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1 **Aerobiology: Experimental considerations, observations and future tools**

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6

7 **Abstract**

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

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25 **INTRODUCTION**

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

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

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

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

57

58 AEROSOL GENERATION, SAMPLING AND POST-PROCESSING 59 CONSIDERATIONS

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

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

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

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

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

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

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

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

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

167 AEROSOL TRANSPORT AND PHYSICAL PROCESSING

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

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

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

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

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

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

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

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

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

243

244 **DETERMINING BIOAEROSOL LONGEVITY**

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

251 Techniques for investigating survival of bioaerosols *in vitro* (Table 2) tend to either
252 maintain the particles in the air column (i.e. 'dynamic bioaerosols') or captured on
253 fine substrate such as spider silk or glue fibres (i.e. 'captured bioaerosols'). The
254 rotating drum is probably the standard procedure used for aerosol longevity studies
255 based on Goldberg and colleagues seminal design (90). Modifications have
256 permitted greater control (e.g. *in situ* monitoring of parameters) and accessibility to a
257 range of environmental parameters (e.g. temperature, UV, volatile organic
258 compounds), and the suspension of larger aerosol particle sizes for sufficiently long
259 periods of time (91-94). Methods based on capturing bioaerosols on microfibers
260 derived from spider escape silk and glue gun fibres have been utilised with success
261 (78, 95-97). Comparative studies on filoviruses have demonstrated that microthread
262 captured bioaerosols decay at a similar rate as those held dynamically within rotating
263 vessels (34, 98).

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

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

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

287

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

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

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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

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

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

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

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

349 How microbes regulate and survive aerosol transport is undetermined. Evidence
350 suggests that the ability for transcription and translation to occur in the environment
351 of an evaporating droplet is reduced (31, 132, 133). Evaporation and rehydration of
352 aerosol particles imparts osmotic and desiccative stress on the microbe reflective of
353 the humidity of the surrounding atmosphere and composition of the particle. The
354 molecular response of many bacterial species to osmotic stress and desiccation is
355 well documented from research understanding survival in food matrices, aquatic and
356 marine systems and terrestrial environments (66). Hyperosmotic stress (i.e.
357 increased a_w) causes a reduction in cytoplasmic volume as water exits the
358 bacterium; concomitantly cell growth and respiration cease as the bacterium adapts
359 to the hyperosmotic conditions. Initially charged solutes (e.g. K^+ ions, glutamate) are
360 accumulated via specific uptake mechanisms (66, 134-136). Interestingly, inability to
361 control efflux of K^+ ions correlated with decreased survival in aerosolised *E. coli* cells
362 (28, 137). Synthesis of compatible solutes (e.g. trehalose) or uptake from the
363 surrounding media (e.g. glycine betaine, proline) stabilises proteins, enzymes and
364 membrane phospholipids enable critical biochemical processes to continue in
365 hyperosmotic stressed bacteria. As the bacterial cell stabilises, a number of proteins
366 are synthesised prompting repair of DNA damage, scavenging of reactive oxygen
367 species and degradation of misfolded proteins (66, 134-136). Osmotically adapted
368 cells often show cross-tolerance to other stresses such as high temperature and

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369 oxidative shock (138). Recently, *E. coli* subjected to rapid downshift in a_w (0.993 to
370 0.960) in media was demonstrated to control protein misfolding by transient
371 expression of the RpoE and RpoH regulons in conjunction with the RpoS regulon to
372 facilitate prolonged adaptation to the hyperosmotic conditions (139).

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

385

386 **NEW TECHNIQUES FOR ADVANCING AEROSOL SCIENCE AND**
387 **AEROBIOLOGY**

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

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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.

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

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

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

445

446 CONCLUDING REMARKS

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

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

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

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474

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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

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		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

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		motion of the bioaerosol during collection. • AGI-30 impaction velocity reaches 265 m·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·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

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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

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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

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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

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