cells to grow into visible colonies while remaining in place on the non-critical surface, thereby providing an estimation of the relative cell density and spatial arrangement of culturable cells on the surface (Fig. 4.9). Excess media that had spread beyond the boundaries of the dish was wiped away upon solidification.

Despite the obvious advantages of this method over swabbing and the use of contact plates, there are several drawbacks that may limit its use as a standard method for assessing surface cleanliness. Firstly, it requires that the solidified media be left untouched to allow for growth of visible colonies, which may be impractical for frequent-use surfaces. As well, the use of this method in areas which should normally be sterile may not be ideal, since it results in a high degree of microbial growth. Nonetheless, this method may prove to be a beneficial addition

to the range of tests that are currently used to investigate surface cleanliness, and may also provide a means to test the efficacy of new antimicrobial compounds and surface treatments.

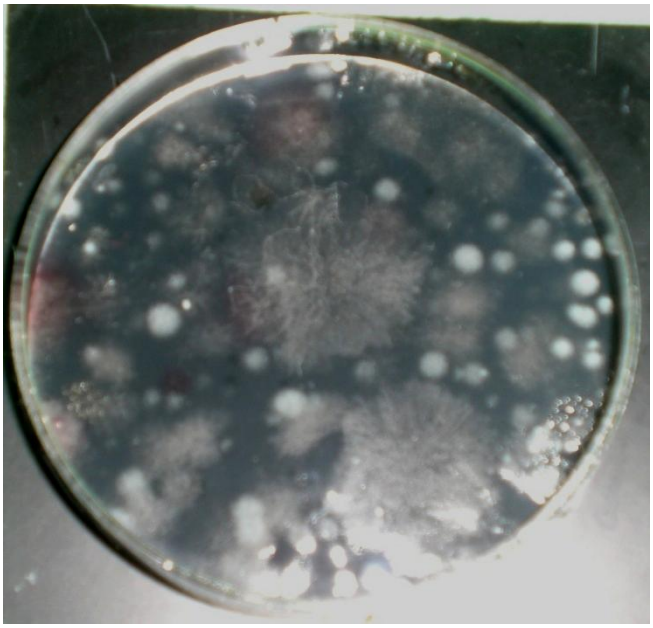


Fig. 4.9: Example of the microbial growth resulting from in-situ surface culturing. In this experiment, non-selective growth media was poured over a non-critical surface and left untouched for six days. A high degree of microbial growth was observed, comprised of both bacterial and fungal colonies. Future use of this method to assess surface cleanliness may utilize an antifungal or antibacterial compound in the growth media to select for the microorganisms of interest.

4.8 Development of an aerosol chamber to simulate bioaerosol generation and deposition

The vast majority of studies which investigate bacterial survival on inanimate surfaces do so by inoculating large droplets (usually 0.05-0.2 mL) onto test substrates. While this is an effective method that allows rapid and reproducible inoculation of a variety of substrates, it does not accurately represent the natural deposition of cells from indoor air onto exposed surfaces. A small number of studies have investigated the survival of deposited bioaerosols (Wilkinson, 1966; Robine et al., 1998, Robine et al., 2000) using some form of aerosol chamber, but none could be found which tested survival of deposited bioaerosols in community scenarios. In this thesis, an aerosol chamber was constructed to address this gap in the literature.

Development of this bioaerosol deposition method required a significant amount of

designing and testing. It was determined that concentration of each test species in the nebulizer should be approximately 10^9 CFU/mL, and an aerosolization time of 10 minutes followed by a settling time of 5 minutes was ideal to achieve starting inoculum numbers similar to those used in the large droplet inoculation method.

The deposition of cells onto glass slides using the aerosol chamber was confirmed with microscopy. Cells of the indoor bacterial flora were obtained by swabbing a non-critical surface and subsequently enriched in broth media, before aerosolization and deposition in the aerosol chamber. Prior to staining, the glass slides were embedded in 1% agarose in order to prevent substantial loss of cells from the surface due to repeated staining and rinsing. Syto9 stain (0.2 µl/ml) was overlaid onto the embedded slides and the staining was carried out in the dark for 15 minutes, after which excess stain was removed by submerging the slides in sterile milliQ H_2O for 15 minutes. Slides were imaged using an epifluorescent microscope (Leica Microsystems, Concord, ON, Canada) (Fig. 4.10).

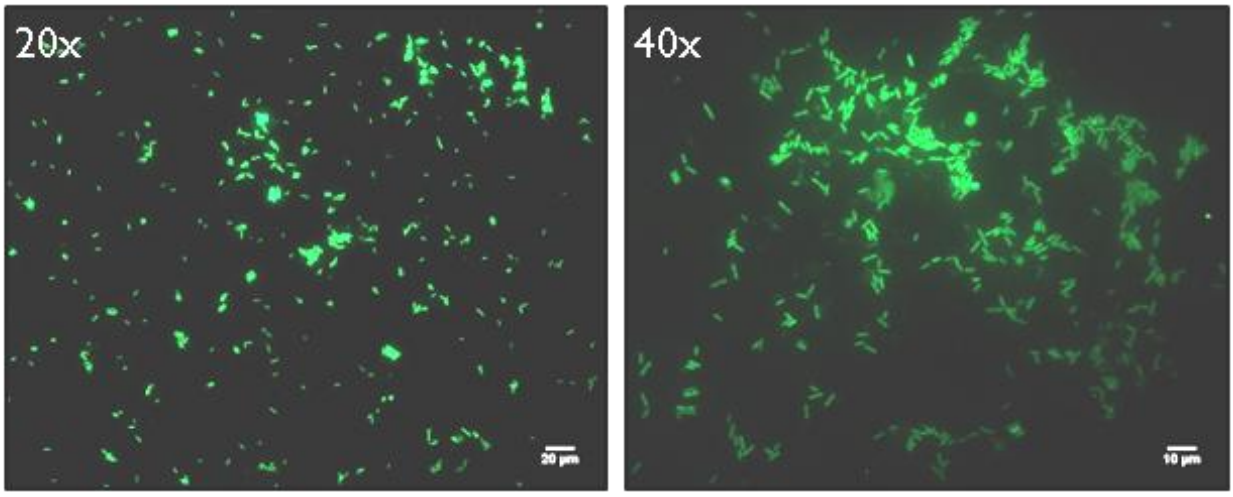


Fig. 4.10: Confirmation of successful deposition of cells in the form of bioaerosols. Mixed community cells were aerosolized for 10 minutes and allowed to settle onto glass microscopy slides. Slides were embedded in 1% agarose and stained with Syto9.

4.9 Modification of the aerosol chamber for testing bacterial redispersal due to airflow

It is believed that everyday housekeeping activities such as bed making, drawing curtains, sweeping, and vacuuming, can disperse bacteria-carrying particles into the air **(**Ayliffe et al., 1967; Beggs, 2003). It is also thought that indoor airflows resulting from ventilation systems or the movement of people through a room may cause redispersal of viable cells from exposed surfaces. The degree of dispersal associated with these activities is unclear and often debated in the literature. In an early study, the total viable count of bacteria in hospital room air rose from a baseline of approximately 560 CFU/m^3 before bed making, to greater than 6,000 CFU/m^3 during bed making (Greene et al., 1960). In a subsequent study, Ayliffe et al. (1967) concluded that only a small fraction of cells can be dispersed through normal housekeeping activities. Likewise, although Hambraeus et al. (1978) acknowledged that these activities likely do cause at least some dispersal, they concluded that dispersal of *S. aureus* from floors does not increase the risk of infection during medical procedures.

More recently, researchers have tried to develop accurate mathematical models for simulating and predicting dispersal patterns. These models however, have so far focused primarily on the redispersal of fungal spores (Buttner et al., 2002), allergen-containing particles (Gomes et al., 2007; Karlsson et al., 1996), general particulate matter (Hu et al., 2007), or freeze-dried bacterial spores (Karlsson et al., 1999). No recent studies could be found which focus specifically on the redispersal of whole vegetative bacteria from indoor surfaces.

The aerosol chamber used in this thesis can be modified in future experiments to assess the potential for deposited cells to be redispersed back into the air column. A number of exploratory experiments were carried out to test several configurations of the aerosol chamber (Fig. 4.11). In general, all redispersal experiments involved aerosolizing a washed bacterial suspension, and allowing bioaerosols to deposit onto test surfaces within the aerosol chamber.

At time points after deposition and drying, redispersal was tested by introducing a stream of sterile air into the chamber for several minutes (usually 10-60 minutes). Airborne particles were collected by bubbling effluent air into 2 mL of 0.9% NaCl, which was subsequently serially diluted and plated. In most of these redispersal experiments, no culturable cells were detected in the effluent air, and only a very small number (i.e. 120 CFUs) were recovered from the chamber air in one of the experiments, despite a high inoculation number on the surface. A lack of redispersed cells in these preliminary tests however, does not necessarily indicate that the phenomenon of redispersal cannot occur. Future experiments should be carried out to determine the best configuration of the aerosol chamber to facilitate accurate estimation of the risks associated with cell redispersal, and to allow for the testing of airflows representative of high-risk indoor environments. As well, the aforementioned experiments focussed on redispersal of cells from sterile surfaces. It is likely that a higher degree of redispersal would be observed if cells were initially deposited onto a soiled surface containing dust, soil, or other organic material.

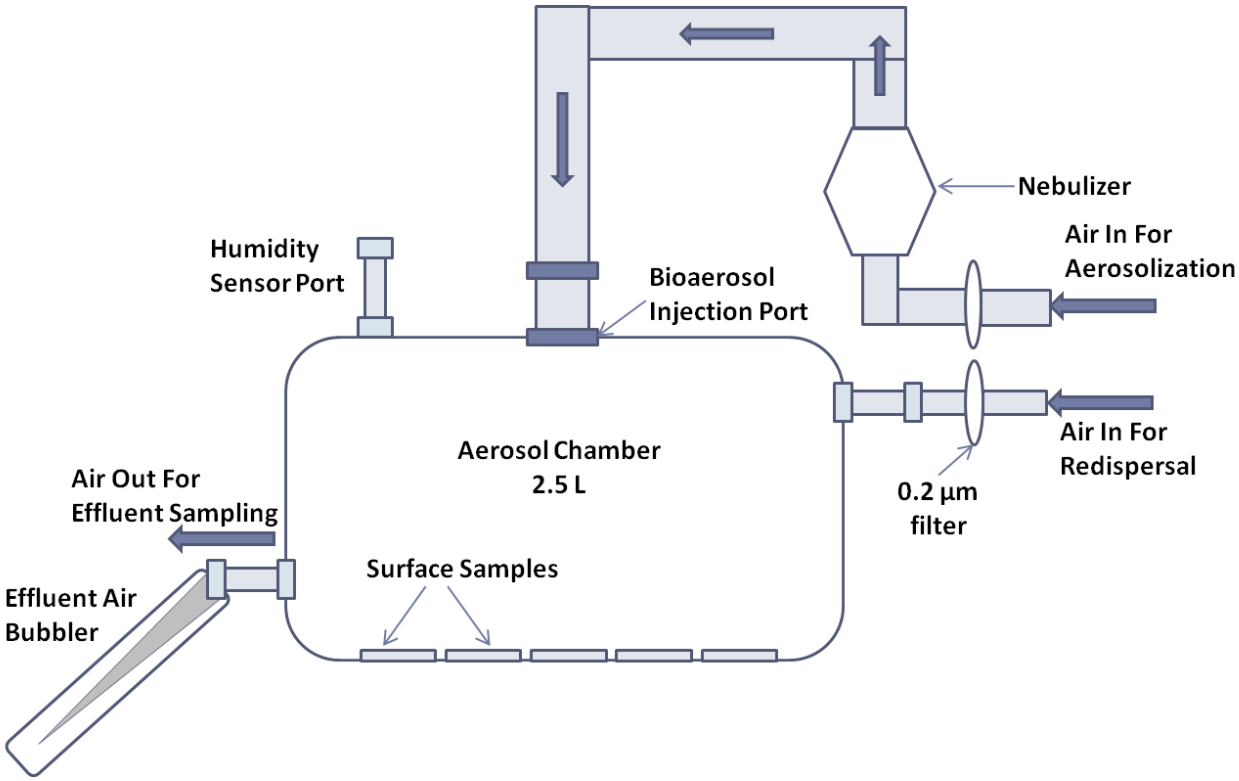


Fig. 4.11: Example of an aerosol chamber set-up used for redispersal experiments. Several variations of the above design were tested, with different locations for the air inlet and effluent sampling ports, although no redispersal was observed in most experiments.

CHAPTER 5: CONCLUSIONS AND FUTURE WORK

The findings presented in this thesis represent an exploratory study into microbial ecology at solid-air interfaces in dry indoor environments. The fate and dangers associated with microbes at solid-air interfaces is poorly understood and typically only receives attention during nosocomial outbreaks. Most of the research that has been carried out studying bacterial survival on indoor surfaces has done so using pure cultures, despite the predominance of mixed communities in the environment. The results of this research suggested that bacterial species within the normal indoor bacterial flora might provide a protective or beneficial environment for extended survival of exogenous pathogens. This is particularly significant in high risk areas such as in clinical environments, where many patients are in a state of weakened immunity and are highly susceptible to infection.

This thesis also highlights the need for new approaches that would more accurately represent the deposition and behaviour of bacteria in indoor environments. In our study, a bioaerosol deposition method was used as an alternative to the traditional large droplet inoculation method, since it can simulate the deposition of cells from the air onto exposed surfaces. In the future, this method can be improved by allowing for greater control over the sizes of aerosol droplets produced in order to more accurately simulate bioaerosol generation by activities such as sneezing or coughing. As well, introducing airflow through the chamber after deposition and during prolonged desiccation could be used to test the effect of natural indoor airflows on bacterial survival and subsequent redispersal of cells back in to the air column. There is also a need to develop standard methods to assess surface cleanliness. In hospitals and other medical settings, surface cleaning regimens likely vary in their effectiveness, and may leave significant numbers of bacterial cells on surfaces even after disinfection.

There are many aspects of this research that deserve closer attention in future studies.

Survival of *P. aeruginosa* appeared to be enhanced when dried in the presence of an isolate (RAI-3) closely related to *Arthrobacter* spp. It would be interesting to repeat these dual-species community experiments using other isolates to see if the trend of enhanced survival is maintained. If better survival is indeed seen in the presence of other desiccation-tolerant isolates, but not when dried with desiccation-sensitive species, it would suggest that in fact sensitive species are able to benefit from the tolerance mechanisms possessed by some community members.

The RAI-3 isolate obtained in this study appears to be a good model organism for testing the long-term effectiveness of novel antimicrobial surface treatments, since consistent cell numbers were maintained on untreated control surfaces during extended periods of desiccation. However, it would be advantageous to first elucidate the desiccation-tolerance mechanisms possessed by RAI-3 cells. This could be partly accomplished by monitoring the production of trehalose and other sugars by RAI-3 cells during dehydration and desiccation. Trehalose production has been observed for *Arthrobacter* spp. during osmotic stress and nutrient starvation (Zevenhuizen, 1992), although no studies could be found which focussed specifically on trehalose production during desiccation. If extracellular accumulation of sugars and other EPS molecules is observed during drying, then this would support the hypothesis that desiccation-sensitive cells require close contact with -tolerant cells in order to exhibit enhanced survival. Furthermore, if trehalose and EPS production is inhibited when RAI-3 cells are exposed to high humidity, then it would further support this hypothesis, since in the present study *P. aeruginosa* did not experience enhanced survival in dual-species community at high humidity.

Molecular analysis of the artificial community during desiccation suggested that community DNA remained intact for the duration of the experiment. This indicates that despite

exhibiting a loss of culturability, cells may be able to maintain viability, and therefore still pose a threat for infection. Subsequent studies could be performed which use other endpoints beside culturability and DNA integrity as a means of assessing bacterial survival. Specifically, viability during periods of desiccation could be tested using live/dead staining and microscopy, which would indicate whether any species have in fact entered the viable but non-culturable state. As well, the metabolic activity of cells during periods of dehydration, desiccation, and subsequent rehydration could be monitored by measuring CO_2 accumulation within experimental chambers. Specifically, the respiration of deposited bioaerosols could be examined by adapting the aerosol chamber described in this thesis and integrating it into a CO_2 monitoring system similar to the CO_2 Evolution Measurement System developed by Kroukamp and Wolfaardt (2009), and using a procedure comparable to the one employed by Boylen et al. (1973), which was used to measure respiration rates of *Arthrobacter crystallopoietes* in the dry state.

Overall, the work presented in this thesis suggests that the ecology of bacteria in desiccated environments in indoor settings is likely more complex than previously believed. A number of questions exist regarding microbial colonization of solid-air interfaces, such as i) do cells survive after drying on surfaces?, ii) how long can they maintain viability?, and iii) what are the factors that influence the survival of bacteria after drying on surfaces? This thesis aimed to address these three questions, but future work should also try to address iv) how are cells able to protect themselves from the harmful effects of desiccation? and v) how can these mechanisms be exploited to prevent the long-term colonization of high-risk surfaces? If nosocomial infections, which continue to plague healthcare systems around the world, are to be reduced and one day eliminated, further studies needs to be carried out to gain a more thorough understanding of the ecology of bacteria at solid-air interfaces.

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