Haddrell, A., & Thomas, R. (2017). Aerobiology: Experimental considerations, observations and future tools. *Applied and Environmental Microbiology*, *17*(83).

DSTL/JA100134

Aerobiology: Experimental considerations, observations and future tools

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Abstract

Understanding airborne survival and decay of microorganisms is important for a range of public health and biodefence applications including epidemiological and risk analysis modelling. Techniques for experimental aerosol generation, retention in aerosol phase and sampling require careful consideration and understanding so that they are representative of the conditions the bioaerosol would experience in the environment. This review explores current understanding of atmospheric transport in relation to advances and limitations of aerosol generation, maintenance in the aerosol phase and sampling techniques. Potential tools for the future are examined at the interface between atmospheric chemistry, aerosol physics and molecular microbiology that could explore heterogeneity and variability at the single droplet and single microorganism level within a bioaerosol. The review highlights the importance of method comparison and validation in bioaerosol research, and the benefits application of novel techniques could bring to increased understanding of aerobiological phenomena in diverse research fields, particularly during the progression of atmospheric transport where complex interdependent physicochemical and biological processes are occurring within bioaerosol particles.

INTRODUCTION

Aerosols injected into the atmosphere from the biosphere (bioaerosols) account for a significant portion of all atmospheric aerosols (1). Despite their low numbers relative to other natural aerosol, bioaerosols (whose sources include microorganisms contained within windblown dust and sea spray) are speculated to impact climate through behaving as efficient cloud condensation nuclei (2-3). Biological aerosols are also important from the perspective of human health being intimately involved in the transmission of many respiratory pathogens (4, 5).

Risk analysis modelling aims to develop predictive models of transmission and infection based on laboratory generation of aerosols containing respiratory pathogens. These experimental models are invaluable for understanding epidemic transmission, developing infection control measures and advising bioterror preparedness for public health (6-8). Effective risk modelling requires an in depth understanding of experimental aerosol techniques and their potential impact on the final outcome, whether that is aerosol decay, transmission rate or infectious dose.

This article reviews the current understanding, advances and limitations in laboratory aerobiological studies where the relationship between microorganism preparation, aerosol generation, evaporation, transport and fate cumulatively may affect the final outcome of inhalational infection or survival in the environment. In this review, the term “bioaerosol” will be limited to refer explicitly to infectious aerosol droplets containing living species, specifically bacteria and viruses; the study of this subset of bioaerosol comes with its own unique set of challenges that need to be recognized and addressed. The PubMed database was searched to identify relevant studies

using the strings: aerosol AND survival, bioaerosol AND generation, bioaerosol AND sampling. The terms bacteria and virus were interchanged for the term survival in the first search string; only published studies were included. References with no relation to bioaerosol as defined as 'infectious aerosol droplets' (e.g. fungal spores, pollen) were generally discarded unless the technology could be applied to the field. Retrieved studies were also reviewed for additional references. Although intrinsically linked to the general theme of this review, the development of inhalational animal models to replicate human disease is considered outside the scope and readers are directed to an extensive literature in this field (9-11).

AEROSOL GENERATION, SAMPLING AND POST-PROCESSING CONSIDERATIONS

Aerosol generation and sampling prior to microbiological analysis are conducted for a range of bioaerosol related research activities (e.g. determination of aerosol decay rates and inhalational infectious dose, efficacy of decontamination strategies, and evaluation of bioaerosol sampling technologies). These dynamic processes can cause damage due to shear forces acting on the microbial cells (12-27). Table 1 outlines some major aerosol generators and samplers used in aerobiological studies and the operating mechanisms. The majority of studies use reflux aerosol generators in conjunction with impingement to collect the generated aerosol. This system can be safely used in biocontainment laboratories for inhalational challenges and aerosol fate studies. However, comparative studies show that refluxing nebulizers produce the greatest loss of physiological function as a function of time in bacteria (16, 19-21, 24). The loss of function has been linked to membrane damage (13, 20, 24), release

of ions into media (e.g. PO_4^{2-}; 28), cell fragmentation (15, 23), reduction in ATP activity (29) and magnitude of associated electrical charge (30) as the bacteria remaining in the nebulizer repeatedly pass through the devices nozzle. Similar effects are observed for viruses (25). Repair of bacterial cells damaged by nebulization appears to be an energy dependent process with a requirement for divalent cations although independent of *de novo* RNA or protein synthesis (13, 31); it is unlikely that repair occurs in viruses due to their reliance on host cell factors for protein transcription and translation. In contrast, it has been reported that damage is reduced in non-refluxing aerosol generators where the microorganisms pass through the nozzle once (16, 24).

Sampling methods for airborne microorganisms include impingement, impaction, filtration, cyclonic separation, and electrostatic precipitation. This review will not cover all bioaerosol samplers, rather selecting the main sampling mechanisms and representative sampler models. The reader is directed to a couple of comprehensive reviews on bioaerosol sampling for further detail (32, 33). Each sampling technique has advantages and disadvantages for sampling microbial aerosols (Table 1) with the potential to cause microbial damage. Dependent on the microbe this damage may be transient: for example, impingement (AGI-30; 15 to 60 min) caused structural damage to *Pseudomonas fluorescens* cells with recovery achieved on non-selective media (15). Aerosol sampling times for determining infectious dose and aerosol decay rates generally range from 1-10 min which minimize the effects of microbial damage (22, 34). However, for infectious aerosols, few comparative studies of the bioefficiency of different sampling mechanisms. Where studies comparing samplers have been conducted, differences between microbial structures influence sampler bioefficiency; for example, infectivity and culturability differences were observed

between bacteriophages and influenza A virions sampled by the SKC biosampler and NIOSH cyclone (25, 35). Similar species dependent effects have been observed for bacteria in sampling bioefficiency; in particular *Bacillus* endospores tend to be less affected by aerosol sampling method (15, 17, 21, 22). One reason for differences in sampler bioefficiency is variation in sampling velocities that for impingement reaches 260 $m \cdot s^{-1}$, ten-fold greater than other samplers (36; Table 1). Secondly, the rapid rehydration that occurs during sampling can be detrimental to microorganisms (37-39).

Minimising stresses occurring during aerosol generation and sampling is hence critical to accurate representation of aerosol decay and infectivity. Aerosol generation stresses can be reduced by using single-pass devices that reduce the probability of a microorganisms being damaged (24). Depending on sampler choice, maximising recovery of microbes can be achieved in a number of ways. Prolonged sampling times is a consistent cause of reduced viability and hence collection times across all types of samplers and should be minimized (22, 40). The cell membrane is a major site of damage for Gram negative bacteria being aerosolised as sampled, demonstrated by increased sensitivity to hydrolytic enzymes (12). Impingement requires collection into liquid which can be optimised to reduce osmotic shock and maximise repair and recovery. For example, addition of compatible solutes and scavenging enzymes (i.e. trehalose, raffinose, polyhydric alcohols, betaine and catalase) can facilitate survival following the stresses associated with aerosol generation, transport and sampling (38, 41-46). Particle bounce and viability loss in impactors for vegetative *Bacillus subtilis* and *Escherichia coli* cells was reduced by applying a thin film of mineral oil significantly enhancing collection efficiency (47). Filtration methods provide high physical collection efficiencies, but bioefficiency can

be dependent on filtration time and post-processing procedures (21, 24, 48, 49). A major problem with filtration samplers is continued drawing of air through the filter desiccates collected microorganisms in a time-dependent manner. However, filtration onto gelatin membranes provides a medium that retains moisture and can be placed into warm media to recover collected microorganisms providing good bioefficiency (21, 24).

Post-sampling enumeration and storage are additional considerations. Enumeration can introduce error as organisms can be sensitive to impaction onto an agar surface (50), sensitive to the plating media (15) and the process of spread plating (51-53). Direct methods such as microscopy or flow cytometry in conjunction with various dyes or quantitative polymerase chain reaction (PCR) can indicate physiological activity of the collected microorganisms (15, 17, 54). Storage temperature, sampling solution and length of time can prompt microbial replication (or death) causing misrepresentation of the actual viability of the sampled bioaerosol (48). Samples should be processed as soon as possible after aerosol sampling; however this is highly dependent on the microorganism as for example, *Bacillus* endospores have been demonstrated to be less affected by storage temperature (4 and 25 °C) compared to *Escherichia coli*; however compared to immediate enumeration, both species had increased counts after extended periods of storage at 25 °C (10 and 24 h for *B. subtilis* and *E. coli* respectively) indicating significant disaggregation and/or multiplication in the collection medium, which in this case was sterile deionized water containing a small quantity of detergent (48).

The data indicates that the method of aerosol generation can damage the microorganism at the subcellular level, at the very least subtly, and influence resultant estimates of microbial viability in the aerosol phase. None of these

mechanisms are entirely representative of the natural transmission mechanisms of respiratory pathogens e.g. coughing and sneezing followed by deposition in the respiratory tract (4, 5). The complexity of fluid fragmentation and droplet formation of oro-respiratory secretions during coughs and sneezes has recently been elucidated with the viscoelastic properties of respiratory secretions playing a defining role in final droplet size (55, 56). Viscoelasticity of respiratory secretions will change with anatomical location (e.g. nasal, bronchial) and disease state (e.g. chronic bronchitis, sinusitis, cystic fibrosis) as a result of changes in mucin content which will also affect droplet sizes (57, 58). Natural aerosol transmission events are likely to be less violent than the aforementioned aerosol generation processes. Therefore, selection and validation of experimental regimes (aerosol generator, spray fluid composition and sampling) to minimize microbial damage, promote maximal recovery and most closely replicate the natural event being modelled, is important for interpretation of aerosol data used in risk analysis models. Based on this review, and more extensive reviews on sampling methodology (32, 33) it is apparent that given the variability in microorganisms responses to the stresses of aerosol generation and collection, then it is advisable to perform method validation for each particular microorganism. Testing a range of aerosol generators and samplers to ensure the behaviour of the microorganism within the system is understood facilitates appropriate selection of apparatus and methodology to maximise recovery during enumeration.

AEROSOL TRANSPORT AND PHYSICAL PROCESSING

The physicochemical properties of bioaerosol particles govern all of the biological processes within. The conditions in a bioaerosol particle that a microorganism will experience can be dramatically different than in bulk liquid; the solute concentrations commonly reach supersaturation (59), while the rate of water transport within the

droplet can vary by orders of magnitude (60). Both of these properties are regulated by total water present in the droplet. Thus a detailed understanding of the hygroscopic properties of a bioaerosol as a function of solute composition (including biological species itself) is critical for understanding and predicting longevity and overall infectivity.

The typical trajectory in RH for a respiratory pathogen would be high at the point of dispersion (>95%) to low during atmospheric transport (ambient relative humidity, RH) to high upon inhalation (>95%) (61). During its lifetime, the water activity (a_w) within a droplet equilibrates with the atmospheric RH through either the addition or removal of water (62). From droplets larger than 100 nm in size, the water activity is equal to the gas phase RH at equilibrium. The rate at which this mass flux occurs and the final particle size attained are a reflection of the temperature and humidity of the gas phase of the aerosol and the droplet solute (63, 64). Importantly, all microorganisms require water for activity as critical enzyme driven biochemical reactions (e.g. respiration). Interestingly, in studies looking at osmotic tolerance in bulk liquid phase, depending on bacterial species, multiplication and growth is inhibited at a_w values of 0.86 – 0.97 with further reductions inducing dormancy or eventually reducing viability (65, 66).

The hygroscopic behaviour of any multicomponent aerosol is dependent on the relative abundance of each chemical species in the solute, where each component will contribute a proportion to the uptake or loss of water (62). This paradigm holds true for bioaerosol, for example it has been shown that the solute concentration affects the hygroscopic growth of aerosolized *B. subtilis* and *Pseudomonas fluorescens* vegetative cells (67). However, to study the hygroscopic behaviour of aerosol where the aim is to generate predictive models, much information about the

solute is required. The relative abundance of each component within the aerosol is mandatory (68-72), as is a detailed understanding of how the various components within the solute interact with one another (73). While this is somewhat straight forward with regards to non-biological aerosol, it remains a major challenge in bioaerosols. For example, infected individuals coughing and sneezing will produce larger droplets with different concentrations of mucus and other organic and inorganic solutes compared to healthy individuals (58). Similarly, in laboratory studies, microbial culture conditions (liquid broth, solid agar and nutrient composition) and growth phase affect the concentration and types of nutrients present in the spray suspension and these factors influence aerosol survival (25, 74-78). Indeed, survival of a viral simulant, the bacteriophage MS2, differed in human derived saliva, artificial saliva and cell culture medium, with greatest decay observed in human derived saliva (79). This has been observed for other viruses and bacteria upon comparing survival after aerosolization from body fluids (natural or synthetic) and culture medium (80-83). This highlights the caution needed in extrapolation of results from the experimental to *in vivo* situations being modelled in risk analysis.

The primary challenge in experimental studies of the factors that regulate the hygroscopic behaviour of bioaerosol is to control and know the complete composition of the bioaerosol droplets. For example, a simple factor such as control of the number of organisms per droplet/particle is not trivial using conventional aerosolization processes. To attempt to address this specific issue in studies of laboratory generated bioaerosols, a particular size is selected for a nebulized and dried bioaerosol sample allowing estimation of the number of species per droplet prior to hygroscopic analysis (16). For more complex (and atmospherically relevant) bioaerosol, the hygroscopic behaviour of anthropogenic bioaerosol has been

estimated indirectly (84, 85). In these studies, the relative growth in bioaerosol particle size with increases in RH was estimated through correlation analysis between the temporal size distributions (aerodynamic diameter) of airborne fungi with meteorological information (RH).

Thermodynamic models to predict the hygroscopic behaviour of aerosol (e.g. Universal Quasichemical Functional Group Activity Coefficients; UNIFAC) have been used for bioaerosols to limited success (59, 86). Generally, these models are able to predict the hygroscopic behaviour of large and complex organic molecules through parameterization of the functional groups present (such as carboxylic acids; 87). Even though, organically, bioaerosol consists primarily of sugar alcohols and highly polar sugars (88), it remains unclear the extent to which these models can be used to predict the hygroscopic behaviour of bioaerosols (89). The reason for this is that even when the relative abundances of functional groups and chemical species within a single bioaerosol droplet are known, the accumulation of noncovalent interactions between these species is not; the presence of cellular membranes within the droplet could kinetically limit the hygroscopic behaviour of all the chemical species within the aerosol.

The limited number of comprehensive studies that explicitly study the physicochemical properties of bioaerosol is problematic. Their absence has constrained the means by which the longevity of suspended bioaerosol can be investigated.

DETERMINING BIOAEROSOL LONGEVITY

Bioaerosol longevity is simply the length of time in which a biological species will remain either infectious or viable while suspended as a single particle. In an ideal experiment, the entire composition of the target bioaerosols would be explored; as discussed in previously sections this is technically challenging due to the selectivity of samplers and the heterogeneity of bioaerosol composition. Despite this, numerous studies on bioaerosol longevity have been published.

Techniques for investigating survival of bioaerosols *in vitro* (Table 2) tend to either maintain the particles in the air column (i.e. 'dynamic bioaerosols') or captured on fine substrate such as spider silk or glue fibres (i.e. 'captured bioaerosols'). The rotating drum is probably the standard procedure used for aerosol longevity studies based on Goldberg and colleagues seminal design (90). Modifications have permitted greater control (e.g. *in situ* monitoring of parameters) and accessibility to a range of environmental parameters (e.g. temperature, UV, volatile organic compounds), and the suspension of larger aerosol particle sizes for sufficiently long periods of time (91-94). Methods based on capturing bioaerosols on microfibers derived from spider escape silk and glue gun fibres have been utilised with success (78, 95-97). Comparative studies on filoviruses have demonstrated that microthread captured bioaerosols decay at a similar rate as those held dynamically within rotating vessels (34, 98).

The methods for retention of microorganisms in the aerosol phase have been used extensively to determine biological decay in the airborne state as a function of time and a range of environmental conditions (Table 3). The aerosol is sampled at time intervals and the number of viable microorganisms enumerated enabling calculation of aerosol decay rate. Sampling method and subsequent microbiological processing and enumeration can alter the number of recovered microorganisms (15, 17, 21, 22).

Therefore it is important to minimize microbial stress during aerosol collection to facilitate accurate calculation of the decay rate. During method validation, it is important to differentiate biological decay from physical losses due to deposition on the walls of the vessel or removal from the microthreads due to turbulence (or the presence of antimicrobial substances on the silk). Physical loss in aerosol systems is determined by using physical tracers that will not biologically decay such as *Bacillus* spores, chemicals (e.g. fluorescein) or polymer beads (21, 99, 100). The decay rates of the target microorganism and the physical tracer can be compared and the true biological decay rate determined.

A disadvantage of these techniques is that they sample bulk aerosol and it is difficult to develop an appreciation of microenvironment heterogeneity occurring within individual aerosol droplets from the physicochemical and biological perspective. For example, each individual aerosol droplet is likely to have a different chemical composition, exacerbated by differences in particle size that manifest themselves biologically on the microorganisms incorporated within the droplets. Such differences may be a source of variability in how microbes respond and survive aerosol transport.

ENVIRONMENTAL FACTORS AFFECTING MICROBIAL LONGEVITY DURING ATMOSPHERIC TRANSPORT AND BACTERIAL SURVIVAL MECHANISMS

A large number of environmental and meteorological factors can influence microbial survival during aerosol transport (Table 3), and to provide greater context for interpretation of results the environmental features of the sampling site should be described. The fate of the microorganism is likely dictated by its physiological status

294 which is a combinatorial consequence of the atomisation process (e.g. spray device,
295 cough, sneeze) with the associated evaporative stresses of aerosol transport and
296 rehydration during inhalation (or sampling into liquid). The mechanisms by which the
297 microorganisms perish have been partially elucidated and depend on the
298 composition of the droplet and surrounding atmosphere.

299 Atmospheric oxidants (e.g. reactive oxygen and nitrogen species, sulphur dioxide,
300 ozone) will impact on microbial longevity by either directly acting on the organism or
301 with constituents within the aerosol droplet (101, 102). Presence of oxygen has been
302 demonstrated to have a deleterious effect on airborne coliform bacteria, particularly
303 at RH less than 40%, and hypothesised to be due to production of reactive oxygen
304 species (ROS) by Maillard reactions (31, 103). Maillard reactions are amino-carbonyl
305 reactions occurring between amino groups on proteins and reducing sugars that
306 cause oxidation of macromolecules and death in microorganisms (104). In airborne
307 microorganisms, these reactions may be the cause of oxidative damage to critical
308 enzymes (44, 105-107), phospholipids and nucleic acids causing metabolic
309 imbalance, destabilisation of membranes and reducing repair activity (31).
310 Interestingly, recently Maillard chemistry has been implicated as a source of organic
311 compounds within atmospheric aerosols altering particle viscosity and hence the
312 diffusivity rate of water and reactive gases (108). Bioaerosols (including virus,
313 vegetative bacteria, spores and peptides) subjected to atmospheric ozone
314 concentrations and variations in RH showed temporal changes in fluorescence
315 spectra related to oxidation and hydrolysis of tryptophan (109-111). Although
316 survival is generally greater at higher RH (>80%), certain values (i.e. 70-85% RH for
317 *E. coli* B; 41, 44) produce a large decrease in aerosol survival (41, 107, 112, 113).
318 Likewise, RH dependent changes in salt concentrations and pH within droplets

influence virus viability causing conformational changes in surface proteins and membrane fluidity affecting infectivity (114).

Solar irradiation and atmospheric pollutant gases (including open air factor; OAF) are two further environmental parameters that can significantly affect longevity in the aerosol phase. Solar irradiation markedly decreased viability compared to control conditions that simulate the night (46, 78, 115-118). Particle size-dependent survival against solar irradiation has been observed with bacterial clusters persisting for longer periods (78, 117). Terrestrial solar spectral irradiance varies through the day, with season and with geographical location (119). The UV wavelengths are of most importance for inactivating microorganisms (116, 117), where UV-A and UV-B reach the troposphere with the potential to cause a variety of DNA genomic lesions and damage to nucleic acids, proteins and lipids due to generation of reactive oxygen species (120-121). It is important that studies using both simulated and natural solar irradiation report variables such as solar intensity as accurately as is reasonably possible to facilitate data interpretation and standardisation between laboratories.

Atmospheric constituents such as various pollutant gases and secondary organic aerosols (SOAs; Table 3) have been demonstrated to have significant deleterious effects on aerosol longevity (31, 93, 122, 123-130). Many of these may contribute to a phenomenon known as ‘open air factor’ (OAF) where aerosolized microorganisms exposed to open climatic conditions decay more rapidly than those in enclosed laboratory vessels subjected to similar temperature and RH (31, 123-125, 129, 130). The precise nature of OAF is not fully understood but is hypothesised to involve a number of highly reactive products (e.g. hydroxyl radicals) from photochemical interactions between ozone and unsaturated hydrocarbons from anthropogenic (e.g. engine-related alkenes) and non-anthropogenic sources (e.g. plant turpenes) (31,

123). The reactive species rapidly oxidise and degrade macromolecules such as lipids, proteins and nucleic acids (31, 131). The effect of OAF is enhanced at high humidity (80-90% RH) for both *E. coli* and *Micrococcus albus* (123). Such humidity effects warrant investigation, possibly relating to the increased water content of aerosol particles at higher humidity.

How microbes regulate and survive aerosol transport is undetermined. Evidence suggests that the ability for transcription and translation to occur in the environment of an evaporating droplet is reduced (31, 132, 133). Evaporation and rehydration of aerosol particles imparts osmotic and desiccative stress on the microbe reflective of the humidity of the surrounding atmosphere and composition of the particle. The molecular response of many bacterial species to osmotic stress and desiccation is well documented from research understanding survival in food matrices, aquatic and marine systems and terrestrial environments (66). Hyperosmotic stress (i.e. increased a_w) causes a reduction in cytoplasmic volume as water exits the bacterium; concomitantly cell growth and respiration cease as the bacterium adapts to the hyperosmotic conditions. Initially charged solutes (e.g. K^+ ions, glutamate) are accumulated via specific uptake mechanisms (66, 134-136). Interestingly, inability to control efflux of K^+ ions correlated with decreased survival in aerosolised *E. coli* cells (28, 137). Synthesis of compatible solutes (e.g. trehalose) or uptake from the surrounding media (e.g. glycine betaine, proline) stabilises proteins, enzymes and membrane phospholipids enable critical biochemical processes to continue in hyperosmotic stressed bacteria. As the bacterial cell stabilises, a number of proteins are synthesised prompting repair of DNA damage, scavenging of reactive oxygen species and degradation of misfolded proteins (66, 134-136). Osmotically adapted cells often show cross-tolerance to other stresses such as high temperature and

oxidative shock (138). Recently, *E. coli* subjected to rapid downshift in a_w (0.993 to 0.960) in media was demonstrated to control protein misfolding by transient expression of the RpoE and RpoH regulons in conjunction with the RpoS regulon to facilitate prolonged adaptation to the hyperosmotic conditions (139).

The molecular studies described above have all been conducted in bulk solution phase and expose the microorganisms to hyperosmotic stress. Microorganisms will be exposed to hyperosmotic conditions within an evaporating droplet (i.e. low a_w conditions), enabling speculation that similar molecular mechanisms play role in bacterial survival within evaporating aerosol droplets. As will be discussed later, advances in atmospheric chemistry and single cell genomic techniques means that investigation of whether similar molecular mechanisms occur in an aerosol droplet as a function of evaporation rate and droplet composition are on the horizon. Importantly, if airborne microorganisms can induce adaptive responses promoting survival then there is the potential that colonisation and infection of the respiratory tract is primed whilst the bacteria are transported in the atmosphere. Any induced virulence factors would offer attractive targets for combating respiratory infection.

NEW TECHNIQUES FOR ADVANCING AEROSOL SCIENCE AND AEROBIOLOGY

Bioaerosols, even when produced under controlled laboratory conditions, are complex. They are generally polydisperse in terms of both physicochemical and biological properties, and the heterogeneity in the nature of the bioaerosol evolves with time and distance from the source. Technological advances in the fields of aerosol science and molecular biology are timely to facilitate multidisciplinary

393 approaches to understand heterogeneity at the single droplet and single
394 microorganism level (including microbial aggregates) and to explore the
395 fundamentals of biological decay and survival in aerosol droplets.

396 Optical techniques such as optical tweezers and electrodynamic balances where
397 single aerosol droplets can be captured and levitated within an electric field for
398 periods of time (seconds to days) have been extensively used in atmospheric
399 chemistry to investigate heterogeneous chemistry, phase separation, hygroscopicity
400 and ice nucleation activity using analytical techniques including Raman
401 microspectroscopy (140-145). Utilisation of these techniques for biological aerosol
402 has been limited to date. However, optically trapped single biological cells in solution
403 produce characteristic Raman scattering signatures (146-149) and *E. coli* exposed to
404 1-butanol resulted in spectroscopic and anisotropic detection of real-time phenotypic
405 changes in fatty acid composition and membrane fluidity (149). Although these
406 studies were conducted in liquid bulk solution rather than aerosol droplets, it
407 exemplifies the power of the technology. Furthermore, such techniques are being
408 used to explore individual aerosol particles containing microorganisms, fungal spores
409 and pollen (150-152). The electrodynamic balance technique has been used to
410 accurately deposit single particles containing respiratory syncytial virus onto airway
411 epithelial cells enabling the cellular response to infection to be analysed (153). This
412 technique enables interaction at the air-cell interface with single aerosol particles, a
413 more representative scenario than the air-liquid interface studies commonly
414 conducted for *in vitro* infection studies. It is a technique that seems applicable
415 although currently rarely applied to understanding the heterogeneity of bioaerosols at
416 the single droplet and microorganism level.

Microbial cells respond to environmental stimuli by regulating gene expression resulting in modulation of the quantities and composition of functional proteins available to combat a particular stressful condition. Transcriptional analysis and insertional mutagenesis have been used to identify bacterial genes regulated in response to stresses associated with aerosol survival such as desiccation, and osmotic pressure (136, 154). Currently, these techniques have not been applied to aerosolised microbial populations, however it can be hypothesised that similar responses may be expected and warrant exploration. The relative abundance of particular proteins critical to aerosol survival will vary from cell to cell. Exploring this heterogeneity at the single cell level is complicated due to the relatively low abundance of stress-responsive proteins. However, the last five years have seen significant advances in molecular techniques enabling exploration of the genomic, proteomic or 155-158). Techniques for isolating single cells such as flow cytometry and microfluidics can be combined with techniques such as PCR and next-generation sequencing for probing the transcriptional response of single cells (159). Indeed, single cell genomic techniques have been applied to understanding airborne metagenomes in urban settings (160, 161). Application to aerosolised populations in a laboratory setting would seem straightforward. However, care in experimental design would be needed to discriminate the true effects of aerosol transport and the stresses of aerosol generation and sampling.

These emerging technologies have the potential to dramatically impact numerous areas of bioaerosol science. They will lead to improved parameterization of the fundamental properties of bioaerosol, such as the interplay between environmental conditions with species longevity and/or gene expression. This data will lead to better predictions of disease dynamics in areas such as general industrial hygiene,

animal husbandry, hospital design and biosecurity. Furthermore, the data collected from these laboratory based instruments will inform conventional research of environmental samples.

CONCLUDING REMARKS

Experimental factors affect the microbiological sample taken forward for quantification of infectious dose or biological decay rate. Therefore a thorough understanding of the sampling and enumeration process is critical to interpretation of the final data set. Furthermore, no single aerosol generation or sampling method is likely to suit all purposes (i.e. size selectivity, species sensitivity), therefore the experimental apparatus should be selected based on the hypothesis and microorganism being tested and the data interpreted alongside the caveats associated with the methodology. For experiments designed to generate data for input into risk analysis determination of human inhalational exposure then it is recommended that aerosol generators, samplers (and collection fluid) be used that cause minimal damage or promote maximal recovery of the microorganisms during collection to prevent underestimation of risk estimates.

Fundamental questions remain regarding aerosol transmission of respiratory pathogens, particularly the underlying mechanisms of survival and/or death during aerosol transport and the role the microenvironment of the droplet plays as it evaporates then rehydrates during inhalation. However, as outlined in this review, advances in distinct scientific fields could support a systematic dissection of the biological response of microorganisms within compositionally controlled aerosol droplets within specific atmospheric conditions. It is envisaged that within the next

ten years multidisciplinary approaches combining existing and novel techniques in atmospheric chemistry, aerobiology and molecular biology will converge and begin to dissect and empirically understand the mechanisms of microorganisms survival and decay in the aerosol state and the effect on infectivity and disease transmission.

ACKNOWLEDGEMENTS

Dr Allen Haddrell and Dr Richard Thomas recognise the funding support of Elizabeth Blackwell Institute for Health Research and the Ministry of Defence respectively. Thanks are extended to Prof Jonathon Reid for critical review of the manuscript.

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993 TABLE 1 Methods used to generate and sample microbial aerosols useful for aerosol fate and inhalational infection research.

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Mechanism	Apparatus examples	Description	References(s)
Aerosol generation			
Reflux nebulization (1-, 3-, 6-jet versions commonly used)	Collison nebulizer, Wells atomizer, TSI 9302, FK-8 aerosol gun, Aeroneb Lab	• Refluxing two-fluid atomizer operating by venturi effect and wall impaction. Liquid recirculation occurs every 6 seconds in the 3-jet version (135). • Increased jet numbers increase the rate of aerosol generation and recirculation. Reservoir evaporation occurs over time causing concentration effects. • Generally used for liquids, although the Wells atomizer was used for dry powders. Particle sizes are small, 0.7–2.2 μm. • Forces associated with reflux nebulization can cause deagglomeration of aggregates causing an observed increase in bacterial concentration in the spray suspension.	14, 16, 20, 23-25, 79, 80, 99, 122, 162-167
Non-reflux nebulization	Single-pass aerosolizer	• Atomisation as above minus wall impaction and recirculation	24
Aerosol bubbling	SLAG[b] and variants	• Liquid dripped onto a membrane is broken into droplets by air flow through the membrane. • Droplets burst due to increased pressure gradient between the inside and outside	16, 24, 26

		of the device generating small aerosol particles.	
Centrifugal atomization	Spinning top aerosol generator	• Centrifugal forces moves liquid applied to a rotating disc towards the edges producing ligands that break into droplets	168
Flow-focussing	FFAG[c], C-Flow nebulizer	• Liquid flows through an orifice forming microjets that break-up into particles by aerodynamic suction of an accelerated air stream. • Good monodispersity of droplets can be achieved.	20, 24, 169
Aerosol sampling			
Impingement	Impingers[d] (AGI-4, AGI-30, Model 7541 AGI); SKC biosampler	• Aerosol accelerates through critical orifice causing inertial impaction into liquid. • Efficiency is affected by physical parameters (e.g. sampling flow rate, nozzle number and angle, distance of nozzle from the liquid, solution type and volume, particle bounce, prolonged sampling time (liquid evaporation, increased damage) and binding of microorganisms to the collection vessel wall. • Reaerosolization can occur due to liquid bubbling. • Addition of glass beads can increase virus collection efficiency • SKC biosampler possesses three angled nozzles creating a gentler swirling	17, 18, 21, 22, 170-178

		motion of the bioaerosol during collection. • AGI-30 impaction velocity reaches 265 $m \cdot s^{-1}$; much reduced in other samplers.	
Impaction	Single or multistage impactors: Andersen, Mercer, Ultimate, MAS-100, Burkard	• Operate at constant flow rates, with air flowing through an orifice causing inertial impaction of particles too large to remain entrained in the air flow; size fractionation possible. • Collection can be onto a range of different substrates (e.g. agar plates, gelatin coated slides or filters). • Substrate choice can affect collection efficiency due to effects on microbial viability and particle bounce. • In the Burkard and 6th stage of the Andersen impactors, impaction velocities reach 12 and 24 $m \cdot s^{-1}$ respectively.	21, 22, 47, 179-181
Filtration/ impaction	Gelatin filter, nitrocellulose, polycarbonate	• Greater physical sampling efficiencies. Biological sampling efficiency may be lower due to sensitivity of the collected microorganisms to air drawn past the filter. • Elution of material from the filter surface (e.g. vortexing, shaking, solution volume and type) can influence efficiency.	21, 22, 48, 49

Direct capture	Microthreads	• Particles collected onto fine microthreads (e.g. spider silk, glue thread) wound on to a frame.	78, 96-98, 123-125
Cyclonic separation	NIOSH cyclonic biosampler	• Air flow drawn into a cylindrical container is rotated causing larger particles to deposit and collect on the walls by centrifugal forces.	25, 35
Electrostatic precipitation	Ionizers e.g. AS150; Model 3100 aerosol sampler	• Airborne particles electrically charged and subjected to electric field causing gentle deposition velocity onto collection substrate. • Bioefficiency for spores greater than for Gram-negative bacteria. • Impaction velocities reach 0.01 - 1 $m \cdot s^{-1}$.	30, 36, 182
Animal inhalation	Rodent, primates	• Aerosol particles regionally deposit due to inertial impaction, sedimentation, diffusion, interception and electrostatic effects in the respiratory tract. • Deposition is a function of airway geometry and particle properties (e.g. size, shape, density, hygroscopicity).	183

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995 [a] = Note that the list is merely representative and not exhaustive. Researchers are recommended to conduct rigorous validation of
996 the aerosol experimental system for each individual micro-organism tested; [b] = sparging liquid aerosol generator; [c] = flow focussing
997 aerosol generator; [d] = all-glass impinger

998 TABLE 2 Examples of experimental techniques used to study fate of microorganisms
999 in aerosol

Device	Mechanism	Aerosol state[a]	Outdoor use?	References(s)
Rotating drum	Rotational speed of drum prevents aerosol from settling for period of time dependent on particle size	Dynamic	N	34, 82, 83, 93, 94, 99, 111, 126, 163, 184
Microthread	Aerosol captured on spider microthreads or glue fibres wound around a metal frame that can be slotted into an exposure apparatus.	Captured	Y	78, 96-98, 123-125, 130
Sphere	Steel sphere with mixing fans	Dynamic	N	124, 185
Aerosol chamber	Large chambers with mixing fans	Dynamic	N	186
Greenhouse	No mixing fan	Dynamic	Y	187,188

1000 [a] = Dynamic refers to particles maintained as a buoyant aerosol, whilst captured
1001 refers to aerosol particles immobilised on a substrate.

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1004 TABLE 3 Atmospheric, environmental and microbial factors affecting survival and
1005 infectivity in airborne microorganisms

Factor	Description	References(s)[a]
Relative humidity (RH)	Generally studies range from 20 to 90% RH	41, 45, 76, 80, 82, 99, 100, 113, 115, 163, 184, 189-193
Temperature	Wide ranges studied from sub-zero to 50 °C	80, 164, 191, 192, 194
Solar radiation	Variability in spectra examined but inclusive of UV-A and UV-B wavelengths	46, 78, 115-118, 188
Oxygen	Generation of ROS[b] during aerosol transport	44, 105-107, 165, 195
Ozone	Reactive with pollutant gases and pinenes	122, 186
Pollutant gases 'Open air factor'	CO, SO_2, NO_2, ethene, cyclohexene SOAs[c] (e.g. alkenes, turpenes[d])	31, 93, 122-126, 127-131, 185
Wet / dry preparation	Droplets or dried particles	76, 112, 163, 189, 196
Growth phase	Exponential, stationary	31, 165
Particle size	Microbial aggregates have greater survival than single microorganisms	31, 78, 130, 195
Aerosol age	Infectivity decreased prior to culturability with extended time in aerosol	197-199

1006 [a] = reference list is reflective and not exhaustive; [b] = reactive oxygen species; [c] =
1007 secondary organic aerosol; [d] = turpenes are volatile cyclic unsaturated hydrocarbon
1008 molecules released by plants