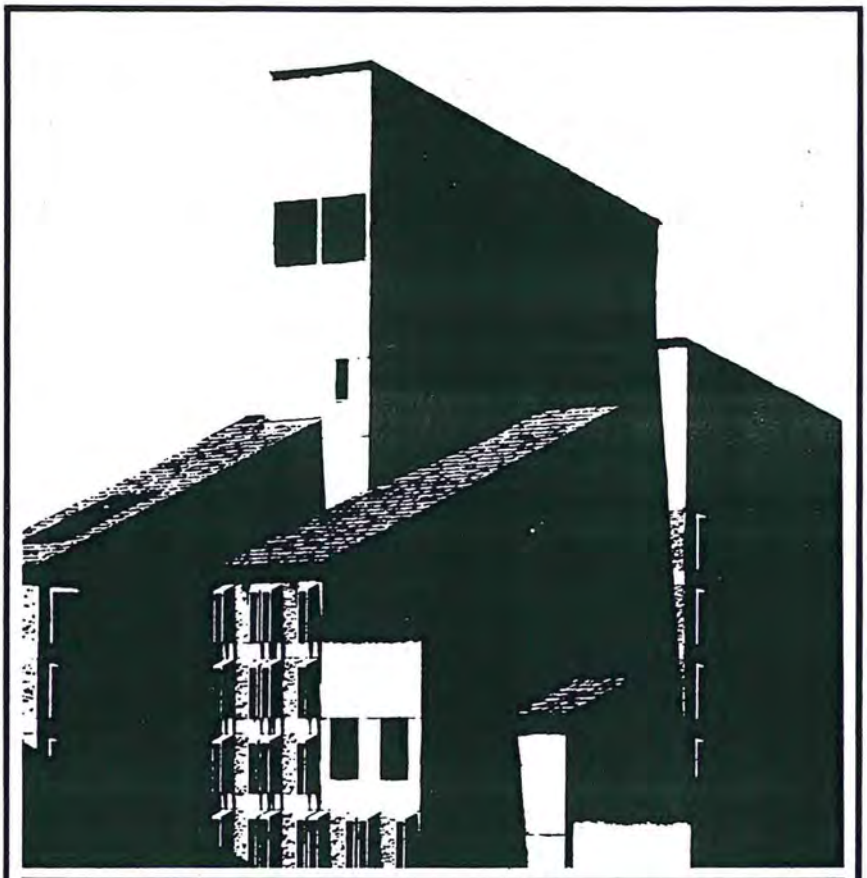

REVIEW of SAMPLING and ANALYSIS
for AIRBORNE MICROBIOLOGICAL
CONTAMIANTION on COMMERCIAL AIRCRAFT

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A Review of Sampling and Analysis Methods for Assessing Airborne Microbiological Contamination on Commercial Aircraft

A Literature Survey and Review

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EXECUTIVE SUMMARY

Exposure to airborne particles of microbiological origin is increasingly being recognized as responsible for infectious, hypersensitivity, and inflammatory lung diseases. Such particles are referred to as biological aerosols, or "bioaerosols," and include but are not limited to bacteria, fungi, their spores and fragments thereof (associated toxins), and viruses. Modern aircraft use pressurized cabins to transport millions of passengers each year. With the occupant levels experienced by the air transport industry, concerns regarding human exposure to bioaerosols have been raised among the traveling public, flight crews, scientific communities, and regulatory agencies. Based on extensive monitoring, professional societies have published guidelines suggesting acceptable ranges for bioaerosol exposures in indoor environments, yet it is not established if such exposure thresholds should apply to commercial aircraft, nor is it established what bioaerosol exposures are experienced by the average passenger during routine air travel.

For the purpose of better characterizing the air quality in commercial aircraft cabins, germane literature from respected sources within the fields of industrial hygiene, environmental microbiology, and medical microbiology have been surveyed and summarized. This survey has focused on a comprehensive characterization of bioaerosols in aircraft using the best available technology, and is divided into the following sections:

I. Bioaerosol collection in currently marketed and research-grade air samplers

II. Assays and analytical methodologies used to characterize airborne bacteria, fungi, spores, viruses, and microbiologically associated toxins following collection

III. Summary of guidelines germane to the microbiological air quality in aircraft cabins

IV. Summary of aircraft air quality case studies that have a significant bioaerosol component

V. Recommendations for future aircraft bioaerosol studies

Bioaerosol Sampling. This survey found that currently marketed bioaerosol samplers are suitable for the collection and recovery of bacteria, fungi and their spores. It appears that liquid-capture samplers, while most difficult to transport, offer the greatest recovery factors and highest yields with respect to the potential for bioaerosol data acquisition. Spurred by the tremendous interest in microbiological warfare agents and occupational exposures, high-volume and research-grade samplers are becoming more portable and economical, such that they are suitable for large-scale field studies of bacteria and fungi on commercial aircraft. Some high-volume samplers, however, have not been subject to long-term testing, and, if used in future aircraft studies, would require careful characterization. Because of unknown capture efficiencies, no bioaerosol samplers are currently considered adequate for quantitation of airborne viruses except filters. Well-tested air filter samplers, such as MOUDI™ impactors and/or simple filter cassettes, offer size discriminating information, can interface with newly developed chemical, immunoassays, genetic characterization methods, and can provide complementary information obtained from liquid-capture samplers. While easiest to transport and operate surreptitiously, solid-phase impactors have the lowest potential for bioaerosol information yield. Further, they subject airborne microorganisms to the largest sampling stresses and cannot be used for complementary analyses. For these reasons, solid-phase sampling is not recommended for future research use in aircraft as the only bioaerosol-collection device.

Bioaerosol Analysis. Five categories of widely accepted microbiological assays were established as germane to characterizing aircraft air quality: culturing, microscopy, immunochemical, chemical, and genetic assays.

Culturing. This survey found that analytical techniques for *culturing* airborne bacteria, fungi and their spores have been widely accepted and successfully applied to characterize aircraft environments. Culturing techniques applied to quantify infective airborne virus particles are less accepted and are limited by sampling techniques. The literature describing airborne virus quantitation is tenuous, and the characterization of airborne viruses under field conditions is at best qualitative.

Direct microscopy is now recognized as a reliable technique to quantify total bioaerosol concentrations (not including viruses), and has been recommended in the recent scientific literature to accompany any culture-based investigations; this is because culture-based techniques have been shown to significantly underestimate airborne microorganism concentrations. The salient theme associated with the bioaerosol microscopy literature maintains that for ecological and epidemiological reasons, it is necessary to determine the total concentrations of atmospheric bacteria and fungi, as well as what fraction of the total is culturable.

Immunochemical techniques have been established to determine the masses of common allergens associated with airborne particulate matter; these include animal danders, insect parts (cockroach and mite), and fungal cell wall components (β-D-Glucans). Immunochemical techniques have been applied to a wide variety of other microbiological components, but their epidemiological and quantitative significance is under debate, and may not be germane to aircraft air quality. Immunochemical assays have recently been applied to study allergens in aircraft environments.

Chemical analysis. Numerous compounds associated with (e.g. bacterial endotoxins), and liberated by microorganisms (e.g. volatile organic compounds - MVOC's, and mycotoxin) can be reliably quantified my standard *chemical* mass-spectrometry and simple sampling methods, and can serve as an indicator of both surface-associated and airborne microorganisms. Many VOC's, however, have both anthropogenic as well as biogenic sources, and differentiating between them makes VOC measurements lose their usefulness as an aerosol biomarker in aircraft. Mycotoxin chemical assays need further development.

Genetic amplification methods (PCR) - Because of its specificity and extreme sensitivity, PCR is well recognized in the literature as a promising forensic technique that has been quantitatively successful in small-scale studies; however, quantitative PCR has not yet been consistently demonstrated in large-scale *field* studies, and may be labile to ubiquitous environmental interferences particular to aircraft.

Aircraft Bioaerosol Case Studies. Over the last 11 years, the results of bioaerosol monitoring on commercial airline flights have been published in a variety of trade publications and government documents. Only four aircraft air quality studies containing a significant bioaerosol component have been reported in peer-reviewed literature. In general, the case studies published to date have reported the same conclusion with respect to bioaerosols: the levels of airborne microbiological contaminants found in airliner cabins are below those found in other indoor environments and are not likely to cause adverse health effects. These studies, however, were scientifically limited by the following factors: (i) low replicate sampling designs, which yield poor variability estimates, (ii) the use of a single assay technique (classical culturing), (iii) the use of "grab" sample paradigms, and (iv) a relatively small number of observations compared to the actual numbers of commercial flights that operate daily. Because of these factors, the conclusions reached by these studies should be considered tentative and should not be generalized to the commercial airline fleet without further study.

Recommendations for Future Studies. The characterization of aircraft cabin air that can better represent bioaerosol concentrations during the routine operations of the commercial aircraft fleet will require larger scale efforts than past studies, and should consider a multidisciplinary approach. This survey precipitated the following recommendations to improve aircraft bioaerosol characterizations:

A statistical rational, with some quantifiable level of confidence, should be developed to determine a minimum number of flights that require monitoring to yield exposure information that can be better extrapolated to the commercial fleet. This monitoring plan should focus on the newest and oldest commercial aircraft in service, and account for the following factors: (i) extremes in recirculation rates and the replenishment of fresh air, (ii) operation of air filtration systems, (iii) flight lengths, and (iv) passenger load factors. The monitoring plan should juxtapose monitoring for "worst and best" case scenarios by combining the above factors.

Analytical assays that can be concurrently executed to provide complementary, quantitative microbiological information. This is to include standard culturing and direct microscopy of bacteria, fungi and their spores, immuno/chemical analysis of selected aeroallergens, and genetic amplification (when appropriate in the future) to identify the presence of selected pathogens. The need for airborne virus quantitation is clear, but it appears that airborne virus analysis will be quantitatively indeterminate for the foreseeable future - culturing viruses from aerosols is not practical in this context and only genetic amplification appears practical for airborne virus quantitation. Genetic amplification is widely acknowledged to hold tremendous promise as a tool for quantitative airborne pathogen analysis and may improve viral detection; this technology has been successful in clinical and laboratory settings, but needs further validation in environmental settings before full-scale application.

Liquid-capture sampling equipment should be used in conjunction with size discriminating filter samplers. Because it would best represent exposures and serve to lower method detection limits, sampling should be continuous or composited over the duration of flights monitored; short-term "grab" sampling paradigms should be avoided. The surreptitious use of sampling equipment should be abandoned if it limits data yield and acquisition. Sampling operations should be designed such that they do not impact passenger comfort, but sampler presence can be acknowledged without ramification (*e.g.*, sampling apparatus placed in overhead storage – pumps may be sound insulated and powered from galley). Since the main emitters of airborne biological agents are the passengers themselves, and past studies have shown the highest airborne particle concentrations in the coach cabin, sampling should be executed in the coach cabins. Personal samplers should not be used because of impediments to crew members' movements, safety factors, and small sample potential (low volume).

Exposure guidelines for other indoor air environments should be critically evaluated for practical applicability to aircraft cabins; the lack of an external reference for aircraft should be addressed. Synergistic bioaerosol exposure factors due to the presence of implicated immune suppressors (*e.g.*, high O_3), sustained low RH, and sustained lowered O_2 partial pressures should be addressed.

Bioaerosol air quality models, previously developed for gasses, smoke, and particulate matter in indoor environments applied to airliner cabins, could be adapted for bioaerosols and executed using currently available literature values. Future observations may be used to verify model outputs for large-scale extrapolation to the different aircraft in the commercial fleet.

SECTION I: REVIEW OF SAMPLING METHODS APPLICABLE TO THE COLLECTION AND RECOVERY OF BIOAEROSOLS FROM AIRCRAFT CABINS

Bioerosols Defined. Bioaerosols, as defined by the American Conference of Governmental Hygienists (ACGIH, 1999), are "airborne particles that are living or originate from living organisms." This definition includes viruses, protozoa, bacteria, fungi and their byproducts, as well as fungal spores, pollens, insect fragments and animal dander. As such, bioaerosols may be viable or nonviable and range in size from less than .01μm to greater than 100μm. This broad range of size and organism characteristics must be considered when choosing a sampling system. There is no one sampling system capable of efficiently capturing all types of bioaerosols.

Bioaerosol Exposure Estimates. Though the dose-response relationship between bioaerosol exposure and health effects is not completely understood (Macher, 1997; ACGIH, 1999), exposure estimates are best described through air sampling. Air sampling may be divided into personal or ambient sampling strategies. Personal samples are taken near the breathing zone of an individual, and usually the sampling device is clipped to the collar of the subject and a portable vacuum pump is attached to their clothing. Personal samples give a better characterization of individual exposure than area samples and may be correlated with individual health effects. Several personal aerosol samplers were discussed in the literature (Kenny et al, 1998; Hauk, 1997; Rubrow, 1987; Kenny *et al.,* 1997; Fangmark *et al.,* 1991), but few samplers have been modified for bioaerosol sampling (Hauk, 1997; Kenny et al, 1998). The prototype samplers and sampling methods discussed in these papers have not been fully evaluated for any bioaerosol. Real-time detectors for bioaerosols are currently being developed but have not been fully characterized for field studies (Hairston *et al.,* 1997). It has been noted by some investigators that area samples generally underestimate concentrations (Crook and Sherwood-Higham, 1997). However, ambient sampling is the most common strategy for bioaerosol characterization as it allows estimates of exposure for general populations (Burge, 1995). Area sampling methods and devices will be discussed in more detail (Section II).

COLLECTION CHALLENGES AND SAMPLING EFFICIENCY

The link between assay and samplers. The challenge in bioaerosol sampling is to remove the biological particles from the air efficiently without compromising their viability, and in a way that complements a chosen assay method (Burge, 1993). For bioaerosol analysis, most samplers currently used rely on microscopic or cultivation techniques. Culturing techniques in particular have been found to lack precision and rely on many sampling variables. Newer assay methods may provide more information with fewer interferences (Henningson and Ahlberg, 1994). In order to fully characterize the aerobiology of a particular environment, the potential sources, organisms, mechanism of capture, and the level of contamination also need to be considered (Willeke, 1993; Hurst, 1997).

The ACGIH has compiled a list of sampling methods with their compatible analysis in their Air Sampling Instruments Manual (ACGIH, 1994), and this is supplemented in their most recent review of Bioaerosols Assessment and Control (ACGIH, 1999). Sampling methods and analysis should be tailored to the organism(s) of interest and to the type of data desired. For example, total microscopic counts are important for interpreting allergen levels, while colony forming units provide information on viable microorganisms. The enumeration of colony - forming units provides at best limited information on viable microorganisms because many viable microorganisms simply cannot be cultured directly from an environmental sample. In addition, certain samplers, designed with multiple collection stages, provide size-discriminate information that may be related to specific health effects in different regions of the respiratory system. Size discriminating samplers and their respective collection efficiency are important because it is the small airborne particles, those less than 5 μm in diameter, which are believed to be most hazardous.

A general idea of the level of contamination in an environment is also important when choosing a sampler, particularly if culturing assays will be used. Samplers provide optimum information at particular ranges of contamination and are affected by the airflow rate through the sampling train. In areas with high contamination levels, samplers that require interpretation

through microscopy or organism growth, such as impactors and filters, may be easily overloaded. In areas with very low concentrations, the airflow rate must be sufficiently high to capture a representative number of organisms and yet must balance losses in viability due to the sampling stresses. Desiccation losses must be considered when solid-phase impaction and filtration are used. Most of the commonly used sampling devices are suitable for sampling environments with intermediate concentrations of bioaerosols (ACGIH, 1999); based on the case studies available from the literature, aircraft cabins would qualify under environments that contain intermediate bioaerosol concentrations. Liquid impingers and wetted cyclones are less affected by ambient concentrations and are ideal for composite sampling paradigms. Samples from liquid-capture devices may be concentrated or diluted to achieve a microorganism concentration range that is sensitive for any chosen analytical technique.

Understanding certain characteristics of the organism(s) being studied is important when choosing a sampling system. The physiology (*e.g.*, cell wall construction or spore-forming response) and overall metabolic resilience of a microorganism will affect its viability after being subjected to sampling stresses such as impaction onto a solid surface, shear forces, and desiccation (Terzevia, *et al.*, 1996). Vegetative bacterial cells are among the most sensitive physiologically, whereas spore-forming bacteria and fungi are the most resilient. The response of airborne viruses to sampling stresses is not well documented. The size distribution of the aerosol is also important because most samplers have lower collection efficiencies as particle size decreases; this is particularly true of impactor-type samplers (ACGIH, 1999).

BIOAEROSOL COLLECTION - PRICIPLES and DEVICES

The principles of bioaerosol collection have been well documented and detailed in recent comprehensive reviews and publications (Henningson and Ahlberg, 1994; Macher, 1997; Pahl *et al*, 1997; ACIGH, 1999). The following brief introduction to sampling principles is meant only to provide a general background for the discussion of sampler selection in airline cabin case studies.

Principles and definitions. The physical principles of bioaerosol sampling are consistent with all airborne particle collection. The goal is to remove particulate matter from the sampler airflow

without changing the physical characteristics and viability of the organism (this "ideal" collection scenario does not exist). The three main sampler qualities that affect sampling are: the sampling efficiency, the collection efficiency, and the biological efficiency. The sampling efficiency is the ability of the inlet of the sampler to draw in all particles regardless of their size. Collection efficiency is a measure of how thoroughly the particles will be captured within the sampler; collection efficiency for a particular size range is defined by a cutoff size where 50% of the particles (of a given size) that enter a sampler, are retained by the sampler (ACGIH, 1999). Biological efficiency is a measure of the sampler's ability to maintain microbial viability (Nevalainen *et al.,* 1992). The most commonly used bioaerosol samplers are inertial devices or filters that allow-volumetric measurement of the organisms sampled (Henningson and Ahlberg, 1994; Macher, 1997; Pahl *et al,* 1997; ACGIH, 1999).

Devices: Impactors. Inertial devices such as impactors and centrifuges use the organism's own inertia to remove it from a sampler accelerated air stream, and deposit it on the collection media (ACGIH, 1994). The major disadvantage of using the particle's inertia as the collection driving force is that small particles such as viruses and single cells do not possess enough inertia to be disentrained from the airflow. Another disadvantage is that sampling times for impactor-type samplers must be short enough to prevent major viability losses due to desiccation or other injury; this is especially important when collecting vegetative cells (Buttner and Stezenbach, 1991). The efficiency for impactors in a particular size range is defined by the cutoff size for each of the capture stages. Impactors have many advantages over other types of samplers. Many portable units are commercially available, and many of the impactors have well-characterized sampling profiles. The Andersen impactors, both six-stage and single-stage, have been suggested as a possible reference sampler(s) (Henningson and Ahlberg, 1994; Lembke *et al.,* 1981; and others); because of the stresses documented to occur during impaction , the use of impactors as a standard is under debate (Terzevia *et al,* 1996). Impactors may be subdivided based on the nozzle and sieve-plate dimensions, usually multi-hole and slit impactors. Multi-hole impactors vary based on the number of stages of impaction (allowing discrete collection of different size particles) and the number and diameter of the holes. Correction factors that consider the density of microorganisms sampled on solid surfaces should be used to obtain the most accurate characterization from impactors (ACGIH, 1999). Slit impactors may differ in

nozzle size, and impaction may be time-elapsed onto a rotating surface. The collection media may be agar plates or an adhesive-coated glass slide. Most impactors are designed for culture-based analysis (ACGIH, 1999); in some cases, impactors have been modified for liquid capture and microscopy (Mocheadreas, 1996), but such modification is not practical for aircraft monitoring.

Devices: Impingers. Impingers use particle inertia and diffusion to impact or wash the organism into a collection fluid, and include swirling capture devices. A dominant capture mechanism of impingers is that organisms diffuse from the bubbles to the collection liquid (Grinshpun *et al.,* 1997). An impinger's collection efficiency is not as influenced by particle size as impactors; thus, impingers have been used for the collection of very small particles, including viruses. Liquid impingers have the advantage of being able to concentrate or dilute microorganisms in the capture media prior to analysis. Impingers also make it possible to homogenize and divide samples for multiple, concurrent analyses. Impingers are less affected by the level of contamination in an environment than are impactors, and have no upper quantitation limit (Buttner and Stetzenbach, 1991). Impingers are also less likely to subject an organism to desiccation stresses; they may allow longer sampling times and therefore higher volumes of sampled air. In certain cases, impingers allow for continuous sampling. Particle collection by impingement is affected by the liquid level and the flow rate. Efficient collection minimizes both particle bounce resulting from low collection fluid levels and re-aerosolization caused by too high a flow rate (Grinshpun, 1999). Lin *et al.* (1997) have shown that there is ~1% of collection fluid loss per minute of sampling for impingers under common RH conditions, and established that there is a critical minimum volume. A major disadvantage to impinger use is controlling the loss of liquid collection medium levels in the field; however, media levels in impingers can be easily replaced during sampling. Because of the low RH environments sustained in airliner cabins, liquid losses must be accounted for when considering liquid capture devices for sampling in future aircraft studies.

The AGI-30 and AGI-4 liquid impingers ha, leading to misinterpretation of results (*i.e.*, cells are particle-associated when suspended as an aerosol *in situ*). Liquid capture efficiencies

vary depending on cell physiology (*e.g.*, some fungal spores are very hydrophobic, which leads to poorer capture efficiency in liquid impingers) (Burge, 1995).

Devices: Centrifugal samplers. Centrifugal samplers use centrifugal forces and particle inertia to impact particles onto the side walls of the sampler. Various samplers use different collection media. Media may be filters, agar, or liquid.

Devices: Filtration. Bioaerosol capture via filtration is attributed to a combination of physical forces: inertial impaction, interception, diffusion, and electrostatic attraction (ACGIH, 1994). Filters are usually capillary pore or porous membranes bound in cassette-type filter holders. Filtration has the advantage of being portable, and may be used with a variety of analyses, including microscopy, culture, and chemical and genetic assays. Filtration methods have also been found to be less size specific than other samplers (Parks *et al.*, 1996). When microscopic analysis is used, the level of contamination in the sampled environment needs to be considered so that the sample does not become overloaded. If culture-based analysis is used, the sampling time needs to be adjusted to prevent high losses due to desiccation. Studies using the Sartorious MD8 sampler combined with gelatin foam filters were found to lessen the desiccation losses and to be as effective as Casella's slit-to-agar sampler (Parks *et al.*, 1996).

SECTION II: REVIEW OF ASSAY METHODS APPLICABLE TO CHARACTERIZING AIRBORNE MICROORGANISM CONCENTRATIONS ON COMMERCIAL AIRCRAFT

Introduction. Griffths and DeCosemo (1994) published a landmark bioaerosol assessment review that described which classical and modern microbiology assays were suitable for bioaerosol analysis; this review presented for the first time a discussion of the applications of modern molecular biology methods adapted for the characterization of bioaerosols. More recent and comprehensive reviews of bioaerosol assay techniques, also summarizing molecular biology applications, have since been provided by Stezenbach (1997) and Macher (1999), and published through internationally recognized professional societies (The American Society for Microbiology (ASM) and the American Conference of Government Industrial Hygienists (ACGIH)). Both these authors served as principal editors who, with many other contributors and associate editors, compiled reviews of various bioaerosol investigations and regulations found in the scientific literature and government documents. These reviews are extensive documentaries, reporting classical and state-of-the-art methods to collect and characterize microorganisms and their associated biopolymers suspended in air. While these comprehensive bioaerosol publications are not specifically directed toward the characterization of aircraft cabin air, the assay methods described are applicable, and many are summarized herein - some redundancy with these comprehensive publications is unavoidable. Because of their size, scope, and publishing time, they do not include the most recent advances contained in archival journals from the molecular biology field, some of which hold tremendous promise for quantitative bioaerosol monitoring of large air volumes; thus, molecular biology methods are given considerable attention herein. This section provides a terse synopsis of bioaerosol assay methods that may be used to characterize microorganisms, their spores, and their associated biopolymers in air samples collected from in-service aircraft. Those methods that were deemed as impractical in the context of sampling commercial aircraft air were given minimal attention or were not included in this review (*e.g.*, gravimetric analysis).

Approaches and Origins. With exception to collection and assay by direct impaction techniques, the microorganism characterization methods used for bioaerosol assays have been predominantly adapted from general microbiology methods that were originally designed to

recover bacteria from aquatic environments. Generally, bioaerosol characterization methods can be separated into two categories; the main distinction among them depends on the use or omission of microorganism enrichment and isolation techniques to obtain individual, pure-culture bacterial colonies or, in the case of virus detection, dense concentrations of viral particles (*i.e.*, culture-based techniques). Non-culture-based assessments do not rely on microorganism growth. Instead, they rely on direct microscopy, extraction, and analysis of biological polymers, and/or genetic or immunochemical probing techniques. With respect to bioaerosol studies, both culture-based and non-culture-based methods have been applied to identify and enumerate microorganisms recovered from environmental and laboratory air samples. To date, there are numerous reports (> 150 archival journal articles since ca. 1974) of environmental or laboratory investigations on airborne microorganism abundance and diversity using only culture-based techniques; though non-culture-based methods are increasing in popularity among bioaerosol researchers, there are relatively fewer reports from quantitative non-culture-based investigations (< 30).

Culture-based enrichment and isolation remain the primary method of environmental sample analysis (ACGIH, 1999); enrichments have been *the only* analytical approach used to characterize the diversity and abundance of airborne microorganisms within commercial aircraft. Numerous authors acknowledge that the selection of bioaerosol analysis methods and samplers are inter. Given the extensive review of sampler performance available in the literature, and the sampler summary in the previous section, no discussion linking samplers and analysis will be presented - this section is dedicated to a summary of analytical methods. Recommendations concerning analytical protocols to characterize aircraft cabin air, and the appropriate sampler types that optimize those protocols are presented in Section V.

Enrichment and Isolation. Culture-based characterization methods rely on enrichment and isolation on or in some type of nutrient-containing media. For bioaerosol studies, microorganisms recovered from air are reported as colony forming units (CFU) or plaque forming units (PFU) grown on solid agar surfaces (or host lawns on agar surfaces in the case of viruses). To relate culturability and airborne concentration, the quantities of microbial colonies and/or viral plaques isolated from air samples are normalized by the volume of air sampled.

Depending on the objectives of any particular bioaerosol study, several different media (or host) types and different antibiotics have been used to cultivate bacteria, fungi and their spores, and/or viruses recovered from air samples. Culture media may be tailored to inhibit and/or optimize the growth of a particular organism, produce differential effects in the appearance of organisms, or be very broad-spectrum in nature. All culturing assays must be optimized for concentration factors such that numbers of the colony forming units grown are within a statistically significant range. And, depending on the air sampling device, isolation is achieved directly or after media transfer according to the following general approaches:

Impaction. For impaction devices, solid agars have been used as both the capture and culture substrate. Impaction methods have been modified for capture in viscous fluid media that can be diluted and transferred to other solid-phase or liquid media (Blomquist *et al.,* 1984). Solid-phase agar impaction was the assay technique used most frequently to characterize and enumerate airborne microorganisms in commercial aircraft (Dechow *et al.,* 1997; US DOT, 1989; Air Transport Assoc., 1994; Lee *et al.,* 1999; Pierce *et al.,* 1999).

Liquid Capture. For liquid capture devices (cyclones and impingers), agars have been used as a transfer and recovery substrate, where liquid aliquots from the samplers' capture reservoir are spread on the agar surfaces. No liquid capture devices have been used in aircraft air quality studies.

Filtration. Microorganisms have been captured and enumerated directly on filter surfaces or subsequently eluted off-filter surfaces, typically with buffers and/or surfactants, and the elutant was spread on agar surfaces. No studies cite filtration as a method to characterize and enumerate airborne microorganisms on commercial aircraft.

An exhaustive reference list regarding bioaerosol *investigations* employing standard culture-based approaches is too numerous to include directly in this text, nor would such a list be germane to aircraft cabin air quality, but a comprehensive bibliography is included for completeness. Analytical protocols for culturing airborne microorganisms are well-established, and specific media formulations and isolation protocols for both defined and undefined media are readily available. It should be noted, however, that many minor modifications to general purpose media (*i.e.,* specific chemical content) and culturing conditions have been reported in the bioaerosol literature - including this level of detail is beyond the scope of this review; where relevant to characterizing airborne biological contamination of commercial aircraft cabins, the details of culturing are presented in the case studies section of this review (Section IV). The

most recent and comprehensive reviews to include detailed culturing protocols and application of culture-based bioaerosol techniques are cited here, with the understanding that they can and have been applied to the aircraft cabin environments in conjunction with the selected sampling technique (all of which have been solid-phase impaction):

- Field Guide for the Determination of Biological Contaminants in Environmental Samples (AIHA,1996)
- Sampling and Analysis of Airborne Microorganisms, In Manual of Environmental Microbiology, American Society of Microbiology Press, Hurst, C.J., Knudsen, G.R., McInerney, M.J. *et al.*, Eds. (1997)
- Methods for General and Molecular Bacteriology, American Society for Microbiology Press (1997)
- Manual of Clinical Microbiology, American Society of Microbiology Press (1999)

Undefined media and airborne bacteria. Using undefined media formulations and standard culturing techniques, numerous bioaerosol studies have quantified the presence of a wide range of heterotrophic bacteria and fungi recovered from environmental air samples. Undefined media, otherwise known as "general purpose" media, have been routinely used for culturing airborne bacteria and their spores; these media contain a combination of protein hydrolysates, mixed carbon sources, vitamin supplements, and buffers. The principal undefined media reported to culture and quantify airborne bacteria exist as solid-phase at room temperature and include the following: Trypticase Soy Agar (TSA), Soybean Casien Digest Agar (SCDA), R2A, and to a lesser extent, Peptone-containing agars (references too numerous to list). According to *Methods for General and Molecular Bacteriology,* these are generally accepted as "broad spectrum" media and are expected to grow the highest amounts of heterotrophic bacteria from any environment. The differential recovery of bacterial colonies, cultured from the same air sample and grown in parallel on different broad-spectrum agars (*i.e.,* TSA and MacConkey agar), has been interpreted as an index of microbial injury incurred during the air sampling process (Terzieva *et al.,* 1996). MacConkey agar has also been used to select Gram negative over Gram positive bacteria in common culture (ACGIH, 1999). The temperature spans for growth are sometimes considered when culturing bacteria on undefined (or defined) media, the separation being operationally defined as mesophilic (near body temperature – 37 °C) and thermophilic (> 50 °C).

Airborne Bacteria Levels on Commercial Aircraft - Undefined Media. Using direct agar impaction techniques, undefined media have been used to cultivate bacteria from air samples obtained from in-service aircraft. R2A, TSA, and an undisclosed "Nutrient" agar have been the only media used to directly culture airborne bacteria in aircraft (Dechow *et al.,* 1997; US DOT, 1989; Air Transport Assoc., 1994; Lee *et al.,* 1999; Nagda *et al.,* 1992; Pierce *et al.,* 1999). Except for Lee *et al.* (1999), the reports from the small body of aircraft air quality literature included genus level discrimination of airborne bacteria that were initially cultured on undefined media. It appears, however, that isolates were transferred to defined media for standard genotypic-based identification (US DOT, 1989; ATA, 1994; Dechow *et al.,* 1997). These reports collectively isolated more than 12 different genera of airborne bacteria present during commercial aircraft flights, which were then subsequently identified on defined media (discussed below). Total bacterial counts on undefined media were reported in the range between 0 and 360 CFU/m^3. Concerning culturable airborne bacteria, a summary of indoor residential air exposure guidelines, their relevance and applicability to aircraft cabin air, and a summary of the concentration levels found on commercial aircraft will be presented in subsequent sections.

Undefined media and airborne fungi. Undefined media formulations have also been commonly used to grow airborne fungi (and their spores), and most fungal growth media used in bioaerosol studies have included antibiotics to suppress bacterial growth. The principal undefined media reported to culture and quantify airborne fungi are Malt Extract agars and, to a lesser extent, Czapek Dox agar; these agars have been the most commonly used as the capture/culture media in impactors and as the transfer/recovery media for filters and liquid capture devices (ACGIH, 1999; Samson, 1994; Chin *et al.,* 1993; Reynolds *et al.,* 1990; Horner *et al.,* 1994; Griffiths, 1994). According to *Standard Methods for the Examination of Water and Wastewater,* these are generally accepted as "broad spectrum media," and are expected to grow the highest amounts of fungi from the environment.

In general, the literature reports on quantitative airborne mycology have been based on culturing assays, although microscopy is included in some investigations (discussed below). Using undefined media, fungi recovered from air samples were often categorized into two main groups

on the basis of specific water requirements and desiccation tolerance: fungi that are relatively less tolerant of desiccation and require higher moisture contents from their growth substrates are termed *hydrophilic*. Those fungal species that are more desiccation tolerant and require lesser amounts of water from their substrates are termed *xerophilic* or *xerotolerant*. There are also intermediate degrees of water requirements that optimize the growth of *mesophilic* fungi. With respect to recent bioaerosol studies, various MEA formulations were the media of choice for quantifying airborne mesophilic and hydrophilic fungi, while DG_{18}, an undefined media with a glucose-peptone-glycerol base, has been used to quantify the presence of airborne xerophilic fungi that survive at low water potentials (ACGIH, 1999; Grant *et al.*, 1989; Hocking and Pitt, 1980; Horner, 1994). The temperature spans for growth are sometimes considered when culturing airborne fungi on undefined media, the distinction being operationally defined as temperature ranges that select for genera which have optimal growth rates near body temperature (37 °C, mesophilic), and those genera that grow best considerably above body temperature (> 50 °C, thermophilic) (Rahnkonen, 1987). In numerous indoor air quality studies, individual fungal genera, isolated on undefined media, have been identified and enumerated by morphological characteristics. This level of identification has required a skilled mycologist and, at times, secondary isolations on defined media; it was reportedly time-intensive and significantly more expensive than nonspecific counts (Reponen *et al.*, 1992; Robertson *et al.*, 1992; Robertson, 1997; Reponen *et al.*, 1996; Horner *et al.*, 1994; Reynolds *et al.*, 1990; ACGIH, 1999; Reponen, 1999; Horner, 1999).

Airborne Fungi Levels on Commercial Aircraft - *Undefined Media.* Using direct agar impaction techniques, undefined media have been used to cultivate fungi, yeast and their spores from air samples obtained from in-service aircraft using either MEA and Sabouraud-4% glucose agar (Dechow *et al.*, 1997; US DOT, 1989; Air Transport Assoc., 1994; Lee *et al.*, 1999; Pierce *et al.*, 1999). Except for Lee *et al.* (1999), the reports from the small body of aircraft air quality literature included genus level discrimination of airborne fungi cultured on undefined media. These reports collectively identified more than 21 genera of airborne fungi and three genera of airborne yeast, present during the commercial aircraft flights sampled. The most prevalent types of airborne fungi enumerated in air samples taken from commercial aircraft were *Cladosporium* spp. and *Alternarea* spp.; apart from these genera, *Aspergillus* spp. and *Penicillium* spp. were

detected on more than 10% of flights monitored. Only one aircraft study reported the culture of potentially pathogenic fungi from cabin air; although no quantities were given, Pierce *et al.* (1999) reported that *Aspergillus niger* and *Paecilomyces variotti,* potential human pathogens, were isolated from air samples taken in commercial aircraft during flight. Most of the fungal species cultured from aircraft cabin air are recognized by aerobiologists as very common mesophilic and xerophilic fungi isolated from the indoor air of residences and commercial buildings in the US, Canada, and Europe (Miler *et al.,* 1988; Flannigan, 1997; Robertson, 1997; Reponen *et al.,* 1996; Horner *et al.,* 1994; ACGIH, 1999). The total levels of airborne fungi recovered from aircraft cabin air on undefined media were below 300 cfu/m^3 in all reports. Although the humidity in aircraft cabins is maintained at low levels, the literature contains no reports on the use of media targeted to recover xerophilic fungi from these environments. Concerning culturable airborne fungi, a summary of indoor residential air exposure guidelines, their relevance and applicability to aircraft cabin air, and a summary of the concentration levels measured on commercial aircraft will be presented in a subsequent section (that summarizes case studies).

Defined and Selective Media. Using undefined "general purpose" media, the aim of many environmental bioaerosol investigations was to collect and grow as many microorganisms as possible from the atmospheric environment in question. Environmental bioaerosol studies have also targeted the recovery and enumeration of pathogenic bacteria and fungi from air samples using specific agar formulations with defined contents - also called "special purpose" or "selective" media. Special purpose media are used to select for specific genera of bacteria and fungi, usually by (i) suppressing the growth of other microorganisms with biocides or antibiotics, (ii) supplying specific substrates, (iii) constraining physical conditions (*e.g.,* temperature), or by using any combination of the above "selection pressures." A review of the literature demonstrates that most bioaerosol sampling using defined media appears to have been executed with direct agar impactors, specifically Anderson types, as well as culturing from liquid aliquots taken from AGI reservoirs. Direct recovery on defined media appears to be performed most often in agriculture or commercial indoor air quality investigations. The clinical microbiology literature and the bioaerosol literature demonstrate that bacteria associated with diseases that can be caused by inhalation are isolatable either directly on selective media or indirectly via transfer

from "general purpose" to selective media (ASM, 1997; ASM 1999). Because of low concentration factors and common culturing interferences (*e.g.*, overgrowth), there are few reports of direct isolation and enrichment of pathogens from aerosols.

The airborne microorganisms most frequently enriched on defined media appear to be fungi rather than bacteria or chlamydiae, although *Legionella* spp., *Mycobacterium* spp. and various enteric bacteria have been recovered on defined, selective media both directly and indirectly (*i.e.*, impaction vs. liquid capture). There are no reports from the aircraft air quality literature of attempts at airborne fungal enumeration using selective media. Further, all selective media used to isolate bacteria from aircraft cabin air have been following initial incubation on undefined, general purpose media. Many bioaerosol studies, using defined media and/or selective incubation, have targeted well-known pathogens. The following list presents some of the major studies reported in the recent bioaerosol literature that quantify known pathogens from air samples using selective media:

(i) Reports of laboratory experiments to isolate different species of aerosolized *Mycobacteria* spp. and *Legionella* spp.: (Riley *et al.*, 1976; Berendt, 1980; Dennis and Lee, 1988; Edelstein, 1981; Dennis, 1988; Falkinham, *et al.*, 1989)

(ii) Reports of bacterial pathogens selectively isolated from "high exposure environments" such as hospitals, agricultural settings, and wastewater treatment operations, including *Mycobacteria* spp. or *Legionella*, spp., and indicators of enteric pathogens (Riley, 1962; Crook *et al.*, 1991; Macher; 1993; Sawyer *et al.*, 1996; Pillai *et al.*, 1996; Fannin *et al.*, 1985; Rahkonen *et al.*, 1987)

(iii) Reports of bacterial pathogens selectively isolated from air following domestic disease outbreaks - *Legionella* spp. (Addiss *et al.*, 1989; Barbaree *et al.*, 1987; Breiman *et al.*, 1990i)

(iv) Reports of bacterial pathogens selectively isolated from air near suspected environmental sources - *Mycobacteria* spp. and *Legionella* spp. (Bollin *et al.*, 1985; Briemann *et al.*, 1990i; Briemann *et al.*, 1990ii; Fallon and Rowbotham, 1990; Saywer *et al.*, 1996; Fennelly ***et al.*, in press**)

(v) Reports of fungi and/or Actinomycetes selectively isolated from occupational or environmental exposures, including *Apergillus* spp. and *Penicilium* spp. (Rahnkonen *et al.*, 1987; Mulhausen *et al.*, 1987; Streifel *et al.*, 1987; Donham *et al.*, 1989; Robertson, 1997; Miller *et al.*, 1988; Reynnolds *et al.*, 1990)

(vi) Reports of airborne *Legionella* spp. culturability response to changes in relative humidity (Dennis and Lee, 1988)

Airborne Bacteria Levels on Commercial Aircraft - Defined or Selective Media. Using direct agar impaction techniques, it appears that only undefined media have been used for the capture and initial cultivation of bacteria from air samples obtained from in-service aircraft. All of the aircraft air quality literature surveyed suggests that no direct cultivation on selective media has ever been attempted, and given the air sampling instruments used, it was assumed that bacterial isolates initially cultured on undefined, broad spectrum media were transferred to defined media for standard genotypic-based identification (US DOT, 1989; ATA, 1994; Dechow *et al.,* 1997; Nagda *et al.,* 1992; Pierce *et al.,* 1999). While species-level discrimination was reported from isolates grown on undefined media, only one aircraft study, Dechow *et al.,* 1997, reported their use of defined/selective media and included the target for the media selections as well as incubation conditions, which are summarized below:

Blood Agar with Aerobic Incubation - no selection presented
Blood Agar with Anaerobic Incubation - selecting for anaerobic bacteria
Xylose-Lysine-Desoxycholate (XLD) Agar - selecting for *salmonellae*
Caseinpeptone-Soyapeptone (CPS) Agar - selecting for thermophilic actinomycetes
Sabouraud - (4%) Glucose SA Agar - selecting mesophilic and thermotolerant fungi

Following enrichment on undefined media (and transfer to selective media), all the aircraft air quality literature reviewed collectively identified 12 different genera of airborne bacteria present on the flights monitored. In some instances, several successful isolations were reported without positive identification; a US DOT report (US DOT, 1989) described some isolates in terms of their gram staining and morphological characteristics (*e.g.,* Gram positive cocci). This report provided a rank order assessment from its culturing survey, and reported that with respect to the bacterial taxa cultured from cabin air, *Micrococcus, Staphylococcus, Anthrobactor, Corynebacterium,* and *Bacillus* were the most frequently identified taxa (US DOT, 1989). No other reports on cabin air quality provided a rank order assessment or attempted to quantify the different bacteria isolated from aircraft; however, a list of bacterial isolates was reported by the

Air Transport Association (ATA, 1994) to include the following species and genera: *Staphylococcus, Acinetobacter lwoffi, Psuedomonas wessicularis, Sphingomonas spiritivorum, Bacillus* spp., *Micrococcus* spp., *Diptheroids, Pasteurella* spp., *Xanthamonas maltophilia, Citrobacteri freundii, Enterobacter* spp., and *Ochrobacterium anthropi.*

Germane to the atmospheric environment in aircraft cabins and the diseases that can be caused by inhalation of bacteria, *i.e.,* the causative agents for some bacterial pneumonias and upper respiratory infections (*Psuedomonia aeruginosa, Streptococcus pneumonia, Streptococcus pyrogenese*), Legionnaire's disease (*Legionella,* spp.), diphtheria (*Corynebacterium diptheriea*), whooping cough (*Bordetella* spp.), and tuberculosis (*Mycobacterium* spp.) can be isolated on selective media or indirectly isolated via transfer from "general purpose" to selective media (ASM, 1997; ASM, 1999). Of particular note is that the literature contains no reports of culturing airborne environmental *M. tuberculosis* other than from direct exhalation TB-infected patients into a collection apparatus containing an aerosol impactor sampler. Other than an undefined *Stapylococcus* species, the literature contained only one report of pathogenic fungi or bacteria cultured from air samples on commercial aircraft; these were *Apergillus niger* and *Paecilomyces variotti* – no concentrations were reported for these potentially pathogenic microorganisms.

It should be noted, too, that culturing techniques cannot generally distinguish between vegetative cells and the spores of fungi and bacteria, and that culturing is a conservative estimate of the culturability of airborne cells regardless of metabolic state (*i.e.,* vegetative or spore). Once airborne, vegetative cells are much more labile to environmental influences than are spores. Early studies of airborne bacterial pathogens provided by Dunklin and Puck (1948) found that the aerosolized pathogens *Pneumococcus* Type I, *Streptococcus hemolyticus* Group C, and *Stapylococcus albus* experienced increased survival rates at humidities below 30 %. This trend has not been confirmed through literature review since the early 1950s. In his extensive review, Mohr (1997) stated that much of the published data [generalizing] the resistance of airborne bacteria to changes in environmental conditions cannot be used for comparing aerosol stability [of vegetative bacterial and fungal cells] because of the presence of several stresses which could

have acted synergistically in a detrimental manner or possibly enhanced survival of vegetative cells.

Generalizations regarding the stability of airborne spores have been made, however, and are important to characterizing aircraft cabin air quality. Aerosolized spores of many bacteria are extremely resistant to variations in oxygen concentrations, extremes in RH, and temperature (Mohr, 1997). According to the ACGIH (1999), fungal and bacteria spores may be assumed to be infectious and viable for as long as they are airborne. The concentrations of spores, bacterial or fungal, in aircraft cabin air have not been reported in any aircraft air quality case study, and no field or laboratory study has attempted to quantify the die-off kinetics of airborne bacteria or fungi under the conditions maintained in aircraft (*i.e.*, sustained lowered O_2 partial pressures concurrent with extremely low humidities (< 15%) and temperatures above 70 °F).

Quantitation and Infectability of Airborne Viruses. Viruses replicate only in other living cells, and a common test system to determine the infectivity of viruses in air is the plaque assay. There are other methods reported to quantify airborne viruses that involve either direct introduction into a host or electron microscopy; these methods, however, are not practical for analysis of aircraft cabin air, and were not included in this review. Genetic amplification methods are applicable to the detection of airborne viruses and may be applied to analyze aircraft cabin air; genetic amplification methods will be discussed in subsequent sections.

Culturing Viruses. Plaque assays are the most common type of virus enumeration technique, although genetic amplification methods have been applied to airborne viruses. Plaque assays measure viruses by their ability to infect host cells, while genetic amplification methods detect and estimate only the amount of specific genetic material from a given virus type in an environmental sample - they do not measure infectivity. Viruses are host specific, and the choice of host dictates which type of virus can be recovered from environmental samples and quantified using plaque assays. Plaque assays are enrichments executed in lab culture, where host cells are immobilized on agar surfaces and mixed with environmental samples containing viruses; thus, virus plaque assays are executed on defined media. When virus particles multiply, they can destroy the host cells they have infected; the disruption and lysis of host cells are visually

quantified as plaques, and the number of plaques formed on a host population can be normalized to the volume of air sampled (PFU/m^3). If a virus does not destroy the host cells, other detection methods must be used. Concerning plaque assays, air samples with unknown virus content have to be screened without regard for speciation, and primary and secondary cell cultures are then required because they allow the widest spectrum of virus species to form plaques (Spendlove and Fannin, 1982; Koneman *et al.,* 1988). Virus culture yields information about the number of infectious virus units in an air sample under culture conditions, and may or may not represent the infectivity of a virus under environmental conditions. In their recent review, Sattar and Ijaz (1997) issued a strong statement regarding the nature of virus disease transfer in air: Proper studies on the airborne spread of many human viruses are not possible because many viruses cannot be readily cultured using standard laboratory techniques.

The methodology for collecting, storing, and assaying of airborne viruses has been reviewed (Spendlove and Fannin, 1982; Ijaz *et al.,* 1987; Sattar and Ijaz, 1987). Sensitive viral analysis requires modifications to the common aerosol sampling techniques that target prokaryotes (Sattar and Ijaz, 1997; Payment, 1997; Spendlove and Fannin, 1982). It appears that most of the commonly used bioaerosol sampling devices are currently considered unsuitable for quantitative virus assay and that the highest recovery factors are from high-volume, silicon-coated (glass) liquid-capture samplers containing bacterial nutrient broth(s) (Sattar and Ijaz, 1987; Stenzenbach, 1992; Brion, 1999). The collection of airborne viruses has been reported using common All Glass Impingers (AGI-30), and the recovery of standard impingers has been improved by placing impingers in series (Sattar and Ijaz, 1987). Large volume (> 1000 m^3/min.) liquid-capture samplers have been reported to recover airborne viruses, but the recovery efficiency was reported to vary with virus type and environmental variables (temperature and RH). The type of liquid used in liquid-capture samplers can greatly affect viral recovery efficiency (Sattar and Ijaz, 1987; Brion, 1999), and several different liquid media have been used to capture virus particles; these media include distilled water, different minimal salts media, nutrient broths typically used for bacterial culturing, and specific phage assay broths. Filter methods have also been reported to recover infectious virus particles from aerosols, requiring elution, subsequent concentration, and final analysis using culturing or genetic amplification (Wallis *et al.,* 1985; Spendlove and Fannin, 1982; Saywer, 1994; McClusky, 1996).

Environmental Factors and Virus Stability. There is a paucity of recent environmental microbiology literature concerning quantitative airborne virus studies. The number of environmental bioaerosol studies reporting on the analysis of airborne viruses is markedly less than those targeting other microorganisms (Stezenbach, 1997). How long any given virus can remain infective once airborne depends on the virus type, environmental conditions (RH and temperature), and exposure to radiation (*e.g.*, sunlight). Prior to 1975, there were several investigations reporting on the culture-based infectivity response of airborne viruses to environmental factors - primarily relative humidity. These studies may provide some insight into the stability of airborne viruses in aircraft cabins that are maintained at low humidities (Loosi *et al.*, 1943; Akers, 1966; Barlow, 1973; Benough, 1969; Benough, 1971). Studies of airborne viruses dating back to the 1940s suggest that RH has a significant influence on the survival (infectivity) of airborne influenza virus. Loosi and coworkers (1943) and Lester (1948) showed infectivity of airborne influenza to white mice was greatest at low humidity (low humidity was defined as RH < 23%). Similarly, measles survival in air has also been show to be maximal at low RH (Hastings *et al.*, 1964). Buckland and Tyrrell (1962) confirmed the previous findings of the low RH stability of human influenza virus; they also showed, however, that other common human viruses, including Parinfluenza, Rhinoviruses, Adenoviruses, and some Enteroviruses, have their highest stability at very high RH (RH > 80%), intermediate stability at lower RH (RH < 30%), and poorest stability at intermediate RH. Most of the human viruses tested lost less than two logs of virus titer on air drying over a 2.5-hour period. Akers and Bond (1966) confirmed this trend with aerosolized members of the Columbia SK group of viruses at various temperatures and humidities. In all the human virus (and phage) studies reviewed, intermediate RH ranges (ca 30% < RH < 80%) appear to provide the least stability for airborne human viruses.

Prior to 1975, relative humidity has been reported to have an inverse relationship with the stability and infectivity of some airborne viruses. Such observations have prompted suggestions in the aircraft air quality literature that human viruses could plausibly remain viable for longer time periods than normally encountered in indoor environments; this is due to the low humidity atmospheres maintained in aircraft cabins (US DOT, 1989). More recent literature has

challenged this generalization based on virus type (enveloped vs. naked). Several comprehensive review papers addressing the aerosol characteristics of viruses have provided the following generalities germane to aircraft cabin environments (Spendlove and Fannin, 1982; Mohr, 1991; Mohr, 1997):

(I) viruses are normally very resistant to inactivation by atmospheric oxygen
(II) viruses with lipids in their outer coat or capsid (enveloped viruses) are more stable in aerosols at low RH than at high RH
(III) when viable viruses can no longer be detected after aerosol collection, the nucleic acid can be isolated and may still be active; this suggests that aerosol inactivation of viruses is not caused by nucleic acid inactivation but by denaturation of coat proteins
(IV) humidification in sampling devices increases the recovery of viruses that are not enveloped by lipids

It has been generalized that viruses associated with airborne routes of infection in humans do not remain infective over long time periods (ACGIH, 1999); this generalization carried no definition of "long time periods." Further, this generalization is in contrast to studies concluding that many (human) viruses are stable in an airborne state, through broad temperatures and extremes in RH, on the order of several hours (> 2.5 hours) (Akers *et al.,* 1966; Akers and Hatch, 1968; Hastings *et al.,* 1964). Most study on the persistence of viral aerosols has focused on the challenge of vaccinated animals with airborne viruses to assess the effects of immunization - a branch of research not applicable to determining human virus exposures in aircraft environments. Some recent reports on the kinetics of airborne viral inactivation included measles virus, influenza viruses, and rhinoviruses. Measles virus has been shown to persist in an infectious state for more than one hour in indoor air (Remmington, *et al.,* 1985; Bloch *et al.,* 1985), and the half-life of a human rhinovirus was reported at 14 hours in indoor air at 20 °C and 80% RH (Karim *et al.,* 1985). The aerosolized rhinovirus studied by Karim and coworkers behaved in ways typical of other viruses that cause human respiratory infection, and these authors concluded that rhinoviruses can remain infectious in air long enough to permit aerial spread. In contrast to this observation, Hatch *et al.* (1976, abstract only) concluded that inactivation of Rhinovirus type 2 proceeds relatively quickly at humidities below 50%. In their extensive review, Hendley and Gwaltney (1988) also reported that rhinovirus will survive in air, but limited information is available on the duration of rhinovirus survival in aerosols. They generalized that rhinovirus survives sufficiently well in aerosols to make it feasible for infectious viruses to reach susceptible hosts via aerosol transfer.

In environmental studies of the airborne survival of viruses unintentionally aerosolized from domestic wastewater, echovirus and coliphages were reported to be apparently more stable than bacteria commonly used as indicators of waterborne pathogens (Fannin *et al.*, 1985; Teltsch and Katzenelson, 1978). These studies provided no estimates of the airborne residence time, and when reported, humidity was between 50-80%. Teltsch and Katzenelson (1978) claimed that wind velocity did not play an important role in the survival or airborne microorganisms. The most striking aspect of these environmental studies was the distances these viruses traveled from their source waters while still remaining infectious: 150 meters in the case of coliphages, 40 meters in the case of echovirus.

Some current medical opinion of the spread of many common human viral diseases, such as influenzas, common colds, varicella (chicken pox), polio, and others, favors hand contact with self-inoculation as the primary means of transmission (Couch, 1984 as cited by Dick, *et al.*, 1987; Hendley and Gwaltney, 1988). In the context of aircraft environments, surface contact(s)/self-inoculation has been implicated as a higher exposure route than air/inhalation exposure (ASTM, 1999). Information on the specific mechanisms of rhinovirus transfer comes primarily from studies of volunteers infected by artificial means. Several investigations, however, of epidemiologically isolated events have confirmed the airborne spread of common human viral diseases without air sampling or viral assays - endpoints for these studies were significant numbers of ill persons with minimal or no common surficial contact. In the following studies, the salient theme presented by the authors is that for many human viruses, airborne transmission may occur more often than previously suspected, suggesting that such transmission may occur aircraft via cabin air.

Riley *et al.* (1978) documented the airborne spread of measles in a suburban elementary school using a model that compared and apportioned infection probability via virus exposure in common rooms, school buses, and from airborne viruses recirculating in the ventilation system. Organisms recirculating through the ventilation system were strongly implicated from a single index case producing approximately 93 infectious airborne measles virus particles per minute. This study assumed that measles virus was exclusively airborne, and a droplet nucleus

hypothesis was endorsed which implied the random distribution of infectious particles in the air of enclosed spaces (this hypothesis is not accepted in the aircraft air quality literature). Riley *et al.* (1978) also assumed that under the environmental conditions observed, decay of airborne human measles virus infectability is insignificant in time frames less than one hour; no environmental factors other than indoor temperature were mentioned.

Moser and Bender (1979) reported an outbreak of influenza on a commercial airliner. The aircraft, grounded and occupied for four hours, produced a high attack rate from one passenger, the apparent index case, who was ill on the plane. Within 72 hours, 72% of the passengers became ill, and the clinical attack rate was shown to vary with the amount of time spent on the plane. The same influenza virus was serologically identified in 20 of 22 ill persons tested. The high attack rate implicated the aircraft ventilation system as responsible for increasing influenza exposures, and discounted surface and near-field contact.

Remington *et al.* (1985) documented the airborne transmission of measles in a public medical office. Using a model based on the airborne transmission of measles virus between children, Remington and coworkers showed that the airborne measles virus remained infective between 60 to 75 minutes following the presence of a contagious host – inadequate fresh air ventilation was implicated as the main exposure factor. Characteristics such as increased warm air circulation and low relative humidity were implicated to have increased the likelihood of transmission. Remmington provided editorial that airborne transmission of viral disease may occur more often than previously suspected. Bloch *et al.* (1985) also documented the airborne transmission of infective measles virus facilitated by a commercial ventilation system in a public medical building. Airflow studies demonstrated that droplet nuclei generated in examining rooms were dispersed throughout the entire office suite; airflow characterizations supported the conclusions that measles viruses can remain infective while airborne longer than one hour at intermediate RH.

Sawyer *et al.* (1994) documented the outbreak of viral gastroenteritis in a hospital setting, most likely a Norwalk-like virus, and implicated its spread through airborne transmission. The explosive nature of this outbreak, in which a common source (*i.e.*, food, water, person-person,

surficial) was not identified, led investigators to conclude that airborne transmission of the virus was the dominant transmission mechanism. No modeling was applied to determine a minimum airborne half-life to sustain the transmission.

Myint (1997) reviewed common colds, asthma, and indoor air quality. This reviewer stated that aerosol transmission of rhinoviruses and coronaviruses - the commonly encountered causative agents for human "colds" - is the most common mode of disease transfer between humans. These viruses are now thought to provoke the majority of asthmatic attacks in children. In opposition to the general summary provided by Sittar and Ijaz (1997), Myint (1997) stated that aerosol transfer of coronaviruses is enhanced by humid conditions (although coronaviruses are enveloped) – no range of RH or references were presented in this context. Citing studies from the 1960s showing that room size and the use of disinfected input air made little difference in "cold" attack rates in offices (Kingston *et al.,* 1962), Myint presented the view that close person-person, but not necessarily direct, contact is the most important route for "cold" disease transfer.

In a thorough review restricted to mammalian viruses, Sattar and Ijaz (1987) summarized studies that challenged human and animal hosts with aerosolized viruses. The studies in this review were from 40 literature reports published after 1970 (included in the bibliography), and the authors generalized these conclusions:

(i) dose response among the various hosts and viruses were indeterminate, and in many cases levels of aerosolized viruses may have been unrealistically high
(ii) in spite of their importance, critical environmental factors such as humidity and temperature were not controlled or monitored
(iii) nearly all studies used laboratory-adapted strains of test viruses that may not represent infectivity in the environment

The reviewers further concluded that meaningful extrapolation from virus challenge studies to actual field conditions was perhaps not determinable, and that the lack of standardization for virus aerosol challenge studies makes comparisons difficult. The literature since 1975 contains few reports that describe the decay of viral infectability under defined environmental conditions, and it appears that the effects of extremely low RH in the range sustained in commercial aircraft during flight (ca 10% - 15%) has not been reported. It is clear that many mammalian viruses have been documented to remain infective while airborne longer than the mean ventilation

residence times in commercial aircraft. Further, the literature describing the response of viruses to environmental conditions (while airborne) is tenuous and incomplete. The literature implicates the need for further research in this area and any extrapolation to airborne viral transmission in aircraft cabins.

MICROSCOPY

Culturing bias and its implications. Traditionally, only the culturable portion of bacteria in the atmosphere has been studied (Tong and Lighthart, 1999; Hernandez *et al.,* 1999; Mocheandreas *et al.,* 1996). In many past bioacrosol studies, the detection and quantitation of metabolically competent microorganisms has been primarily based on plate count assays in which microorganism nutritional requirements and culturability (and associated sample collection methods) bias the results. When not targeting individual species, conventional culture-based approaches have intrinsic limitations for the robust characterization of microorganisms from airborne environmental sources. Because culture-based methods cannot identify slow-growing, non-culturable or inactive microorganisms and fragments thereof, standard plate counts underestimate the true quantity and diversity of airborne microorganisms. Nor can standard plate counts differentiate between spores and vegetative cells as they exist, suspended in the air.

In their extensive review of survival strategies of bacteria in the environment, Roszak and Colwell (1987) discuss the application detection methods that are sensitive to bacteria in all life states, and conclude that many bacteria could be existing in a nonviable or viable but non-culturable (VBNC) state. Colwell and coworkers (1986, 1987) have showed that human pathogens, including *Vibrio cholera*, *Camplyobacter* spp., and *Legionella* spp., exist in an VBNC state and have demonstrated conversion from a culturable state to a VBNC state in environmental samples. In this context, Tong and Lighthart (1999) state that for ecological and epidemiological reasons, it is now necessary to explore the total concentrations of atmospheric bacteria and fungi and what fraction of the total is culturable. Discrepancies between the abundance and diversity of microorganisms - collected by identical methods from aquatic, terrestrial, and atmospheric biospheres but enumerated separately by culturing, direct microcopy, and genetic probing - suggest that less than 1% of active bacteria present in these environments are capable of being cultured using classical methods (Woese, 1987; Amann, 1995; Pace, 1996).

Non-culture-based characterization methods include both direct and indirect techniques. Indirect methods are defined here as including some type of extraction or amplification step and, with respect to successful bioaerosol monitoring applications, include the following techniques: bioluminescence, lipid analysis, immunoassay, and genetic probing coupled with polymerase chain reaction (PCR) or sequence targeted PCR. Direct microscopy, using either phase contrast lenses (no stains), conventional or fluorescent stains, or genetic stains, remains the only true direct technique. Because the indirect non-culture-based methodologies are relatively new with respect to their classical counterparts, there are relatively few reports of bioaerosol monitoring using these methods. A summary of each method follows.

The relationship between actual airborne microorganism concentrations and the microorganism numbers and diversity cultured from air samples is not well understood, and undoubtedly varies widely in any given atmosphere. The artificial environment maintained within aircraft cabins is probably not exempt from inaccuracies due to culturing artifacts, and, based on prior reports of other indoor commercial and residential environments, total airborne microorganism numbers in aircraft are likely to be higher than those previously reported using culturing techniques. Thus, the most robust approach to determining the microbial ecology of airborne microbiological contaminants in aircraft is to use both culture-based and non-culture-based methods. Several case studies of both indoor and outdoor environments have confirmed that total numbers of microorganisms enumerated by direct methods are significantly (orders of magnitude) higher than those recovered by culturing; this confirmation has been provided primarily by direct microscopy.

The earliest report comparing methods for the quantitative determination of airborne bacteria and evaluation of total viable counts was reported by Lundholm (1982). While the methods used in this investigation were predominantly culture-based, Lundholm (1982) demonstrated that the number of viable microorganisms that could be cultured from commonly used air samplers varied significantly, and this differential culturing effect was *not* solely due to sampling stresses intrinsic to equipment. Lundholm (1982) concluded that the adhesive nature of some airborne microorganisms caused the true number of viable particles to be significantly underestimated by

culturing from air samples collected by commonly used equipment (Anderson sampler, slit-to-agar samplers, glass impingers, and filter samplers). The numbers of viable bacteria (on nonselective media) were indexed to the size of airborne particles collected from cotton mills, sewage treatment plants, and skin fragments.

Palmgren *et al.* (1986) were one of the first to report the use of epifluorescent microscopy to directly quantify airborne microorganisms in environmental samples. In a modification of the membrane filtration technique developed by Hobbie *et al.* (1977), the total number of airborne microorganisms collected on nucleopore filters was determined by acridine orange staining - a DNA intercalating agent that binds to all genetic material regardless of biological origin. The method used was termed the CAMNEA method (Collection of Airborne Microorganisms on Nucleopore Filters, Plate Count Analysis and Total Count Estimation Using Acridine Orange Staining). The viable fraction (determined by culturing) of the total microorganism numbers varied significantly when actinomycete and fungal spores from different environments were stored on filter for a week. A high correlation between viable and total microscopic counts was observed from environments where the airborne flora was dominated by fungal spores, while a low correlation was found for airborne bacteria. Peak values of the total counts registered in some of the work environments monitored varied between 10^7 - 10^{11} cells/m^3; viable counts were between 7% and 91% of the total cultured from airborne dusts from different occupational exposures (*e.g.*, pig houses, hay, grain, wood chips, straw). The authors used morphological designations (*e.g.*, coccoid, rod-shaped) and size measurements in four ranges (< 1.5 µm; 1.5-5.0 µm; 5.0-10.0 µm; > 10 µm) to microscopically discriminate between spores, bacteria, and fungi. The authors concluded that this study showed the "utmost importance to combine viable counts with total counts in the study of exposure to [airborne] microorganisms in work related situations."

Eduard *et al.* (1990) reported on the concurrent use of scanning electron microscopy (SEM), light microscopy (LM), epifluorescence microscopy (FM), and culture-based methods to assess the concentration of filter-captured airborne microorganisms from heavily contaminated work environments, including pig barns, grain elevators, and saw mills. Bacteria, actinomycetes, fungi, and fungal spores were discriminated by size and morphology (for microscopy) and

culturing on specific media. Concentration estimates by SEM and LM were similar, but those determined by FM and culturing were smaller. Counts obtained by culturing were always smaller than those obtained by SEM or LM, but they sometimes exceeded those obtained by FM – this is the only report in the bioaerosol literature in which airborne microorganism concentrations determined by microscopy were less than those determined by culturing. Yet the authors concluded that overall direct microscopic counting suggested that between 0.1-68% of the total airborne bacteria and between 3-98% of the total airborne fungal spores were viable as determined by culturing. Microscopic size measurements suggested that the size distributions of all types approximated a log-normal distribution, although single spores and small aggregates of bacteria were perhaps underrepresented in their analysis. The precision of counting spore-containing molecules by LM and SEM was better than counting individual spores, but because of the dispersion technique used, no difference in precision was observed using FM (to count spore-containing particles and individual spores). While the electron microscope counts provided confirmation of the other direct microscopic techniques and yielded some morphological information not otherwise available, the SEM protocol outlined by the author required special preparation and handling techniques that are not practical or economical for the large sample numbers that would be required in aircraft studies.

Rautials *et al.* (1996) used the CAMNEA method to demonstrate the increasing indoor air concentrations of airborne fungi and bacteria during the repair of moldy buildings. Concentrations of culturable airborne fungi captured on an Anderson six-stage cascade impactor were in the range of $10^3 - 10^5$ CFU/m^3. Using acridine orange staining, concurrent epifluorescent counts of filter-captured airborne fungal propagules showed concentration increases in the range between $10^5 - 10^6$ #/m^3. Concentrations of total and viable bacteria were also documented to increase during building repair. The authors used morphological designations (*e.g.*, coccoid, rod-shaped) and microscopic magnification factors (x400 vs. x1000) to microscopically discriminate between spores, bacteria, and fungi. Airborne microorganism concentrations using direct microscopy were always an order of magnitude (or more) greater than those determined by culture-based methods.

Mocheandreas *et al.* (1996) reported a modification to adapt a common six-stage Anderson sampler for direct microscopy of airborne microorganisms; this method used sterilized water instead of culture medium in each of the petri dishes mounted in the Anderson's stages. After sampling, the water was passed through 25 mm nucleopore membrane filters; the microorganisms retained on the filter surfaces were stained with acridine orange and counted with an epifluorescent microscope. A Reuter centrifugal sampler was also tested in parallel with the modified Anderson sampler. To test if the precision of direct count methods was significantly different from that of conventional culturing, the authors obtained more than 100 samples from inside (and immediately outside) six commercial buildings and developed corellations between direct microscopic counts and counts of colony forming units. As determined by ANOVA using simple regression analysis, in all cases, direct counts were significantly higher than colony forming units at a 95% confidence level.

Using the Anderson sampler: Total count = 140 + (0.72 x culturable count)

Using the Reuter sampler: Total count = 111 + (0.98 x culturable count)

These authors reported that total microorganism concentrations could be reported within an hour after sampling. No significant interferences were discussed.

In a small-scale chamber study, Terzieva *et al.* (1996) compared four methods for the detection and enumeration of airborne bacteria collected in liquid impingers. Direct epifluorescent microscopy, using the fluorescent dyes acridine orange and the proprietary BacLight in conjunction with an aerodynamic aerosol-size spectrometer, were used to measure the total bacterial cell concentrations in impinger collection fluids and in the air. Unlike DNA intercalating agents, the proprietary BacLight is marketed to assess microbial viability *in situ* on the basis of membrane integrity. Results from direct microscopy were compared with plate counts on selective (MacConkey agar) and nonselective (Trypticase Soy Agar) media, and the bacterial injury response to aerosolization and sampling stresses was determined. The authors reported that bacterial clusters in the air deagglomerated during impingement, thus increasing the total bacterial count using both dyes. The BacLight staining technique was also used to determine the changes in viable bacterial concentrations during sampling. The percentage of viable bacteria determined by microscopy decreased markedly over a 60-minute sampling period, suggesting that bacteria are stressed and die during extended reflux in AGI-30 liquid

impingers. The differential recovery of bacterial colonies, cultured from the same air sample and grown in parallel on different broad-spectrum agars (*i.e.,* TSA and MacConkey agar), has been interpreted by these authors as an index of microbial injury incurred during the air sampling process. Concurrent counts on MacConkey agar and TSA suggested that structural damage occurs during liquid impingement but that most of the bacterial cells injured during sampling could recover. This suggestion has broad implications for interpreting results of culture-based bioaerosol studies (*i.e.,* the bias introduced from extended sampling time in liquid impingers as well as representing the state of airborne microorganisms *in situ*). The comparison of data on the percentage of injured bacteria obtained by culturing and the percentage of nonviable bacteria obtained by BacLight staining showed good agreement. In all cases, the total number obtained by acridine orange staining was between 20-50% greater than concurrent counts using BacLight, and 50-80% greater than colony-forming units. No significant interferences were discussed.

Hernandez *et al.* (1999) developed and tested a combined fluorochrome method for quantitation of metabolically active and inactive airborne bacteria. In this large-scale chamber study (36 m^3), liquid impingers were filled with a fluorescent viability dye that immediately stained actively metabolizing airborne bacteria as they were collected from room air. Both active and inactive airborne bacteria were then counterstained with a fluorescein derivative and counted with an epifluorescent microscope. The choice of fluorochrome stains allowed concurrent identification of metabolically active and inactive bacteria in the same microscopic field. Airborne bacterial numbers determined by microscopy were compared to standard, nonselective colony counts cultured from impingers operated under identical conditions. Direct microscopic counts of total airborne bacteria were higher than concurrent plate counts by an order of magnitude on average. Counts of bacteria that were actively metabolizing while airborne were lower than counts determined by culturing, suggesting that metabolic competence and repair ability cannot be determined by activity stains. Hernandez and coworkers noted that current detection limits for the quantitation of airborne bacteria by direct counting are quite high, limiting the application of direct microscopy to environments with high concentrations of airborne microorganisms. The authors recommended that for practical use in field studies where bioaerosol concentrations are typically lower (such as aircraft cabin air), the detection limits of direct microscopic sampling methods must be lowered into a more useful range. They suggested that lower detection limits

for direct microscopy may be accomplished by (i) increasing sample collection time, (ii) using smaller filters for microorganism retention prior to staining, and (iii) using high-volume liquid sample equipment.

Tong and Lighthart (1999) investigated the diurnal distribution of total and culturable atmospheric bacteria at a rural outdoor site using epifluorescent microscopy and culture-based methods. Using high-volume, wet cyclone samplers, atmospheric samples were taken bi-hourly for 11 days. Using a modification of the CAMNEA method that included a prefiltration step, acridine orange was used to enumerate total airborne bacteria. The geometric mean of the total airborne bacterial concentrations was about 27 to 222 times greater than that of culturable airborne concentrations from atmospheric samples. The prefiltration step used by these authors undoubtedly introduced some error into the concentrations of total airborne bacteria reported; the actual total concentrations were most likely higher. The authors stated that the much higher total concentrations suggested that atmospheric concentration. This is the only aerosol microscopy study that discussed the issue of significant optical interferences due to nonspecific background fluorescence of both nonbiological particles and primary biological material (that was not of microbiological origin). These interferences can be formidable and are most likely encountered in air samples from outdoor environments.

Other recent research reports, that did not have direct microscopy as their focus, or used direct microscopy to support other non-culture based assays, acknowledge that viable counts severely underestimate microbiological numbers (Henningson et al, (1997); Flannigan (1997) and Macnaughton et al, (1994)). Direct microscopy is now recognized as a reliable technique to quantify total bioaerosol concentrations (not including viruses), and has been recommended in the recent scientific literature to accompany any culture-based investigations; this is because culture-based techniques have been shown to significantly underestimate airborne microorganism concentrations. The salient theme associated with the bioaerosol microscopy literature maintains that for ecological and epidemiological reasons, it is necessary to determine the total concentrations of atmospheric bacteria and fungi, as well as what fraction of the total is culturable.

MOLECULAR TECHNIQUES, GENETIC PROBING, and GENETIC AMPLIFICATION METHODS as APPLIED to MONITORING MICROBIOLOGICAL AIR QUALITY:

All viable microorganisms carry genetic material, DNA and RNA, that may be analyzed for identification purposes. DNA and/or RNA analysis can yield valuable information on the identity, distribution, and abundance of microorganisms in many environments, including air. Many pathogenic viruses and bacteria have genetic sequence information, suitable for identification purposes, professionally catalogued and available through government supported databases: (**Genebank, Ribosomal Database Project**). The detection and matching of DNA/RNA from environmental samples to catalogued genetic sequences can provide information to support exposure assessments.

Several reviews have been published since 1993 that describe the potential application of genetic-based molecular biology methods to the analysis of airborne microorganisms. Mukoda (1994), Heinsohn (1994), Griffths and DeCosemo (1994), MacNeil *et al.* (1995), Crook (1996), Buttner *et al.* (1997), and ACGIH (1999) have provided editorial and reviews of the scientific literature that have focused on the feasibility of applying genetic molecular techniques, particularly amplification, to characterize bioaerosols. These reviews discussed the general biases and potential limitations of genetic probing and amplification technique as applied to aerosol systems; the latter reviews synthesized seven studies that demonstrated the use of genetic amplification technique to detect target species of airborne bacteria in both outdoor and indoor (chamber) environments. To date, the literature contains only demonstration studies and qualitative reports on (*i.e.,* the presence or absence of) selected airborne microorganisms. Only recently have their been reports conclusively demonstrating the ability of genetic amplification techniques to be refined for quantitative analysis with reliable detection limits in ranges useful for exposure assessment. There are several methods used to compare sequences of genetic material recovered from environmental samples to internal standards or existing genetic databases. The most useful of genetic characterization methods that have been applied to detecting microorganisms in aerosol systems include the following: (i) direct genetic probing, (ii) restriction endonuclease analysis, and (iii) genetic amplification methods (which may be combined with (i) and (ii)).

Nucleic Acid Probes. Nucleic acid probes can detect specific genetic sequences through genetic hybridization - the processes of annealing two complementary sequences of genetic material. Genetic probes themselves are usually small pieces of DNA (oligonucleotides on the order of 20 to 40 single-stranded DNA bases) that recognize and bind specifically to complementary sequences of DNA or RNA from targeted microorganisms. Oligonucleotide probes are conjugated to a reporter molecule to detect specific DNA/RNA sequences once the probes hybridize with their targets; reporter molecules vary depending on the type of analysis and required sensitivity. Often, nucleic acid probes are bound to radioactive phosphorus (P^{32}) for scintillation counting, or fluorescent markers for microscopy and/or image analysis. Oligonucleotide probes can be applied to membrane-immobilized DNA extracted from environmental samples, or to whole cells fixed on membrane filters or slide surfaces. Nucleic acid probes have been developed using randomly determined DNA fragments, specific genes coding for ribosomes (protein making functions), virulence factors, and phage and other viral DNA. Comprehensive reviews of genetic probing theory and technique have been provided by Woese (1987), Amann *et al.* (1996), and Pace (1996).

Direct Genetic Probing of Bacteria. The DNA sequences used for genetic probes are phylogenetically adjustable, *i.e.,* they can be designed to target a single species or strain of microorganism, or to encompass many genera and phyla. For bacteria, ribosomal RNA (rRNA) has been considered the most suitable genetic target for culture-in studies because it contains both conserved and species specific DNA sequences (Woese, 1987); genetic "conservation" in this context means DNA sequences are shared among microorganisms. rRNA (specifically 16s RNA) is currently being used as a genetic chronometer to type and catalogue bacteria, and new bacterial probes targeting 16s rRNA are continually being developed. Hundreds of bacterial genetic probe sequences (too numerous to list here), for many phylogenetic levels, are available through the literature and compiled in government-supported genetic databases (Alm *et al.,* 1996). Both direct DNA extraction and whole-cell techniques are applicable to identifying and quantifying bacteria from aerosol samples. Using modifications to direct microscopic techniques described in earlier sections, epifluorescent microscopy can be used to enumerate whole cells hybridized to nucleic acid probes with a well-established protocol known as Fluorescent In Situ Hybridization (FISH), first introduced by DeLong and coworkers (DeLong et al, 1987). Many studies have reported the successful quantitative use of FISH-probing technique in a wide variety of marine, aquatic, and terrestrial environments.

The literature contains only one report of direct gene probe use to characterize and quantify bacteria from environmental air samples. Neef *et al.* (1995) reported on the detection of microbial cells in aerosols using nucleic acid probes. FISH methods were evaluated for the identification of microorganisms in mixed bioaerosols. Culture-dependent methods were compared to direct whole-cell FISH. After sampling aerosols by filtration, special processing of filter-captured cells preceded hybridization with fluorescently digoxigenin- or enzyme-labeled oligonucleotide probes. Group, genus, or species affiliation of collected cells were analyzed with rRNA-targeted probes. Using nucleic acid probes directed against the multiple cloning sites, plasmid-bearing *Escherichia coli* colonies could be differentiated from wild-type colonies. The microbial aerosol concentrations ranging from less than 10^1 to greater than 10^9 cells/m^3 air could be analyzed with appropriate hybridization formats: whole-cell hybridization was only applicable to dense aerosols, and colony hybridization yielded best results with lower concentrations. After a short incubation period (several hours), a combination of both formats could be used to rapidly determine the fraction of culturable cells within an aerosol sample. When applying these techniques to the monitoring of aerosols generated by common aerosolization equipment, low concentrations of airborne *Escherichia coli* cells (between 1-450 m^3) could be detected. The authors concluded that compared to conventional air monitoring techniques, hybridization with nucleic acid probes should allow more rapid and reliable detection of airborne microorganisms, including genetically engineered microorganisms. This study has been met with the critique that the ribosome content of assayed cells must be sufficiently high (*i.e.*, cells must be metabolically active) to provide an analytical signal.

Fungi. Genetic probes for fungi are also continually being developed, but have primarily been applied in aquatic and terrestrial environments. As with bacteria, ribosomal RNA is often used as a genetic target for fungal identification (MacNeil *et al.*, 1995). While genetic probing of Eukaryotes often relies on 18S rRNA (the eukaryotic analogue of the prokaryotic 16S rRNA), Irisbaez *et al.* (1991) proposed a universal method for generating genus-specific fungal probes. Genetic probes for fungi are not limited to rRNA genes and have been reported for many pathogenic genera of fungi, including those implicated to cause human disease via airborne routes: *Aspergillus* spp., *Alternaria* spp., *Fusarium,* spp., and *Penicillium* spp. (Girardin, *et al.*, 1993; Irisbaev *et al.*, 1991; Spreadbury *et al.*, 1993; van Belkum, 1994). MacNeil *et al.* (1995) compiled a list of genetic probes targeting sequences

associated with pathogenic airborne fungi and bacteria from the scientific literature up to 1995; no subsequent publications have added to this list or compiled a similar one. There are fewer reports and catalogued sequences of ribosomal probes for fungi catalogued with the major genetic databases, and as in the case of bacteria, the literature contains no reports of direct gene probe use on fungi from environmental air samples.

Other than direct whole-cell probing, many practices used for microorganism identification extract and immobilize DNA on membranes and probe for sequences associated with the ribosomes of bacteria and fungi, or probe for unique sequences of DNA or RNA of viruses. In eutrophic environments with adequate sample size, this approach has been successful without artificial amplification (Raskin *et al.*, 1994a,b, Wagner, 1995). In oligotrophic environments such as aerosols, direct probing of DNA or RNA extracts has not been reported.

Polymerase chain reaction - PCR: This amplification method has its origins in molecular biology, and was first applied to a sensitive diagnostic test for sickle cell anemia. Polymerase Chain Reaction (PCR) is a technique for the *in vitro* amplification of specific nucleic acid sequences. DNA extraction methods are well-established, but because individual cells contain only femtograms of DNA (10^{-15} g), the amounts of genetic material recovered from environmental samples is often too small to be of analytical value. The detection of unique DNA sequences in environmental samples often requires the artificial reproduction of many DNA copies using the genetic material from collected microorganisms as a template; no culturing is required in the process. The *accurate* reproduction of DNA is termed "amplification," and is achieved by synthetically polymerizing selected or random DNA sequences using a reaction catalyzed by a purified bacterial DNA Polymerase enzyme (in a PCR). DNA sequences targeted for amplification are bound and identified using genetic "primers," each complementary to opposite strands of the region of target DNA. Primers can be randomly selected, or can be chosen to amplify regions of DNA that are unique to any microorganism or conserved among a unique group of microorganisms. PCR primers for many microorganisms have been designed and synthesized from genetic sequence information available in the literature or from genetic databases; primers for a variety of known airborne pathogens are available.

During the amplification process, the PCR product - unique genetic sequence(s) - accumulate exponentially, and with currently marketed PCR equipment, it is reliably estimated to do so by a factor of 10^6 (Saiki *et al.,* 1988, 1989; Gibson *et al.,* 1996; Piatak *et al.,* 1993; Bassler *et al.,* 1995). The exponential amplification of the original sample nucleic acid(s) is the defining feature of PCR; in theory, quantities of target DNA so minute that they cannot be detected by any direct microscopic or culturing method can be amplified to levels that are well above detection limits using simple reporter assays. PCR sensitivity has been demonstrated to co-amplify two different genetic targets from a single sperm cell (genome) suspended in water (Li *et al.,* 1988), suggesting that detection limits are on the order of single cells. A rational for using non-culture-based analysis of disease-causing aerosols has been presented in previous sections, and the value of PCR-based genetic amplification in this context is its potential for very low detection limits. This has been acknowledged in several review publications that discuss the application of genetic techniques for microbiological air quality characterization (Mukada *et al.,* 1994; Heinsohn, 1994; Griffths and DeCosemo, 1994; MacNeil *et al.,* 1995; Crook, 1996; Buttner *et al.,* 1997; ACGIH, 1999). Most reports of PCR applications to pathogens in aerosols target *Legionella* species; however, there are recent reports of the successful application of PCR to detect *Mycobacterium tuberculosis* species.

PCR can be used for detection of individual microorganism species, provided that genetic sequence information is available for primer design, and genetic methods can be used to confirm PCR amplification products. Electrophoresis, the isolation of discrete DNA sequences in a gelatinous media, is typically used to analyze PCR products once amplified. When coupled with gradient electrophoresis techniques (Denaturing Gel Gradient Elcctrophoresis), multiple organisms of interest may be concurrently assayed from the same sample. Protocols for the electrophoretic separation of bacterial ribosomal DNA using DGGE (Denaturing Gel Gradient Electrophoresis) are well-established (Microbial Insights, 1998), and have been used successfully in water and wastewater. To date, the time frames for PCR execution and analysis are on the order of hours to a day, and the skill level required for its accurate execution is reasonably high. The commercialization and licensing of the PCR process and equipment has stabilized in recent years, and maintain this technology as somewhat more expensive than conventional culturing, microscopy, or immunochemical methods. Presence or absence assays using PCR are widely accepted, provided careful sampling practice is followed.

There are hundreds of publications on PCR amplification of eukaryotic, bacterial, and viral genomes in the medical microbiology literature. Compared to culturing and microscopy techniques, the body of literature describing PCR techniques for environmental applications is relatively new and limited; less then ten have been dedicated to the characterization of microorganisms in aerosols. Investigations applying PCR to aerosols have typically included collection and concentration of airborne microorganisms on filters or liquid impingers, and, to a lesser extent, wet cyclones. This section reviews successful applications of PCR to detect aerosol-associated microorganism aerosols, and a discussion of general bias and specific interferences encountered with PCR aerosol analysis is included.

Concerning airborne pathogens, it became possible to design PCR primers and genetic probes to identify *Legionella* spp. when MacDonell and Colwell (1987) identified unique sequences of the 5s ribosomal RNA of catalogued *Legionella* spp., and several investigators have detected *Legionella* in environmental samples using different DNA probes. Starnbach *et al.* (1989) determined that PCR of target DNA sequences combined with hybridization using a DNA probe labeled with P^{32}, and reported that PCR and gene probes could be used to detect as few as 35 CFU of *Legionella pneumophila.* PCR has been used to discriminate between *Legionellae* and non-*Legionellae* from culture plates (Edelstein, 1986); to subtype cells from a single colony of *L. pneumophila* (Gomez-Luz *et al.,* 1993); to detect *Legionellae* in environmental water sources; and to detect *Legionellae* in air (Heinsohn, 1994). From these studies, comparisons of the PCR method and culture showed that the 5S rRNA primers for *Legionella* spp. had a higher level of sensitivity than culture and that the PCR method was reliable and reproducible.

Majbubani *et al.* (1990) was one of the first studies to demonstrate the utility of PCR to detect pulmonary pathogens in environmental samples, and compared the specificity of PCR to immunochemical techniques. PCR was used to detect and amplify DNA from 15 (serogroups) of *Legionella pneumophila;* the specificity of PCR amplification was demonstrated using 5S rRNA and macrophage infectivity potentiator (*mip*) genes as targets. *Pseudomonas* spp. that exhibited antigenic cross-reactivity with *Legionellae* in serological (immunochemical) detection tests did not produce positive signals with a concurrent PCR-gene probe method. DNA sequences that were amplified by PCR were confirmed with radiolabeled oligonucleotide probe hybridization. While the media in this

study was environmental water sources, it demonstrated that single gene amplification and subsequent detection could be achieved using PCR and genetic probing methods against an airborne pathogen.

Koide *et al.* (1993) investigated the presence of *Legionella* spp. in water circulating in cooling towers by PCR using three different sets of primers concurrently (two of the PCR primers were previously reported by Majbubani *et al.,* 1990); two primers were directed at *L. pneumophila,* and one was directed at all catalogued members of the genus *Legionellae.* Of 27 cooling water towers tested, 25 were PCR-positive for *Legionella* spp., and 14 contained *L. pneumophila.* The authors concluded that a comparison of the PCR method and culturing showed that primers targeting rRNA had sensitivity greater than that of culturing, and that those primers targeting specific infectivity genes had sensitivity approximately equal to that of culturing. No discussion of interferences was included.

Gomez-Luz *et al.* (1993) compared PCR, multiple endonuclease digestion, and immunochemical analysis for subtyping *Legionellae.* Cells from single colonies could be subtyped by arbitrarily primed PCR within a few hours. The discrimination between strains of *Legionella pneumophila* was equivalent to that of monoclonal antibody analysis and ribotyping (endonuclease digestions).

Concerning other airborne pathogens, primers have been developed to detect Mycobacteria in clinical specimens and environmental samples using PCR technique. In clinical specimens and artificial media, Boddinghaus *et al.* (1990) reported a method detection limit of less than ten *Mycobacteria* cells by amplifying ribosome-coding DNA sequences that included all bacteria in the genus *Mycobocteria*; once amplified, the rRNA sequences were confirmed with genetic probes and gel. Also in a clinical setting, Altamirano *et al.* (1992) reported a method detection limit for Mycobacteria at less than 20 cells using PCR combined with conventional dilution and gel electrophoresis. Nolte *et al.* (1993) also used PCR to detect *Mycobacterium tuberculosis* in the sputum of patients diagnosed with active tuberculosis. Nolte and coworkers (1993) used purified Mycobacteria DNA as an internal control and demonstrated that feemtogram levels of target DNA could be detected; results were confirmed with genetic probes using electrophoretic techniques. In all these clinical and environmental investigations, no significant interferences were reported.

Zhai *et al.* (1993 - limited translation from Chinese) reported the PCR detection of *Francisella Tularensis* in aerosols by PCR and compared the results to selective plate counting on defined media. PCR was reported as more sensitive than culturing to determine the total numbers of *Francisella tularensis.* An undefined semi-quantitative PCR recovery was reported within three hours of aerosol sampling, while traditional plate counts required at least three or more days for significant quantitation.

Pillai *et al.* (1993) reported on PCR-based ribotyping of airborne bacteria and pathogen indicators following the land application of sewage sludge. Over a four-month period, glass impingers (AGI-30) were used to collect air samples near a desert site where anaerobically digested sewage sludge was applied to dry soils under varying climatic conditions. DNA corresponding to ribosomal genes was PCR-amplified from four *Clostridium* spp. isolates recovered from air samples, as well as from nine isolates recevered from the sewage sludge. Upon digestion with restriction enzymes, the authors concluded that airborne *Clostridium* spp. isolates were closely related to each other but not to isolates obtained from sewage sludge. While the results were qualitative, the authors demonstrated the forensic use of PCR to trace the origins of airborne bacteria in an environmental setting. No significant environmental interferences were discussed.

To analyze field and laboratory chamber samples for *Legionella* spp., Heinsohn (1994) used the Enviroamp™ PCR assay system, then licensed by Perkin Elmer Cetus Corporation. The Enviroamp™ assay used multiplex PCR (several different primers simultaneously used in the same PCR analysis), developed by Majbubani *et al.* (1990), to identify *Legionellae* in air samples. Enviroamp™ primers and gene probes have been optimized for broad specificity to members of the genus *Legionellae,* as well as to the species *L. pheumophila.* At the time of this publication, Enviroamp™ used an internal positive control DNA sequence that was co-amplified with species- and strain- specific primers for *Legionellae.* A colormetric dot blot assay was used to detect PCR amplification products, and the sample signal was compared to an internal control. Sample results from the colormetric Enviroamp™ reporter were compared to the control signal as "more-than" or "less-than" on the basis of a visual assessment. Heinsohn reported positive results for aerosol recovery of *Legionellae* using a common glass cyclone and PCR with Enviroamp™ primers. Despite the inclusion of an internal positive control, however, Heinsohn (1994) concluded that the Enviroamp™ system was semi-quantitative. A maximum of 18% of an ambient, culturable *L. pneumophila* was recovered using the cyclone sampler,

and the culturability loss was attributed to sampling stress. Air samples from cooling water towers that had positively cultured *Legionella* spp. did not consistently test positive using Enviroamp™; the author implicated that specific (petroleum hydrocarbons and heavy metals) and perhaps unknown agents interfered with PCR amplification.

Alvarez *et al.* (1994 and 1995) provided chamber and full-scale environmental study of PCR for bioaerosol monitoring. In these studies, the sensitivity of PCR and its susceptibility to environmental interferences were assessed, with *Escherichia coli* DH1 as the target organism. Air samples were collected with AGI-30 samplers and seeded with *E. coli* DH1 cells. Parallel studies were performed with cells seeded into the samplers prior to the collection of air samples to determine the effects of possible environmental inhibitors and sampling stress on the PCR assay. Baseline studies were also performed without environmental challenge or sampling stress to compare protocols used for cell lysis. Amplification of target sequences resulted in a detection limit of a single bacterial cell by the freeze-thaw and solid-phase methods within 5 and 9 hours, respectively. With a genomic target, the sensitivity of the solid-phase method was tenfold lower than that of freeze-thaw. Samples which contained between 10^3 and 10^4 CFU/m^3 of environmental organisms inhibited amplification; however, dilutions of these samples resulted in successful amplifications. No difference in the sensitivity of the PCR assay was obtained as a result of sampling stress, although a tenfold decrease in culturability was observed. A field validation of the protocol with genomic primers demonstrated the presence of airborne *E. coli* and/or *Shigella* spp. in outdoor samples. The authors concluded that the PCR method for detection of airborne microorganisms is rapid and sensitive, and can be used as an alternative method for air quality monitoring; however, significant interferences were reported , including those from non-target DNA.

Mukoda, *et al.* (1994) reported on *Legionella pneumophilia* solutions aerosolized into a small-scale laboratory chamber (ca 250 L), and air samples were collected using AGI-30 impingers and polycarbonate membrane filters with an average pore diameter of 0.45 um. Samples were eluted, homogenized, and concurrently analyzed using the Enviroamp™ PCR kit (as previously discussed by Heinsohn, 1994), standard plate counts on undefined media, and direct epifluorescent microscopic counts using acridine orange (AODC). These authors concluded that viable plate counting techniques consistently underestimated aerosolized *Legionella* concentrations as compared with PCR or direct

microscopic counts. They concluded that while they had demonstrated the feasibility of PCR for bioaerosol analysis, further studies were necessary, particularly to evaluate the limits of PCR detection for bioaerosols. No discussion of interferences were included. Mukoda and coworkers also provided a summary of genera that are present in indoor air and are clinically significant, and for which DNA sequence information is available to develop primers for PCR. At the time of publication, that list included the following genera: *Legionella, Pseudomonas, Micrococcus, Mycobacterium,* and *Corynebacterium.* To date, no such list has been compiled for viruses.

Mastorides *et al.* (1997) reported on the detection of airborne *Mycobacterium tuberculosis* by air filtration and polymerase chain reaction. Using a low-flow laboratory pump (2 l/min.) and a simple filter-cassette apparatus, Mastorides and coworkers immobilized *Mycobacterium* tuberculosis containing particles on polycarbonate membrane filters in a negative pressure Tb isolation ward. Room air was filtered for six hours prior to analysis. The membrane filter was aseptically recovered and was strongly positive within 24 hours of collection. The method detection limit was defined as ten Mycobacteria particles lysed in the PCR mix using visual confirmation by electrophoresis. No discussion of interferences were included.

Nugent *et al.* (1997) reported the successful use of PCR for personal exposure monitoring to genetically engineered microorganisms (GEM). In this study, genetically modified *E. coli* strain (strain XL1-B) was aerosolized directly from growth media to simulate an unintended GEM release. A wet cyclone sampler and a personal sampler (IOM personal Inspirable Aerosol Sampler) were used to collect genetically modified and unmodified *E. coli* cells in a well-characterized test chamber. The IOM personal sampler reported better recovery and analytical sensitivity for the PCR process. The authors reported lower detection limits for exposure to an aerosol containing 1.7×10^4 cells/m^3 (as determined by microscopy), with results being obtained within four to five hours after sample collection. The PCR technique employed could detect as few as eight *E. coli* cells in the PCR reaction, but because of aerosol sampling and analytical artifacts, the authors determined that the *effective* PCR detection limits were determined by: (i) the concentration technique prior to PCR amplification, and (ii) the genetic target, either the main bacterial chromosome or a plasmid unique to *E. coli.* Although plasmids may exist in multiple copies, the detection limits determined using primers directed toward

the main bacterial chromosome were lower than those determined using plasmid DNA as the target. No significant interferences were reported.

To detect airborne *Mycobacterium tuberculosis,* Schafer *et al.* (1999) reported the use of PCR coupled with a colormetric reporter system, which was developed by Roche Dioagnostic Systems, Inc., and licensed under the tradename *Amplicor*™. Schafer and coworkers used Anderson and MOUDI™ impactors to determine the size ranges of aerosolized *Mycobacterium tuberculosis* H37Ra (an avirulant *M.tb* surrogate) in a small-scale laboratory chamber (ca 1.4 m^3). DNA was surfactant-extracted from MOUDI™ filters and amplified using the *Amplicor*™ primers, which target all members of the *Mycobacterium* complex. An avidin/peroxidase-conjugated DNA probe, specific for *Mycobacterium tuberculosis,* was used to confirm and quantify the presence of *M. tuberculosis* DNA using a colored-producing substrate recognized by peroxidase. The experimental design included additions of lysed mycobacteria to the PCR mixtures as internal controls. The authors concluded that this PCR method was not truly quantitative given that there was no correlation between the quantity of Mycobacterium H37Ra DNA or whole cells added to the PCR mix and the intensity of color measured as the analytical endpoint. Direct phase contrast microscopy was used to quantify *M.tb* cells aerosolized into the chamber, and measurements showed that prior to aerosolization, *M.tb* cells were on average 6.5 um in length. MOUDI™ - PCR analysis showed, however, that the airborne particle size was in the range between 0.3-2.0 um; this was concurrently varified by culturing *M.tb* on Anderson six-stage impactors. PCR analysis was completed in 1.5 days, while culturing *M.tb* using Anderson samplers required five weeks. No significant interferences were reported.

PCR and airborne virus detection. There are five reports in the main body of medical and environmental microbiology literature on the use of PCR to detect the presence of airborne viruses; all were from clinical or laboratory settings with low potential for environmental interferences. Using membrane filters to sample airborne particulate matter, Sawchuk *et al.* (1989) and Kashima *et al.* (1991) used PCR to provide confirmation that human papilloma virus DNA could be detected in the air of an operating room following destructive surgical procedures. In a controlled laboratory environment, Saksena *et al.* (1991) provided a qualitative demonstration of PCR to detect airborne viruses. In this study, sterile water was exposed to aerosolized virus particles and subsequently analyzed by PCR. The collection technique used was liable to the limitations of differential settling

and collection, yet it was one of the first that demonstrated the ability of PCR to detect airborne viruses using a liquid capture medium.

Sawyer *et al.* (1994) reported on the detection and semi-quantitation of Varicella-Zoster (VZV) virus in air samples from hospital rooms using PCR. Semi-quantitation is defined here as a minimum concentration with no upper bound (*e.g.*, > 1000 DNA copies in the original bioaerosol sample). The authors collected and desiccated airborne droplet nuclei on cellulose membrane filters with an average pore diameter of 0.45 um; air samples were obtained from hospital rooms of patients with active VZV infections, rooms containing patients with herpes zoster infections, and outside patient isolation rooms. VZV DNA was detected in 82% of 78 air samples taken from rooms with active VZV infection, and was detected in 66% of air samples taken outside the hospital VZV patient isolation rooms. In terms of infections, the VZV DNA detected in these nosocomial situations could represent amounts of virus insufficient to produce infection or DNA from inactive viruses. Attempts to cultivate the viruses in this study were unsuccessful, and the proportion of VZV DNA amplified from active virus particles was indeterminate. The sensitivity of amplification was determined between 10^2-10^3 copies of VZV DNA genome per sample; however, no quantitative justification was given for this detection limit.

Using filtration-based air sampling methods similar to the above clinically-based virus study of Varicella-Zoster (VZV), McClusky *et al.* (1996) detected DNA sequences unique to cytomegalovirus in hospital rooms housing immunocompromised patients.

Quantitative PCR: PCR has been used for the detection of specific microorganisms in many environmental matrices, and has also been used successfully to detect both bacteria and viruses in air samples. Quantitation is defined here as identifying a discrete number of particles, or concentration range of particles, containing a target DNA sequence with some sampling/analytical variation. Sensitivity is defined here as the lower method detection limit, including the report of target DNA sequences above a method detection limit (*e.g.*, > 1000 units). Only recently have genetic amplification methods been reliably quantitative when applied to environmental matrices. Although PCR amplification technique offers increased ecological sensitivity over culturing in environmental matrices, concerning aerosol applications, PCR has some analytical artifacts that may introduce analytical bias. While the theoretical relationship between the amount of starting target sequence and

amount of PCR product has been demonstrated under ideal conditions, this does not generally apply to clinical and environmental samples, specifically because of the differential efficiencies and kinetics of the PCR process which depend on the abundance of target sequence. The numerical sensitivity of PCR, termed "quantitative PCR," has been intensely researched, and its accuracy has been under debate in the recent literature. Initially, PCR applications were not reported to have accurate quantitative power, *i.e.,* the ability to determine the number of target DNA sequences in an original sample could not be determined directly from the quantity of PCR product. The literature reviewing PCR as a quantitative technique is tenuous. Ferre's 1992 review reasoned that the quantitative power of PCR was compromised by both predictable and unknown interferences with exponential amplification. The first attempts to use PCR quantitatively involved the comparison of PCR amplification targets and products to more classical DNA quantitation using membrane techniques (*e.g.,* slot and Northern blots). Using indirect methods, Noonan *et al.* (1990) and Murphy *et al.* (1990) reported relative differences in the amounts of DNA targeted rather than the real number of nucleic acid copies.

Direct PCR product measurements have been compared to the numbers of original copy templates using internal references for co-amplification. Wang *et al.* (1989), Kellog *et al.* (1990), and Neubauer *et al.* (1990) reported the use of known DNA templates as internal standards for the PCR amplification process with varied results. Fluorescently labeled primers have been used, and minor differences in amplification efficiency have been reported to possibly induce large and unpredictable differences in PCR product yield (Langraf *et al.,* 1991; Clementi *et al.,* 1993). Sample preparation, nucleic acid purification procedures, nonspecific inhibitors, and equipment performance have been implicated to introduce unknown bias in many studies (Dickover *et al.,* 1990; Piatak *et al.,* 1993; Heinsohn, 1994).

A competitive PCR approach was reported by Gilliand *et al.* (1990) and Noonan *et al.* (1990), as cited and practiced by Piatak *et al.* (1993). Known quantities of a template(s), nearly homologous to the target sequence(s), were added to PCR for analysis in parallel with target sequences. The nearly identical template served as a competitor at all steps in the amplification process. In comparison to the competitor, the amounts of target sequence present in the original sample can be determined directly or by interpolation. Validation requires demonstration that the competitive control template amplifies with equal efficiency as the targets. This approach has been reported to have excellent sensitivity and

accuracy, given stringent internal controls, when applied to quantifying HIV in clinical samples (Piatak *et al.*, 1993). The design of an efficient competitor and the validation of amplification efficiencies require significant and skilled effort, greatly lengthening analytical times and cost (Heid *et al.*, 1996).

Another competitive method was reported by Holland *et al.* (1991) and Livack *et al.* (1995), as cited and practiced by Bassler *et al.* (1995). This competitive assay used a modification of standard PCR by the addition of fluorescently labeled oligonucleotide gene probe that specifically binds to the target sequence DNA. Fluorescent signal from the gene probe requires activation, and this activation occurs each time DNA polymerase copies the target sequence DNA during the PCR process. The change in reporter dye intensity has been reported as directly proportional to, and quantitative for, the amount of PCR product and target. Bassler *et al.* reported detection limits as low as 50 CFU with crude cell lysates of *Listeria* spp. This fluorgenic technique is licensed under the trade name *TacMan* by Applied Biosystems, Foster City, CA. Both specific primers and oligonucleotide probe sequences must be carefully specified for this technique such that the oligonucleotide probe binds to a genetic sequence within the amplified region of the target DNA. Before they can be applied to environmental or clinical samples, the design, labeling, and synthesis of the fluorescent oligonucleotide reporters used in this method require special methods and technical considerations, as discussed by Livack *et al.* (1995) and Bassler *et al.* (1995). The fluorescence reporting systems improve analysis times over conventional electrophoretic and staining methods; Bassler *et al.* (1995) reported analysis time of crude cell lysates of three hours.

Analytical modifications to the TaqMan-based PCR quantitation have included reports of real-time DNA measurements (Heid *et al.*, 1996; Gibson *et al.*, 1996). The use of a sequence detector coupled with a laser, computer, CCD camera, and image analysis software has been reported to allow continuous measurement of fluorescent spectra within PCR thermocycler equipment.

In the only successful report of quantitative PCR used for bioaerosol field monitoring, Heinsohn (1994) modified the commercial Enviroamp™ system to enumerate *Legionellae* in environmental samples. The colormetric reporting system of the Enviroamp™ kit was replaced with S^{35} labeled streptavidin; this modification was quantitative for the PCR amplification product of *Legionellae* RNA and another gene target over the range between 10^3 and 10^7 DNA copies. The signals from S^{35}

radioprobing of PCR product was logarithmically regressed to fit a model with a correlation coefficient of 0.973 for *Legionella* spp. and 0.993 for *L. pneumophila.*

Analytical interferences to Genetic Amplification and PCR. Although the tremendous sensitivity and quantitative potential of PCR has been generally agreed upon, the literature suggests that bioaerosol collection, DNA extraction techniques, and associated interferences may be limiting factors for microbiological air analysis using genetic amplification techniques. Many PCR studies have been executed under controlled laboratory conditions, and it is well-documented that the extreme analytical sensitivity of PCR amplification may give rise to analytical artifacts due to reaction inhibition or contamination (Nugent, 1997). Several reports acknowledge that there are significant environmental interferences that may impair accurate PCR characterization of microorganisms in environmental samples, including those recovered from aerosols. Some interferences may be prophylactically mitigated, while others remain unidentified or unpredictable impairments to PCR analysis. These interferences are:

1. Presence of trace concentrations (< 1 mg/L) selected heavy metals, particularly zinc, in collection and analysis fluids (Heinsohn, 1994); trace concentrations of iron and aluminum have also been implicated as possible agents interfering with the PCR process
2. Presence of selected particulate matter comprised of, or containing, polynuclear aromatic hydrocarbons in collection and analysis fluids (Heinsohn, 1994)
3. Presence of humic substances in collection and analysis fluids (Jacobsen and Rasmussen, 1992; Tsai and Olson, 1992; Tebbe and Vahjen, 1993)
4. High concentrations of both target and non-target DNA (Alvarez *et al.,* 1995)
5. Contamination due to handling and laboratory practices (Kwok and Higuchi, 1989; Heinsohn, 1994; Mukoda, 1994)

No reports in the literature have addressed the possible synergy of compounds that have been implicated to interfere with PCR amplification and analysis.

Heinsohn (1994), in an extensive PCR-based field study targeting airborne *Legionellae,* reported on interferences that were likely drawn into the collection fluids of cyclone samplers in the field. The field component of this study was subject to industrial cooling waters, where the potential for

interferences from many compounds was present. Heavy metals and PAH-containing compounds were isolated as probable interferences to using quantitative PCR, and presumptive evidence that zinc was responsible for the inhibition was presented. This report is the only bioaerosol study to execute experiments to determine thresholds for possible interferences. The author states that such interferences do not prevent PCR application to field bioaerosol analysis, and suggests that an analytical mitigation can be incorporated to PCR processing of aerosols to alleviate these interferences; no specific references are provided, however.

Alvarez *et al.* (1995), citing studies of PCR applied to soil microorganisms, stated that trace amounts of humic substances, noncharacterized coextracted substances, and high concentrations of non-target DNA can inhibit PCR and cause false-negative results. Any or all of these interferences have been reported to decrease or completely occlude the PCR amplification of genetic material extracted from microorganisms in environmental samples. Alvarez *et al.* (1994) also acknowledged that too much target or non-target DNA inhibited the PCR amplification process, and applied simple dilution technique to produce useful results.

Nugent *et al.* (1997) processed bioaerosol samples by boiling prior to PCR, and reported this as a simple procedure which had several advantages over the PCR preparation methods reported by Alvarez *et al.* (1994, 1995) and Heinsohn (1994). While Nugent *et al.* (1995) did not discuss interferences to the PCR process, the discussion in this study suggested that the boiling protocol has the potential to reduce interferences, and compares more favorably for both detection sensitivity and processing time than those reported in other bioaerosol genetic amplification studies.

Kwok and Higuchi (1989) addressed the problems of false-positives due to improper sample handling and processing of samples for PCR. Heinsohn (1994), Sawyer *et al.* (1994), and Mukada *et al.* (1994) were the only PCR-based bioaerosol studies to explicitly address and mitigate false-positive results from aerosol samples. Contamination (the introduction of target DNA that is not present during actual sampling) is not a true interference, but must be considered as part of the sampling and analysis for PCR; physical confinement methods, aliquoted reagents, and multiple negative controls included with analysis is recommended to avoid and/or detect sample handling and laboratory-based PCR contamination (Sawyer *et al.*, 1994; Mukada *et al.*, 1994).

Aircraft cabin air has been documented to carry petroleum hydrocarbons and a wide variety of volatile organic compounds and semi-volatile organic compounds (VOC's) (ASTM, 1999: van Netten, 1999; Balouet and Winder, 1999). The presence of airborne petroleum hydrocarbons and other VOC's in aircraft cabins has been attributed to the introduction of contaminated outside air (*i.e.,* aircraft engine exhausts), direct release from passengers, housekeeping practices and cleaning chemicals used in aircraft interiors, and to the leakage of jet engine oils and hydraulic fluids and their associated pyrolysis products (ASTM, 1999: van Netten, 1999; Balouet and Winder, 1999). Other studies of aircraft cabin air quality have reported respirable particle concentrations (< 10 μm average diameter) in the range between 5-138 $\mu g/m^3$ (Lee, 1999); the chemical nature of these particles was not reported. Heavy metals that have been implicated to cause interferences with PCR have not been analyzed in aircraft cabin air samples. Because of their sources and physical/chemical nature, however, some airborne contaminants present in aircraft may chelate, sorb or otherwise associate with heavy metals. As was reported by Heinsohn (1994), PCR applied to characterize aircraft cabin air may incur interferences due to the collection of petroleum hydrocarbons, metals, and other unidentified materials in conjunction with the collection and analysis of target microorganisms.

Bioluminescence. Stanley (1989) reported the use of an Adenosine Triphosphate (ATP) assay suitable for quantitation of microorganisms captured in air samples. ATP was chosen because it is universally used as an energy-carrying compound in all cells and can be rapidly and inexpensively quantified using photometry. Further, cellular ATP content was assumed to be proportional to cellular activity. Microorganism enumeration was based on signals emitted from a well-characterized enzyme catalysis reaction using the light-yielding enzyme luciferase, isolated from common fireflies. ATP fuels this reaction, and the light released when ATP extracted from air samples was mixed with luciferin was standardized to active cell numbers. ATP bioluminescence assays are phylogenetically nonspecific and only account for actively respiring organisms in the air sampled. The technique, as applied by Stanley, had relatively poor sensitivity, requiring 105 active microorganisms per ml of capture solution.

MICROBIOLOGICALLY ASSOCIATED FRAGMENTS AND BIOPOLYMERS OF SIGNIFICANCE TO BIOAEROSOL EXPOSURE ASSESSMENTS

Toxins associated with airborne bacteria: Endotoxin. Airborne gram-negative bacteria are ubiquitous, and many gram-negative species have been isolated from aircraft cabin air. The outer membrane of gram-negative bacteria contains unique, heat-stable, lipopolysaccharide components known to confer bacterial endotoxin. A special class of fats, termed "Lipid Type A", that are conjugated to long carbohydrate chains, presents Endotoxicity. This conjugation increases the base lipids' solubility over that of most other classes of lipids. In theory, it is this increased solubility that confers toxicity to eukaryotic cells introduced to this unique class of molecules, and thus the term "endotoxin". Endotoxin expression, both in quantity and composition, varies widely among the gram-negative bacteria; this variability governs the quality of toxicity any given endotoxin can confer to a host. Endotoxins, like mycotoxins, may be released from cells during growth, or may be aerosol- liberated upon lysis; airborne endotoxins are often associated with dust. Reviews of environmental endotoxin exposures, like mycotoxins, have focused on occupational exposures. A terse review of airborne endotoxin was provided by Olenchoch (1997) who concluded that endotoxins are not confined to traditionally "dusty" occupational exposures, but that endotoxin can be found in many environments that are considered to be "nondusty" or "less dusty" such as commercial buildings with operating humidification systems, and in industrial environments with water spray humidification systems.

Reviews provided by Walter et al, (1994), Milton (1999), and Olenchoch (1997) concluded that a significant portion of environmental endotoxin occurs in the form of micro vesicles, and is associated with airborne (or settled) dusts. It is widely accepted that endotoxins remain relatively stable when associated with dust, even when conditions do not support the survival of gram-negative bacteria that produce endotoxin. Milton (1999) provided an excellent review of endotoxin exposure effects and monitoring and concluded that low-level endotoxin exposures,

only slightly in excess of the normal outdoor background level, have been associated with increased severity of asthmas and other hypersensitivity disease.

Endotoxin has been reportedly measured by the following two methods: (i) potency demonstrated against a defined biological system (toxicity) and, (ii) fatty acid analysis of extracted lipopolysaccharides. Standard methods to measure and quantify exposure to environmental endotoxin have not been accepted. Using chromatography and mass spectrophotometric techniques, the quantification of a conserved region of gram-negative lipopolysaccharides (3-hydroxy fatty acids) was reported by Walters et al (1994), applied for a cross-validation of sampling and analysis of airborne environmental endotoxin. Walters et al (1994), proposed a standard procedure for airborne environmental endotoxin based on membrane filter capture and modification to a popular proteinase assay using enzymes extracts from the blood of the Horseshoe Crab (*Limulus* amebocyte lysate assay (LAL)). In an extensive review of endotoxin monitoring methods, this approach, among others using LAL as the analytical endpoint, was carefully criticized by Milton (1999). Milton (1999) warned of the high variability observed in LAL results from many past endotoxin studies and surmised that LAL based studies were labile to environmental interferences and analytical artifacts due to the following factors:

1. Endotoxin recovery from environmental samples is highly variable mostly due to the differential sampling efficiency of currently marketed bioaerosol samplers – polycarbonate filters appear to have the highest recoveries for sampling airborne endotoxin.
2. Stability of endotoxin activity during sample storage may vary depending on the sample matrix; this suggests that stability factors must be established for each sample type.
3. The proteinase enzymes activated in the LAL assay are labile to biopolymers other than gram-negative lipopolysaccharides.
4. Currently marketed LAL reagents vary widely in their sensitivity to environmental endotoxin.
5. Results are difficult to interpret and compare because of the many different filter media, extractants, and reagents used in past studies – no reasonably consistent analytical protocol has apparently been followed.

Every airliner air quality case study reported in the peer-reviewed literature to date, that has taxonomically classified isolated recovered from air samples, has, not surprisingly, identified gram-negative bacteria; thus, the potential for airborne endotoxin to be present in airliner cabin exits. The literature contains some reports of significant associations of mean personal inhalable endotoxin levels and gram-negative bacteria (Alwis et at, 1999; Milton, 1999). No aircraft cabin air quality case studies however, have reported attempts to measure concentrations of airborne or dust-associated endotoxin. Some gram negative bacterial species, that have been associated with airborne endotoxin (e.g. *Acinetobacter* spp., *Psuedomonas* spp.) have been isolated from in-flight air samples taken during operations of commercial airliners, but no concentrations of these species were reported (US DOT, 1989; Air Transport Assoc., 1994; Pierce et al, 1999).

The literature suggests that high airborne endotoxin exposures are occupationally and industrially associated with artificial humidification devices and relatively high RH conditions. Certain agricultural exposures have also been documented as "high", and are also associated with the presence of organic dusts; these exposures too have been under high humidity climates (e.g. cotton mills, swine barns, and grain handling). While it appears endotoxin may be present in some quantity on commercial aircraft, the imprecision of available environmental endotoxin assays renders exposure assessments unreliable. Based on occupational and humidity related endotoxin exposure studies, it appears that relative low-levels of endotoxin would most probably be encountered in operating aircraft; however there are no observations reported to date.

Toxins associated with airborne fungi: Mycotoxin and β-(1_3)-D-Glucans.

Mycotoxin. Some fungi have been documented to produce secondary metabolites that are harmful to humans when ingested or inhaled; some of these secondary metabolites are included

in an ill defined group of alkaloids, proteins, terpenes, and other compounds collectively termed mycotoxin. In general terms, secondary metabolites are produced when a cell of a toxigenic fungi has grown and exhausted available nutrients; this results in an accumulation of primary metabolites that are then available for subsequent breakdown (Miller, 1992). Mycotoxins are usually potent eukaryotic cytotoxins that interfere with cellular processes: some are vasoactive and can disrupt the central nervous system, and some are potent carcinogens; individual mycotoxins have been documented to elicit more than one type of toxic effect in humans (Burge and Ammann, 1999). The World Health Organization recognizes more than 100 mycotoxins that are produced from what are considered "common fungi". Mycotoxins may be released from fungal cells, be associated with, or sequestered within a fungal cell or spore; often mycotoxins are associated with dust. Mycotoxin analysis is often performed on collected dust.

Adverse human health effects can result from exposure to airborne mycotoxins. Literature reports involving mycotoxin-induced disease are often associated with agricultural and industrial workers that have high occupational dust exposures (e.g. grain industry or wood workers). Mycotoxin can cause both long-term and short-term health effects that range from immediate toxic and allergenic responses (toxic and allergic alveolitis) to potential long-term carcinogenic and teratogenic effects. Schiefer, (1990) reviewed mycotoxins in indoor air and reported that mycotoxins generally have low volatility, suggesting that mycotoxin inhalation routes are primarily associated with particulate matter (dust) or fungal cell constituents (and spores).

Yang and Johanning, (1997) and Burge and Ammann (1999) and Miller (1992) provided terse reviews of airborne fungi and their associated mycotoxins. These reports summarized important toxigenic fungi from a public health standpoint. Their summary included the more than 150 fungal species circumscribed by the following six genera:

Penicillium spp. *Apergillus* spp.

Stachybotrys spp

Claviceps spp.

Alternaria spp.

Fusarium spp.

Other fungal agents, which have been associated with mycotoxin induced human hypersensitivity pneumonitis, allergies, and organic toxic dust syndrome, were also presented in these reviews, and included the following organisms:

Auerobasidium pullulans

Botrytis cinerea

Cryptostroma corticale

Farnai rectivirgual

Serpula lacrymans

Mucor stolinfer

Burge and Ammann (1999) reviewed state-of-the-art environmental assays used for mycotoxins, and concluded that all of the currently available mycotoxin analytical methods were designed for agricultural products, and the crossover for use on air samples from indoor environments may be problematic where mycotoxin concentrations are low. Stating that mycotoxin samples were usually collected from dusts and surfaces, Burge and Ammann (1999) review, cited only two studies that attempted to analyze airborne mycotoxin using filters to collect air samples (Pasanen et al, 1993, 1994 as cited by Burge and Ammann (1999)). Two methods have been reported to identify and quantify environmental occurrence of mycotoxin: Chromatography and Immunoaffinity (antibody assays). For chromatography, samples must be carefully extracted using a suite of organic solvents to minimize interference. Burge and Ammann (1999) suggest that other than use in research labs, use of these techniques is limited because of necessary reference standards, expensive analytical equipment, and specialized expertise. Economical, immunochemical-based analytical kits are available for mycotoxin analysis; however, commercial assays exist for only a few agriculturally significant mycotoxins.

No aircraft cabin air quality case studies have reported attempts to measure concentrations of airborne or dust-associated mycotoxin. Some fungal species, that have been documented to generate mycotoxin(s), *Aspergillus spp., Penicillium spp.* and *Alternaria spp.*, have been isolated from in-flight air samples taken during operations of commercial airliners, but no concentrations of these species were reported (US DOT, 1989; Air Transport Assoc., 1994; Pierce et al, 1999).

β-(1_3)-D-Glucans. Glucans are glucose-based polymers that comprise a major structural component of most fungal cell walls. As specific class of these structural biopolymers, β-(1_3)-D-Glucans, may exist as branched or unbranched molecules and form large, stable helices (Williams et al (1996), as cited by Burge and Ammann, (1999)). Several research reports suggest that β-

(1_3)-D-Glucans can remain relatively stable when associated with dust, even when conditions do not support the survival of vegetative fungi cells (Flannigan (1997); Miller (1992)). β-(1_3)-D-Glucan is an inflammatory agent that depresses antibody formation and is known to react synergistically with endotoxin (Fogelmark, et al, 1992, 1993, as cited by Alwis et al (1999). Indoor air studies have shown dose-response relationships between levels of β-(1_3)-D-Glucans and eye and mucous membrane irritation, and respiratory inflammations (Alwis et al, (1999); Flannigan (1997); Miller (1992)).

β-(1_3)-D-Glucans assay methods. Aerosol exposure to β-(1_3)-D-Glucans has been documented using bioassays and immunochemical assays (Burge and Ammann, (1999); Flannigan (1997, 1992)). The sampling method most often reported for Glucan bioaerosol analysis was filtration collection on a polycarbonate membrane filter (Flannigan, (1997)). Filter immobilized Glucans were extracted by autoclaving in saponin in most studies. A single literature report discussed the use of an inhibition enzyme immunoassay to measure β-(1_3)-D-Glucans in occupational and home environments from dust samples (Douwes et al, 1996). Rabbit antibodies were produced by immunization with bovine serum albumin-conjugated β-(1_3)-D-Glucans and purified by affinity chromatography on epoxy-Sepharose. Several β-(1_3)-D-Glucans and β-(1_6)-D-Glucans were studied to determine sensitivities and interference: laminarin, curdlan, and pustulan. The laminarin-based calibration curve in the inhibition EIA was 40-3000 ng/mL (15-85% inhibition). Another β-(1_3)-D-Glucan, (curdlan) showed a similar inhibition curve but there was 5 times less reactive on a mass basis. Pustulan, presumed to be a β-(1_6)-D-Glucan, showed a parallel dose-response curve at concentrations 10 times higher than that of laminarin. Control experiments, with $NaIO_4$, and β-(1_3)-D-Glucanase treatment to destroy both β-(1_3)-D-Glucan and β-(1_6)-D-Glucan structures, indicated that the immunoreactivity of pustulan in the assay was due to β-(1_3)-D-Glucan and not to β-(1_6)-D-Glucan structures. Other polysaccharides, such as mannan and other β-(1_6)-D-Glucans, did not react in the inhibition EIA. β-(1_3)-D-Glucans extraction of dust

samples in water (with mild detergent) was performed by heat treatment because aqueous extractions obtained at room temp did not contain detectable β-(1_3)-D-Glucan levels. The assay detected heat-extractable β-(1_3)-D-Glucans in dust samples collected in a variety of occupational and environmental settings. On the basis of duplicate analyses of dust samples, a coefficient of variation of 25 % was observed.

Burge and Ammann (1999) reported that β-(1_3)-D-Glucans act similarly to endotoxin in some LAL assays; thus, β-(1_3)-D-Glucan quantitation is labile to the some of the same limitations as endotoxin assays when quantified by this method. Further, the LAL assay is 10^3 times less sensitive to β-(1_3)-D-Glucan, than to bacterial endotoxin (see bacterial endotoxin section). Flannigan's 1997 review of air sampling for fungi in indoor environments, like Milton's (1999) critique of airborne endotoxin assays, reported that although β-(1_3)-D-Glucan analysis appears to have more relevance to health-related indoor air quality than traditional methods, more research into its use (i.e. β-(1_3)-D-Glucan as a toxic aerosol biomarker) is required. Further, Flannigan (1997) noted that because the availability of glucan specific LAL is restricted, studies of indoor in which it has been applied are limited in number and design, and dose-response experiments with individual species and mixtures are lacking, fuller evaluation is clearly needed.

IMMUNOCHEMICAL ASSAYS FOR SINGLE SPECIES, MICROBIALLY ASSOCIATED TOXINS, AND COMMON ALLERGENS.

Immunochemical assays have been employed in some relatively recent bioaerosol studies to quantify targeted pathogens (usually single species), common allergens, and microbially associated toxins in both laboratory chambers and environmental samples. All immunochemical assays use a primary antibody stock that is raised and tested against single antigen, or a particular set of antigens, that are uniquely presented by a target species (e.g. *Legionella* sp.) or allergen (e.g. Fel d 1 - a major cat dander epitope) or toxin (e.g. β-(1_3)-D-Glucan). A secondary antibody stock, that

recognizes and binds to the primary antibodies, is conjugated to a reporter molecule, and provides signal necessary for quantitation. The specificity of the primary antibody-antigen binding governs the stringency of any immunoassay. The most common use of immunochemical assays is to quantify the presence of dust-associated animal dander allergens, cockroach allergens, and dust mite allergens (ACGIH, 1999).

There are two general types of antibody stocks - polyclonal and monoclonal - that have been used in environmental aerosol microbiology, and it appears, that in the more recent bioaerosol studies, monoclonal antibodies have been used against airborne microorganisms and selected allergens – of particular note is the effort directed toward raising antibodies directed against *Legionella* spp (Barbee et al 1987; Dennis and Lee, 1988). An excellent review, condensing several reports on immune assays directed against *Legionella* spp., was provided by Fields (1997). Fields reported that direct fluorescent antibody methods to detect Legionellae are limited by the number of specific antisera that can be used. Reports on the sensitivity and specificity of direct fluorescent antibody testing of environmental specimens vary greatly according to Fields (1997), with most studies indicating that the test is relatively insensitive and non-specific.

With respect to the microorganisms and allergens germane to bioaerosol studies, such immunochemical assays have many variations, the following of which have been applied:

Colorimetric Reporters and Enzyme-Based Immunoassay: Enzyme Linked Immunosorbent Assays (ELISA) are widely used to quantify allergen concentrations in dust-samples (ACGIH, 1999). ELISA assays use a secondary antibody stock to provide signal choices (e.g. fluorescence, color or radioactivity) and adjust sensitivity. The secondary antibodies irreversibly bind to specific regions on the primary antibodies not involved with recognition of the target antigen. For aerosol studies, secondary antibodies are often conjugated to an enzyme that generates color or emits

radiation and thus produces a sensitive signal that can be measured on a spectrophotometer, film, or scintillator. Dust samples are sieved to obtain the fine dust fraction, soluble proteins are extracted from the dust in an appropriate buffer, and the extracts are serially diluted. The same process is executed for an allergen standard. When ELISA was applied to aerosol samples, some type of liquid capture and centifugal antigen concentration was required (AQS, 1997). Usually this type of assay was executed in standard microtiter plates coated with capture antibodies (primary antibody). Dust, microorganisms, and/or other extracts of aerosol samples were concentrated via centrifugation and distributed in 96 well microtiter plates that are treated to bind antigenic determinates or proteins. Assays for more than one allergen can be performed on the same dust suspensions by substituting appropriate antibodies. One of the first techniques to measure aerosol-associated allergens was competitive radioimmunoassay. This is a modification to the ELISA technique that measures the antigenic activity in extracts of aerosol and dust samples by the degree to which it *inhibits* binding of antibodies to the same antigen immobilized on a solid phase (often a plastic microtiter plate) or membrane.

In a review that focused on immunoassays and allergens, C.M. Luczynska (1997), summarized allergens and fungi for which there are monoclonal antibody stocks available, and for which immunoassay may succeed in aerosol samples provided enough antigen were captured. Detection limits vary widely depending on reporting technique and target.

Organism	Reference
Dermatophagoides pteronyssinus (dust mite)	Sporik et al (1994), Arlian (1991)
Dermatophagoides farinae (dust mite)	Heymann et al (1986)
Blatella germanica (cockroach)	Arruda et al (1995)
Aspergillus fumigatus	Stewart (1994)
Alternaria alternata	Kleine-Tebbe et al (1993)

Immunochemical assays applied to aircraft air quality studies. There are no reports in the peer-reviewed literature on the use of immunochemical assays for common allergens encountered in aircraft environments. As part of the Harvard-designed survey on the *Comparison of the Environments of Transportation Vehicles*, Dumyahn et al (1999) reported the use of immunoassays to quantify animal, cockroach, and fungal epitopes in surface and dust samples taken from aircraft interior surfaces (e.g. seats and carpets) – results from this study will be presented in the proceedings for ASTM Symposium on Air Quality and Comfort in Airliner Cabins, New Orleans, LA, 1999. The results from this study are key to determining the validity for practical application and potential for information yield of immunoassays when applied to exposure assessments in aircraft. ***Immunochemical*** techniques (based on antibody assays) have been established to determine the masses of common allergens associated with airborne particulate matter; these include animal danders, insect parts (cockroach and mite), and fungal cell wall components (β-D-Glucans). Immunochemical techniques have been applied to a wide variety of other microbiological components, but their epidemiological and quantitative significance is under debate, and may not be germane to aircraft air quality.

Microbial Volatile Organic Compounds (MVOC's) as an Indicator of indoor Microbial Contamination. The metabolism of microorganisms can release volatile organic chemicals (MVOC's). Of interest in health-associated air quality studies are volatile organic metabolites associated with some toxigenic fungi and their mycotoxins (Pasanen et al (1996)). MVOC's have been used in indoor air quality building investigations as an indicator of excesses of indoor microbial growth. Many MVOC's have odor thresholds in the parts per billion and parts per trillion ranges. MVOC's are able to penetrate vapor barriers such as polyethylene plastic, nylon, and vinyl wall coverings (Strom et al, 1994). MVOC's considered specific to microbiological growth include ketones, such as 2-heptanone, alcohols such as 1-octen-3-ol, and 2-methyl-butanol, and furans such as 3-methylfuran. Alcohols, particularly eight carbon alcohols, have been have

been implicated to be relatively ubiquitous fungal VOC biomarkers that have a high affinity for porus surfaces and have relatively long half-lives indoors because of surface association. Conversely, furan-based VOC's have a low affinity for surfaces the occurrence of airborne furans and are thought to be a general indicator of active microbiological growth (Air Quality Sciences, 1997).

For MVOC analysis, indoor air is collected on a solid sorbent media using a low-flow sampling pump. Analysis is performed using gas chromatography combined with mass spectrometry (GC/MS) according to widely accepted methods (AIHA, 1996). GC/MS analysis has been reported to provide a detection limit of 0.5 nanograms per approximately 50 L of air sample (Air Quality Sciences, 1997). Concentrations of individual MVOC's are summed to provide an estimate of the total microbial VOC value. From a cross-section of 139 schools, public buildings and commercial facilities, MVOC values were elevated 50% above those measured in outdoor locations at the same site – outdoor samples, obviously, are essential for comparison.

MVOC results interpreted concurrently with classical culture-based approaches have been reported to suggest that MVOC's are a reliable indicator of past moisture problems in buildings (Air Quality Sciences, 1997). Many VOC's, however, have both anthropogenic as well as biogenic sources, and differentiating between them makes VOC measurements lose their usefulness as an aerosol biomarker in aircraft.

AIR QUALITY MODELING AND BIOAEROSOLS

Sequential box models and computational fluid dynamics (CFD) models have been developed and applied to describe the distribution of a wide variety of airborne pollutants in indoor environments. Indoor air quality models are often based on simple mass-balance equations, which attempt to account for incoming and outgoing materials. Indoor air quality models, of various levels of complexity, have been developed

and validated in residential and commercial buildings using tracer gasses and direct measurements of environmental tobacco smoke (ETS), O_3, Rn^{222}, and CO_2 (Hernandez and Ring, 1982; Afonso et al, 1986; Ozkaynak et al, 1982; as cited by Miller et al, 1997). A single well-mixed room is the most common representation of indoor environments for analysis and modeling of air pollutant concentrations. Many indoor environments, such as aircraft however, are not well-mixed, and several modeling approaches have been suggested for predicting indoor concentrations in situations where mixing is incomplete, including the application of computational fluid dynamics. Another approach to model situations where mixing is incomplete has been validated where indoor environments are represented by two or more idealized reactors, each independently well mixed (Nazaroff and Cass, 1986; Miller et al, 1997; Ryan et al). Under the multiple reactor scenario (AKA: sequential box models), pollutant concentrations in each reactor are coupled by airflows between them.

Prior Aircraft Air Quality Models. Sequential box models have been applied to assess the air quality in commercial aircraft. Ryan et al, (1988) applied a model to aircraft cabins consisting of several sequential compartments. Each compartment contained source and sink terms for ETS, CO_2, and water vapor as well as leakage and air transfer from adjacent compartments. Modeling compartments (zones) were assigned to the flight deck, 1st class, business class (where appropriate), coach, and lavatories. The model was applied to predict air quality in the passenger cabins of Boeing 727, 767, and MD80 aircraft during the cruise phase of a typical flight. The multi-zone models were configured to account for recalculation rates and ventilation systems unique to these aircraft. Results of the modeling simulations suggested that, (i) different zones within all the aircraft studied have widely varying pollutant concentrations; (ii) flow may not be unidirectional in all aircraft; (iii) as recirculation rates increase, pollutant levels rise throughout aircraft; and (iv) pollutant concentrations decrease nearly linearly as occupancy decrease.

Other aircraft cabin simulation studies were recently reported at the ASTM meeting on aircraft cabin air quality (ASTM, New Orleans, LA, October, 1999). Arnold et al, (1999) presented the effects of

recirculation on aircraft cabin air quality using box-modeling approaches. The focus of the model was the prediction of CO_2, RH, and O_2 concentrations in the passenger cabin and flight deck. The simulation results were compared with actual in-flight data to validate the model-predicted findings. The results showed the dependence of breathing air constituents on varying passenger loads, varying recirculation rates, and different cabin-pressures. Baker et al, (1999), reported the use of computational fluid dynamics for characterizing the distribution of ventilation air in commercial aircraft passenger cabins. CFD models were introduced for their usefulness to predict and manipulate comfort levels and draft ratings.

Bioaerosol Air Quality Modeling Applications. More recently, air quality models have been adapted to predict the infectious concentrations of airborne pathogens in high-risk, indoor environments. In a series of articles, Miller, Nazaroff, Nicas, and coworkers published the first attempts to apply sequential box models to predict the movement of *Mycobacterium tuberculosis* in indoor air (Miller-Leiden et al 1997; Miller et al, 1998; Nazaroff et al, 1998; Nicas and Miller, 1999). These articles built on the fundamental mass balances used by previous IAQ models, but presented a unique analytical framework for evaluating measures to control airborne tuberculosis under realistic conditions. Further, some of these models were designed to describe the response of airborne mycobacteria to engineered removal systems (i.e. air filtration and ventilation) and *in-situ* disinfection systems.

Results of two modeling studies executed by Miller-Leiden et al, (1996) and Nazaroff et al, (1998) suggested that air filtration in conjunction with dilution (ventilation) can yield good reduction in airborne bacterial contamination; however, these authors suggested that in high-risk settings, filtration coupled with ventilation should not be relied upon as the primary means of infection control (Miller-Leiden et al, 1996).

A multi-zone model was applied by Nicas and Miller (1999) to evaluate the efficacy of upper room air ultraviolet germicidal irradiation of airborne tuberculosis. A three-zone representation of a TB patient isolation room, equipped with a germicidal UV disinfection system was developed. The UV disinfection

system was located in the upper-room zone and was modeled for complete inactivation of airborne *mycobacteria*; the unirradiated lower zone of the room was configured with an internal near-field zone surrounding the TB patient. Infectious particles were generated in the near-filed zone and their transport was modeled by the airflow between zones. Like other sequential box models, each zone was modeled independently as a well-mixed compartment; the whole room however, was not well mixed as configured by airflow and bacterial transport between zones. When compare to single-box models, the three-zone model showed the UV disinfection system to be far less effective in reducing exposure to active airborne pathogens in the near-field. Near-field exposure intensity is relevant because health-care workers are usually in close proximity to the TB patients they attend. These results are germane to aircraft environments because of the very close proximity passengers are required to maintain with each other, because of seating arrangements.

Accepted direct microscopy and culturing methods are being used to evaluate the *in situ* response of airborne *Mycobacteria* and spores to upper room UV irradiation (Miller and Macher, (1999); Xu, et al, (2000)). The sequential box models reported under this review are currently being validated using airborne bacteria in full-scale experiments at the University of Colorado (CDC contract 200-97-2602). It is anticipated that results will be available in the peer-reviewed literature during calendar year 2000 and 2001.

In light of the documented disease transfer of tuberculosis and influenza on aircraft (Moses and Bender, (1979); Kenyon et al, (1996)), and the association of "near-field" transmission risk being a function of proximity on aircraft, these modeling results may be considered for directing future bioaerosol modeling efforts in modern airliners.

Modeling Summary. The environmental situations in aircraft pose a unique environment to which sequential box models and CFD have been applied. The results of past epidemiological studies applied to disease transfer in aircraft have strongly suggested that proximity to pathogen sources (infected passengers) are a key factor to passenger exposure levels on aircraft. Multi-zone models have the capability to address and simulate the near- and far- field effects experienced on aircraft, and account for the various ventilation system configurations in the commercial fleet. Given that HEPA and other filtration systems have been added to some modern commercial aircraft, and that one-pass disinfection systems have been proposed, existing models should be modified to simulate exposure to airborne pathogens in different airliner cabin configurations and operating modes (i.e. recirculation rates, fresh air exchange rates). Given the potential power of these modeling tools, and the growing interface between environmental microbiology and aerosol physics, bioaerosol modeling of aircraft environments should now be considered a valid and conservative approach for bioaerosol exposure assessments of airline passengers.

In general, the interpretation of contaminant concentration data, weather it pertains to chemicals or biological agents, depends on the specific model of room air mixing that is assumed appropriate. The recent applications of sequential box models and CFD to simulate airborne contaminant concentrations, oxygen levels, and RH in aircraft environments, has demonstrated the utility of these tools for economic evaluation of non-microbiological human exposures (Ryan et al, 1988; Arnold et al, 1999; Baker, et al, 1999). Further, indoor airborne contaminant modeling has been successfully extended to include pathogenic bacteria and their response to *in situ* disinfection systems, ventilation dilution, and filtration. With appropriate modifications, these models may be extended to study the distribution and abundance of airborne pathogens and in aircraft cabins.

SECTION III: SUMMARY OF GUIDELINES APPLICABLE TO BIOAEROSOL SAMPLING AND BIOAEROSOL EXPOSURE IN AIRCRAFT ENVIRONMENTS

General guidelines. Because of the wide range of organisms studied in a general bioaerosol investigation, one sampler or method will rarely be adequate to characterize the bioaerosol component of the environment. Few official guidelines have been stated for any bioaerosol sampling, mainly because the relationship between dose and health effects is not well-characterized. In the United States, OSHA proposed an Indoor Air Quality Standard in 1994 that is currently under revision; as of 1999, comments and testimony regarding this standard are still under review and the fate of the standard is unknown. In Canada, the Health and Welfare Department provides no numerical limits for indoor bioaerosol exposures; rather, qualitative exposure control recommendations are provided (Health and Welfare Canada, 1987). In Europe, the Commission of the European Communities (CEN) is considering standardization of bioaerosol sampling methods. Other organizations such as the AIHA and the ACGIH as well as several prominent investigators have suggested sampling methods, but the trend in concentration guidelines has been away from actual numerical limits and towards relativistic guidelines. The interim air quality guidelines for Hong Kong contain a numerical action level of 1000 CFU/m^3, and this limit has been cited as criteria for acceptable microbiological air quality on commercial airliners based in Hong Kong (Lee *et al.,* 1999).

US Professional Society Guidelines for Indoor Air. The 1992 OSHA Technical Manual section on Indoor Air Quality Investigations stresses the importance of identifying the prominent taxa, especially fungi, and suggests levels of colony forming units that indicate contamination. The National Institute of Occupational Safety and Health (NIOSH), recommended in the 1998 NIOSH Manual of Analytical Methods (NMAM), interpreting bioaerosol data by comparing indoor and outdoor levels, or symptomatic areas with asymptomatic areas. In 1989, the ACGIH proposed guidelines for bioaerosols based on comparing a case area with a controlled environment with similar parameters, such as an office with suspected bioaerosols and a "clean" office. Another suggested technique was comparing the indoor environment with the outdoor environment. Significant results were those where the two areas' total counts differed by an order of magnitude, where the ecology of the airborne microorganisms were different, or where the indoor environment

concentrations were greater than the outdoor concentrations (Rao, unsourced). ACGIH recommends collecting samples in the breathing zone as well as at the supply and return airstreams of the buildings ventilation system(s). The 1989 proposed guidelines also specified upper limits of acceptable contamination. As of 1999, ACGIH no longer recommends using numerical values to interpret data (ACGIH, 1999). The City of New York, in 1993, set limits of 1000-10000 CFU/m^3 for Stachybotrys atra levels in indoor environments. In a 1997 article, Macher recommended collecting three or more simultaneous samples for each trial and comparing the results to reference areas. Many investigators propose the Andersen impactors and All Glass Impingers as reference samplers (Henningson and Alhberg, 1994; Lembke *et al.,* 1981; Ding and Wang, 1997).

Internaltional Guidelines. Internationally, a few countries and organizations have set, or are developing guidelines for airborne microbiological contamination. In Europe, the CEN is considering standardization of bioaerosol sampling methods. The Commission of the European Communities (CEC) in Report 12*: Biological Particles in the Indoor Environment* reported ranges of values, from very low to very high for particular bioaerosol contaminants: dust mites, fungi in homes, fungi in non-industrial buildings, bacteria in homes, and bacteria in non-industrial buildings. The values were compiled from ranges of contaminants found in studies of indoor environments. In Canada, The Federal-Provincial Advisory Committee on Environmental and Occupational Health, developed the *Indoor Air Quality in Office Buildings: A Technical Guide* (Nathanson, 1993). This guide recommends limits of fungal colony forming units based on the number of species present, the correlation between indoor and outdoor species, and the season. Earlier guidelines were established by the Canada Mortgage and Housing Corporation in 1988. These guidelines, for the *Determination of Fungal Propagules in Indoor Air*, set limits for action based on the number, diversity and species of fungi (as referred by Rao, unsourced). Similarly, the World Health Organization, in 1988, set indoor air quality guidelines on biological contaminants determined by the same criteria. The Interim Indoor Air Quality Guidelines for Hong Kong (as referred by Lee et al. 1999) set an Action Level of 1000 CFU/m3. In the Russian Federation the Maximum Allowable Concentration (MAC) for biological contamination ranges from 1000-10000 cells/m3, depending on the species (as referred by Rao, unsourced).

Peer-Reviewed Guideline Suggestions. Many prominent investigators have also recommended various limits and sampling protocol for biological contamination. Miller et al. (1988), recommends further investigation if there are more than 50 CFU/m3 of a single species, or more than 150 CFU/m3 of a mixture of species. If the species are common fungi, up to 300 CFU/m3 was considered normal. Reponen et al. (1990), found that more than 500 CFU/m3 fungi in the winter is abnormal. Reynolds et al. (1990) found that indoor sources are indicated if the indoor counts are significantly higher than the outdoor counts. Yang et al. (1993) recommends species identification for understanding effects of contamination and recommends a limit of 200 CFU/m3 for fungal spores. The trend in setting limits for bioaerosols seems to be heading towards relativistic guidelines and away from numerical limits.

European Union Guidelines for Indoor Air. The Commission of the European Communities (1993, Report 12) states that the most important variable in bioaerosol sampling is comparability between samples. The report recommended that the same type of sampler be used throughout a study and that the same sampling protocol be followed for every sample. The report also listed ranges of values for different bioaerosol contaminants corresponding to very low to high levels of contamination based on results from past studies of nonindustrial settings. It also recommended particular sampling protocols for various contaminants, such as dust mites, bacteria, and fungi.

Sampling guidelines germane to aircraft environment characterization are summarized below:

Dust mites. These arthropods are mainly important to human health as allergens (CEC, Report 12). They are mostly found in bedding, but may also be in upholstery and carpeting (Pollart *et al.*, 1988). Allergies to dust mite antigens range from 45-85% among asthmatics, and as few as 500 mites per gram of dust may provoke acute asthma attacks (Platts-Mills and de Weck, 1988). Dust mites are rarely found in industrial or nonindustrial workplaces, but have been found in low numbers in schools, daycare facilities, and hotels (CEC, Report 12). Low levels of dust mite allergen might be found on aircraft. Air sampling for dust mites is done by cascade impaction or high-volume filtration with membrane filters. Air sampling is hypothetically more representative

of respiratory exposure, but requires a long sample time, 2-24 hours, and there is evidence that high-level short-term exposures may be more important when assessing health risks (Platts-Mills and deWeck, 1988). Air sampling for dust mites is not recommended. Bulk dust samples, collected with a vacuum cleaner retrofitted with a paper filter (38cm, 6um pore size) and a known suction rate, are preferred for collecting dust mite particles. With this collection method, designed by the International Workshop of 1987 and endorsed by the CEC, one square meter area of upholstery and carpeting are sampled for two minutes per site. The dust samples are prepared for analysis by microscopy or immunochemical assay. Microscopy allows the identification of species and the different life-cycle stages of the mites, but visual counts of disintegrating mites and fecal matter are difficult. RAST and ELISA immunochemical assays are used to categorize total mite allergen and specific types of mite allergen, respectively. The CEC Report 12 recommends analysis with the ELISA method.

Animal allergens. Due to the low number of cats and dogs traveling in the aircraft cabin, studies, sampling, and analysis methods regarding animal dander have been omitted in this review. Animal allergens are rare in most places where dogs and cats are not kept (CEC, Report 12). However, a recent aircraft environment study sampled for animal allergens (*Harvard Transportation Vehicle Environment Study,* Dumyahn *et al.,* data presented at the ASTM meeting on aircraft air quality, New Orleans, LA, October, 1999).

Fungi. Fungi are important to humans due to their allergenic and toxigenic properties (CEC, Report 12). Approximately 2-30% of people with respiratory allergies are allergic to fungi and their spores (Gravesen, 1979). Sampling methods for fungi should be tailored to collect and quantitate both viable and nonviable fungal particles and spores because both are important when considering possible health effects. Identification of particular genus and species may also provide critical information. Viable sampler results are dependent on cutoff size, time, the volume

collected (Verhoeff *et al.*, 1990), and the relative air disturbance during sampling (Hunter *et al.*, 1988). Comparability between samples and results is very important and should be maintained throughout the study by using one type of sampler and sampling protocol consistently. Duplicate, parallel samples are also recommended (CEC, Report 12, 1993). The influences of the ventilation system can be studied by taking samples at the air supply vents while the ventilation system is running and comparing them to samples taken with the system off (CEC, Report 12, 1993).

Bacteria. The main sources for bacteria are humans and animals (CEC, Report 12, 1993). Disease transmission via aerosolized bacteria has been documented in several studies. CEC Report 12 recommends using one sampler and sampling protocol consistently throughout collection. When using a sampler that collects by impaction onto agar, the sampling time should be less than ten minutes to prevent losses due to desiccation, and an antibiotic should be added to the agar to prevent fungal growth; this practice has been followed in past aircraft environment studies.

Viruses. Viruses may be transported via fomites and aerosol droplets as well as by direct and indirect contact. They may be sampled in the air by impaction onto agar and transferred to cell culture medium (ASTM, 1990), or by impinging directly into a liquid culture medium. Because of poor collection efficiencies, air sampling for viruses has been designated by many researchers as quantitatively indeterminate (Sattar and Ijaz, 1997), but quantitative genetic amplification approaches are under serious field evaluation.

Specific Guidelines on Airliner Cabin Air Quality. Guidelines concerning aircraft cabin air quality are mandated under the ASHRAE Standard Ventilation for Acceptable Indoor Air Quality (ANSI/ASHRAE, 62-1989). This Standard does not address microbial contamination, but focuses and sets limits on carbon monoxide levels and ventilation rates. Manufacturers' specifications of aircraft airflow suggest rapid dilution of airborne microbiological particulate matter in pressurized cabins. Under different cruising conditions, the theoretical number of air exchanges is between 5 and 42 cabin volumes per hour; this is near or above the exchange rates suggested by the CDC for hospital rooms for patients with active tuberculosis. However, in an airliner air quality case study,

Hocking (1997) found that some airlines do not meet the ASHRAE ventilation recommendation of 7 L/sec per passenger. ASHRAE is currently working on a new proposed standard: 161P: Air Quality within Commercial Aircraft - which will consider airborne microbiological contaminants. The Federal Aviation Regulations (in the US) and the Joint Aviation Requirements (in Europe) state that the cabin air ventilation system must provide enough fresh air to accommodate reasonable comfort and contain no harmful vapors or gases; no specifics on airborne microbiological agents are provided (Flight Safety Foundation, 1998).

Comparison of Culturing Results from Aircraft Air Quality Studies to Proposed or Existing Bioaerosol Exposure Guidelines

YEAR	AUTHOR	DATA INTERPRETATION/GUIDELINES
1999	Pierce et al; [ASHRAE; 957-RP]	Compared to bacteria and fungi typical in buildings
1999	Lee et al.	HKIAQ guidelines: 1000 CFU/m3
1997	DeChow et al.	Compared to guidelines for operating rooms in hospitals (<50 CFU/m3) and intensive care units (<150 CFU/m3)
1995	Wick and Irvine	Compared to common city locations: buses, shopping mall, sidewalk, and airport departure lounge
1994	ATA	NIOSH Recommended Exposure Limit: 1000 CFU/m3*
1989	Nagda et al.; [DOT-P-15-89-5]	Rank Order Assessment; comparison

*This reference was unsourced

SECTION IV: BIOAEROSOL EXPOSURE ASSESSMENTS ON MODERN AIRCRAFT – A SUMMARY OF CASE STUDIES

The literature to date contains 13 aircraft cabin air quality studies that collected and reported data on microbiological contamination and human disease. Six studies have taken air samples from in-service commercial aircraft to enumerate culturable bacteria, fungi and their spores using classical culturing methods. A seventh study, which compares the environments in several different types of public transportation vehicles during 1994 and 1995, included a significant bioaerosol component but has not been published (27 flight segments were sampled for bioaerosols; however, data are not yet in peer-reviewed literature or other public domains - *Harvard Transportation Vehicle Environment Study,* Dumyahn *et al.,* presented at the ASTM meeting on aircraft air quality, New Orleans, LA, October, 1999). Apart from enumeration assays of air samples taken during commercial flights, airborne microorganism levels have been shown to exceed concentrations required to produce human disease using epidemiological methods.

Disease transfer in modern commercial aircraft environments has been documented in four case studies that consolidated solid epidemiological evidence. These case studies implicated aerosol disease transfer as the facilitating mechanism, although in one case, the ventilation system was malfunctioning. Moser *et al.* (1979) used widely accepted epidemiological means to report an influenza outbreak aboard a Boeing 737 aircraft. The aircraft was not configured with a typical commercial passenger configuration, having a forward freight compartment and 56-seat passenger seat compartment aft. The aircraft, grounded and occupied for four hours with a malfunctioning ventilation system, produced a high attack rate from one passenger, the apparent index case, who was apparently ill with influenza while on the plane. Within 72 hours, 72% of the passengers became ill, and the clinical attack rate was shown to vary with the amount of time spent on the plane. The same influenza virus (Influenza type A - /Texas/1/77) was serologically identified in 20 of 22 ill persons tested. The high attack rate implicated the aerosol-mediated transfer of the virus within a four-hour period, and the improperly operating ventilation system was implicated as responsible for increasing influenza exposures. Results from detailed interviews and questionnaires discounted surficial and near-field contact as dominant transfer mechanisms. Two other reports with less epidemiological evidence have suggested that measles virus has been transmitted aboard both international and domestic flights (Bloch *et al.,* 1982; CDC, 1982).

Driver *et al.* (1994) investigated the potential transmission of *Mycobacterium tuberculosis* from an airline crew member diagnosed with active tuberculosis. This study covered a five-month period during which the crew member flew on many different aircraft. Depending on the aircraft, the air-exchange rates varied between 5 and 29 cabin volumes per hour in the passenger cabin. Recirculation systems were present in some aircraft, and the proportion of recirculated air ranged between 24 to 66%. All aircraft that recirculated air were equipped with high-efficiency particle filters. For at least three months, the infected crew member worked on aircraft that did not recirculate air 72% of the in-flight time. Passengers (59) and crew (212) who were exposed to an infected crew member were voluntarily screened by tuberculin skin tests. Among domestic comparisons and contacts with the index case, 4 of 59 frequent flyers were skin-test positive conversions. As judged by Fisher's exact, Chi-squared and Students' t-tests, the authors concluded that *M.tb* infection risk increased with hours of exposure to the index case. Further, the documented conversion cases corresponded to the time period when the crew member had been working on aircraft that did not recirculate air 72% of the time. The data in this study supported the conclusion that *M.tb* was transmitted from an infectious crew member to other crew members regardless of aircraft ventilation conditions. Because of the clustering of exposure response, the authors concluded that transmission of *M.tb* to passengers could not be excluded.

Kenyon *et al.* (1996) used widely accepted epidemiological means to demonstrate the transmission of multiple-drug resistant *Mycobacterium tuberculosis* during four commercial flight segments on Boeing 747-100 series (international transpacific - duration, ca eight hours) and Airbus A320-200 series (domestic - duration, ca two hours) aircraft. Both aircraft were equipped with air recirculation systems that included high-efficiency particle (HEPA) air filters. According to manufacturers' reports, approximately 50% of the air is recirculated on both aircraft types, with air exchange rates reported in the range between 6 and 20 air changes per hour. A single passenger (index patient) diagnosed with active tuberculosis was on these aircraft, each of which transported 925 other persons. Passengers and crew were identified from airline records and were notified of their exposure, asked to complete a questionnaire, and screened by tuberculin skin tests; previous infection, vaccination, and other risk factors were incorporated into the results. As judged by Fisher's exact, Chi-squared and Students' t-tests, the authors concluded that passengers seated

within two rows of the index patient were more likely to have positive turberculin skin tests than those in the rest of the coach sections. Three of the four flights produced mycobacteria response in other passengers; the authors suggest that prolonged exposure to *M.tb*-containing aerosol droplets from the index patient plays a role in the exposure response documented by tuberculin skin test conversions considering proximity and dose-response effect.

McFarland *et al.* (1993) published a *Lancet* article on exposure to *Mycobacerium tuberculosis* during air travel. A contact investigation, following a pulmonary tuberculosis case of a person who traveled on a single flight from London to Minneapolis, was summarized. Using accepted epidemiological approaches, including questionnaires and Mantoux and Heaf tests, the authors retained a relatively low yield data set: Of US citizens, questionnaires were completed for 81%, and test results were obtained for 61%; of non-US citizens, 22% returned questionnaires, and 8% returned useful test results. Although the source-case was considered highly infective, results of this investigation did not demonstrate evidence of *M.tb* transmission. The authors concluded, however, that the risk of exposure during air travel exists; although short-term, such exposures could be intense and result in transmission.

Using classical culturing and enumeration methods, six case studies have attempted to characterize microbiological air quality, among other chemical parameters, in operating commercial aircraft. A seventh case study has been executed, but not yet published in peer-reviewed literature (*Harvard Transportation Vehicle Environment Study*, Dumyahn *et al.*, data presented at the ASTM meeting on aircraft air quality, New Orleans, LA, October, 1999).

Nagda and coworkers (DBA: GEOMET Technologies, Inc., 1989) were contracted by the US Department of Transportation to survey air quality in operating commercial aircraft. This large-scale investigation included analysis of airborne microorganisms on 23 flights that allowed smoking and 69 flights that did not allow smoking. Bioaerosol sampling was executed with multiple-hole impactors containing broad-spectrum media, and employed simple time bracketing techniques to ensure sensitive measurement ranges; heterotrophic bacteria and mesophilic fungi were the media targets. Air samples were collected at two locations in the coach cabins of non-smoking flights and a single location on smoking flights. Grab samples were drawn prior to

descent, near the end of the flights. The results of the bioaerosol sampling were reported as CFU/m^3, and several different bacterial genera were isolated and identified by accepted taxonomic methods (Burge *et al.,* 1987). Similar approaches but different media were used to characterize airborne fungi. While microscopy was stated as a method, it is unclear how it was used, and total microorganism counts were erroneously reported as CFU/m^3. Culturable airborne concentrations of bacteria averaged approximately 100 CFU/m^3, and airborne fungi averaged about 10 CFU/m^3. A rank order assessment of the most commonly cultured bacteria and fungi was provided (previously summarized in Section II), and some of the bacteria and fungi cultured are recognized pathogens. Culturable fungi cells and spores were below detection limits on approximately 10% of the flights sampled (but detection limits were not specified). The authors accounted for the following operating factors: (i) load factors, (ii) wide- or narrow-body aircraft type, (iii) threshold air-exchange rate (recirculation rate < 20), and (iv) air recirculation. The authors concluded that load factors had the strongest association with bioaerosol concentrations, although the standard deviations reported would not support that conclusion, and it appears no statistical tests were applied. Further, the results suggested that airliner cabin concentrations of bacteria and fungi, as well as the prevalence of the taxa identified, were not indicative of significant potential for illnesses associated with some indoor environments. It appears this conclusion was based on literature comparison of bacteria and fungi cultured from homes in three large residential studies. This study was not published in a peer-reviewed archival journal.

The Air Transport Association of America (1994) contracted Consolidated Safety Services, Inc., to conduct a comprehensive survey of air quality in commercially operating aircraft. For culturing of colony-forming bacteria and fungi, discrete short-duration air samples were taken at the early and late phase of flight on 35 randomly selected flight segments. Four aircraft were targeted for sampling. Boeing 727 and McDonnell-Douglas DC9 were chosen to represent older aircraft with low fresh-air replacement and without air filtration devices. Boeing 757 and McDonnell-Douglas MD80 aircraft were chosen to represent newer aircraft with high fresh-air replacement and high-efficiency particle filters. Bioaerosol sampling was executed with multiple-hole impactors and RODAC samplers containing broad-spectrum, solid-phase media. It is not clear if the method used employed simple time bracketing techniques to ensure sensitive measurement ranges; heterotrophic bacteria and mesophilic fungi were the media targets. Both the first class and coach cabin air was

sampled. Bacteria were cultured from the air samples in the range between 0-360 CFU/m^3, and fungi were cultured in the range between 0-110 CFU/m^3. No pathogens were cultured during this study. The microorganism counts were erroneously reported as total airborne bacteria and fungi (as CFU/m^3). The number of observations below detection limit was not reported, nor was any standard deviation reported. This study was not published in a peer-reviewed archival journal.

Dechow *et al.* (1997) summarized aircraft bioaerosol exposure data collected under a study designed by the Medical University at Lubeck, Germany. The concentrations of airborne microorganisms on 14 intercontinental flights were measured on two airbus models: the A310 and A340. The A310 was chosen because it used 40% recirculated air with vents restricting airflow to the corresponding cabin zone; A310 models monitored in this study had in-line filters on air transfer lines which removed 90% of particles at 0.5 um. A340 aircraft monitored in this study had central recirculation fitted with HEPA filters at 40% recirculation rates. 100 L air samples were drawn through slit-impactors with an effective D_{50} size cutoff of 0.65 um. Approximately 180 cultures were used to identify pathogens or pathogen indicators on selective media. The experimental design included sampling the cockpit, galleys, and lavatories, in addition to all classes of the passenger cabins. Concentrations of culturable bacteria were in the range between 0 and 1703 CFU/m^3, with a geometric mean of less than 100 CFU/m^3 (transatlantic A340 flights) and between 168-483 CFU/m^3 (transmediterranean A310 flights). Concentrations of culturable fungi were in the range between 0-15 CFU/m^3 in all cases. The geometric mean for bacteria in the cabin supply air was determined to be 76 CFU/m^3 on A310 aircraft and 28 CFU/m^3 on A340 aircraft. In all cases, the culturable airborne bacteria concentration was higher in air samples taken from the coach cabin than samples taken from any other part of the aircraft. In all cases, the cockpit air samples had the lowest mean concentrations of any location on all flights (less than 80 CFU/m^3). A time series of culturability measurements taken during some of the flights showed that the highest airborne bacterial concentrations were sampled early in-flight and at deboarding. The authors concluded that this is due to the efficiency of filtration/ventilation systems. The majority of cultured bacteria were nonpathogenic gram-negative cocci. While this was published in peer-reviewed literature (*Chemosphere,* Elsevier Science, Ltd.), the main body of data and supporting statistics was presented in a medical school report at the Institute for Hygiene and Environmental Medicine, Medical University of Lubeck.

Lee *et al.* (1999) reported the concentrations of culturable airborne microorganisms on three Cathay Pacific flights during 1996 and 1997. A business class sampling location was used aboard Boeing 747-400 series aircraft, which were equipped with HEPA filtration equipment and had occupancy levels below 78%. Air samples were taken during boarding, immediately after takeoff, and twice per flight using a Burkard air sampler with a reported method detection limit of 10 CFU/m^3. Airborne bacterial counts ranged between 3 and 93 CFU/m^3, while airborne fungi ranged between 17 and 107 CFU/m^3. Airborne microorganism counts were reported to be slightly higher at the beginning and end of the flight, but the highest culturable microorganism during boarding and deboarding. It was implied that the predominant bacterial species cultured were those typically shed from human skin, and concentration reported was said to be within the normal range found in schools and office buildings, with no specific references given. The authors extrapolated their observations to state, "This implied that biological contamination levels on Cathay Pacific aircraft were very low." This report appeared in the peer-reviewed literature.

Pierce *et al.* (1999) reported monitoring bioaerosol concentrations on eight Boeing 777 aircraft during 1998. Four domestic (between 1000 and 1500 miles) and four international (greater than 3000 miles) flight segments were monitored. These aircraft were equipped with HEPA filters on their air recirculation systems operating with 50% fresh-air replacement during flight. The mean load (occupancy factor) for the flights was 91% during the period of observation. Sampling was performed using a RODAC portable compact air sampler (media not documented) taking an undefined number of grab samples in the aircrafts' coach sections. Culturable airborne bacteria ranged in concentration between 50-244 CFU/m^3, and airborne fungi were below detection to 37 CFU/m^3. The highest concentrations were observed during the boarding and deplaning process. No bacterial pathogens were identified on the media used. Two fungal isolates that are considered as potential human pathogens, *Apergillus niger* and *Paecilomyces variotti*, were cultured from aircraft air samples. In reference to a previous aircraft environment study executed by Wick and Irvine (1995), the authors concluded that airborne bacteria and fungi concentrations were relatively low when compared to those levels typically found in buildings. This report was from peer-reviewed literature.

CASE STUDY SUMMARY

Air travel is undoubtedly increasing in volume. As of 1993, more than 13 million non-immigrants enter the US by air annually. Because persons with tuberculosis and other airborne infectious diseases can have long infectiousness periods, it is reasonable to assume that contagious individuals will occasionally, and perhaps not uncommonly, travel by air into and through the US (McFarland *et al.,* 1993). While the sampling equipment used in past aircraft cabin air quality studies was well-characterized, solid-phase impaction is recognized to provide lower yield than comparable volume liquid-capture equipment. In many studies, airborne bacteria and fungi concentrations were erroneously reported as "total bacterial concentrations". Short duration grab samples were taken (perhaps erroneously) as representative of the entire flight exposure with only culture-based methods, and in many cases the variability in the measurements was not reported. Further, many of the conclusions provided were not peer-reviewed.

In terms of sampling systems, both the DOT (1989) and the ATA (1994) studies used the Surface-Air-Sampler (SAS) bioaerosol collector developed by Pool Bioanalize Italiana. The SAS is a multiple-hole impactor that samples onto agar plates. SAS sampler performance has been reviewed (Griffiths, 1994; Henningson, 1994; Nevailenen, 1992; Buttner, 1997; ACGIH, 1999). The instrument was chosen for the DOT study because it allowed portable and unobtrusive collection of both vegetative and hardy bacteria. The SAS sampler has been found to consistently underestimate CFU counts by ~50% when compared to the Andersen N-6 (single-stage) impactor, the RCS centrifugal air sampler, and a slit-to-agar sampler (Smid *et al.,* 1989). The collection efficiency for the SAS was found to be close to 100% for particles with a minimum of 4 um diameter, and 50% for particles with a 2 um diameter (Lach, 1985; Jensen, 1995). In the Wick and Irvine (1995) study, the Reuter Centrifugal Air Sampler (RCS), made by Biotest AG, Dreieich FRG was used. This instrument is portable and battery-operated; it works by impelling particulates onto agar strips. Placencia *et al.* (1982) found that the RCS had higher yields than slit-to-agar samplers. Higher yields were also noted by Casewell *et al.* (1984), who compared the RCS to the Casella slit sampler; they also found that the RCS's efficiency for small particles was poor. Clark *et al.* (1981) found the RCS good for particles with an aerodynamic diameter greater than 5 μm; they also found that the actual sampling volume varies significantly from the manufacturers' values and is therefore not ideal for quantitative estimates (Clark *et al.,* 1981; Macher and First, 1983).

SECTION V: RECOMMENDATIONS AND AMMENDMENTS FOR FUTURE AIRCRAFT MICROBIOLOGICAL AIR QUALITY STUDIES

A more robust characterization of aircraft cabin air that can better represent bioaerosol concentrations during the routine operations of the commercial aircraft fleet will require larger-scale efforts than past studies. Such studies should consider a multidisciplinary approach, including applied statisticians, environmental microbiologists, industrial hygienists, air-frame manufacturers, and industry representatives. This survey precipitated the following recommendations to improve future aircraft bioaerosol characterization studies:

Sampling Plans

Previous case studies have reportedly sampled cabin air in-flight to estimate human exposures to bioaerosols. Since 1988, a total of 152 flight segments have been sampled for microbiological contamination (another 27 flight segments have been sampled for bioaerosol, but are unaccounted for at this time because data are not yet public domain - *Harvard Transportation Vehicle Environment Study,* Dumyahn *et al.,* limited data presented at the ASTM meeting on aircraft air quality, New Orleans, LA, October, 1999). Air samples on commercial flights have been predominantly collected as discrete "grab" samples because of equipment considerations (short durations were required to avoid overload on solid media impactors). The assay methods used in the different case studies are not uniform, but there is some overlap between the culture methods and media used. Only one case study (DeChow *et al.,* 1977) reported a time series of microbiological sampling to discriminate between cruising altitude concentrations and boarding/on-ground situations.

Sample Size Considerations. The case studies reviewed under this survey analyzed bioaerosols from approximately 179 commercial flight segments during an 11-year period. Since the absolute number of flights sampled is extremely low compared to the number of daily commercial departures (ca. > 10 000 flights/day), a statistically based rational should be used to determine a minimum number of flights that need monitoring to yield meaningful exposure information. No such statistical analysis of existing exposure information has been reported to date.

The sample (monitoring) pool must be large enough such that it can be extrapolated to the commercial fleet with some quantifiable confidence level. This rational should be applied to develop a monitoring

plan to determine both the *number* and *type* of aircraft that should be sampled from the commercial fleet should a larger-scale study be executed. Or, alternatively, if validating the number of past observation is sufficient to draw meaningful conclusions. Statistical criteria, including the choice of appropriate models, acceptable confidence levels, and final interpretation of results, should be agreed upon prior to future studies.

Future monitoring plans should focus on the newest and oldest commercial aircraft in service, and account for the following factors: (i) extremes in recirculation rates and the replenishment of fresh air, (ii) operation of air filtration systems, (iii) flight lengths, and (iv) passenger load factors. The monitoring plan should provide for "worst and best" case scenarios by combining the above factors. Load factors for future sampling events can be predicted based on historical occupancy data archived with airline rate designers. Some consideration should be given for the increasing number of regional jets and commuter typed flights and the fraction of commuter-type air hours should be appropriately weighted against longer-haul flights based on passenger flight hours.

Sampling Paradigm Considerations. Using discrete grab samples with sampling durations typically less than three minutes (a maximum of 270 L), previous case studies have taken airliner cabin air samples using variations of solid-phase impactor equipment. Compared to the duration of flight, the sampling periods executed in past case studies represent only a small fraction of the in-flight exposure periods. Only one case study reported time series analysis using multiple grab samples (Dechow *et al.*, 1997). Because of the wide range of effective air exchange rates and mixing regimes reported for commercial aircraft, recovery factors and human exposures would be better represented by composite or continuous air sample paradigms. Composite sampling is defined here as the pooled analysis of semi-continuous sampling over an observation period; multiple-grab samples taken into the same sampler medium at predetermined time intervals constitute a composite sample. Composite sampling has been widely practiced in the water pollution field to characterize water quality in large volumes supplied to, and recovered from municipalities. As opposed to solid-phase impactors, liquid-capture and filter-capture devices offer composite capabilities because they cannot be overloaded for their respective analysis (filter capture is not recommended for culturing analysis but for size discrimination, microscopy, and/or genetic analysis). While extended sampling times have been shown to damage collected microorganisms in some liquid impingers, data yield and exposure representation can be greatly improved by "pacing" composite

sampling throughout the flight time. Liquid-capture air sampling has been discounted in past case studies because liquid handling makes them "difficult to use unobtrusively on aircraft" (DOT, 1989). However, liquid-capture samplers have become more compact since the execution of past air quality studies, and even though liquid media is more difficult to handle, these samplers offer the flexibility of composite sampling as well as the option for culturing, direct microscopy, and immunochemical and genetic assays from the same sample. Filter-capture devices can offer the same analytical flexibility, but because of desiccation, effects cannot be used for culturing.

SAMPLER RECOMMENDATIONS for LIQUID CAPTURE

1. SpinCon® Advanced Air Sampler/Concentrator, MidWest Research Institute (MRI).

   The SpinCon is a portable, self-contained sampler that can sample up 800 Lpm. The sample is collected in a liquid medium (can use various liquids) - up to 15 mL of liquid may be used. Samples are compatible with most analytical methods. The SpinCon is efficient at collecting a wide range of particulate matter (mean aerodynamic diameter .2μm and larger).

   Size: 12.5 in. X 8 in. X 18 in.

   Weight: 35 lbs

   Power Requirements: 120 VAC

SAMPLER RECOMMENDATIONS for FILTER CAPTURE

2. Gillian AirCon-2 Constant Flow Collection System Area Sample, Sensidyne, Inc.

   The is a pump and filter cassette sampler that was originally developed for lead and asbestos monitoring. This unit may be easily adapted for composite sampling of bioaerosols using polycarbonate membrane filter casettes (25 or 47 mm dia).

   Size: 6" X 8" X 13"

   Weight: 21 oz

   Power Requirements: A battery is required for operating this sampler.

   Pump: 2 - 30 l/min with full-scale accuracy div @ +/- 20%

Assays for microbial fractions: mycotoxin, endotoxin, and Glucans. Review of the state-of-the-art environmental assays used for mycotoxin, endotoxin, and Glucans, concluded that all of the currently available analytical methods were designed for high concentrations of these compounds associated primarily with organic dusts and agricultural products. The crossover for use on air samples from indoor environments may be problematic where mycotoxin, endotoxin, and Glucans concentrations are low. Mycotoxin, endotoxin, and Glucans based studies may be labile to environmental interferences and analytical artifacts due to the following factors:

(i) Recovery from environmental samples is highly variable mostly due to the differential sampling efficiency of currently marketed bioaerosol samplers
(ii) Stability of toxin activity during sample storage may vary depending on the sample matrix; this suggests that stability factors must be established for each sample type.
(iii) The proteinase enzymes activated for these assay are labile to biopolymers other than their targets
(iv) Currently marketed reagents in proprietary kits vary widely in their sensitivity to these environmental toxins.

Where microbiologically associated toxins were involved, Burge and Ammann (1999) suggest that other than use in research labs, use of these techniques should be limited because of necessary reference standards, expensive analytical equipment, and specialized expertise. Until the enzyme and chemical assays for mycotoxin, endotoxin and Glucans are better developed and there is better evidence to suggest that these compounds could potentially present exposure problems at the levels expected in commercial aircraft environments, future aircraft air quality studies should not include theses assays.

Common Allergens – immunochemical assays. There are no reports in the peer-reviewed literature on the use of immunochemical assays for common allergens encountered in indoor environments. As part of the Harvard-designed survey on the *Comparison of the Environments of Transportation Vehicles*, Dumyahn et al (1999) reported the use of immunoassays to quantify animal, cockroach, and fungal epitopes in surface and dust samples taken from aircraft interior surfaces (e.g. seats and carpets) – results from this study will be available in June, 2000 in the proceedings for ASTM Symposium on Air Quality and Comfort in Airliner Cabins, New Orleans, LA, 1999. The results from this study are key to demonstrating the validity for practical application and potential for information yield of immunoassays when applied to exposure assessments in aircraft.

BIOAEROSOL ASSAYS

Culturing and Microscopy - Bacteria and Fungi: Culture-based enrichment and isolation remains the primary method reported for environmental sample analysis of airborne bacteria and fungi (ACGIH, 1999). It is now widely accepted in the environmental microbiology community that culturing techniques consistently underestimate both the total and viable numbers of bacteria, fungi and their spores. In recent studies of both indoor and outdoor atmospheric environments, this underestimation has been reported to span up to four orders of magnitude. While direct microscopy was mentioned in the introduction and methods section of one aircraft air quality report (DOT, 1989), no estimates of total airborne microorganisms using direct, non-culture-based techniques have been reported in aircraft bioaerosol investigations to date; all bioaerosol studies of aircraft cabin air have exclusively used classical culture-based methods to assess the presence of airborne bacteria and fungi. Further, some of the aircraft air quality literature has improperly interpreted culture-based results as Total Bacteria (CFU/ m^3) and Total Fungi (CFU/ m^3). Although no standards yet exist for indoor aerosol bioburdens based on total microorganism numbers, there are an increasing number of bioaerosol reports and comprehensive publications suggesting the importance of direct techniques and total microorganism counts for the following reasons:

(i) culturing biases are intrinsic to plate counts regardless of the media used
(ii) that some pathogenic microorganisms can exist in a viable but non-culturable state (*e.g., Legionella* spp.), and the importance of noninfectious airborne diseases caused by non-culturable, slow-growing, or nonviable microorganisms (*e.g.,* asthma and other hypersensitivity diseases)

In response to the limitations of culture-based techniques used in previous aircraft studies, this review precipitates the recommendation that future aircraft biological air quality research include direct characterization techniques for the express purpose of determining total aerosol bioburdens; experimental designs should include microscopy where appropriate. The analytical technique for determining total numbers of airborne bacteria and fungi are now well-established in the bioaerosol literature and can be easily applied using well-characterized and inexpensive air sampling equipment (see sampling equipment recommendations). DNA intercalating agents and/or cell-wall stains can and should be used for epifluorescent microscopy to discriminate and enumerate airborne bacteria, fungi and their spores. Since the introduction of the CAMNEA method (Palmgren *et al.,* 1986), many researchers have successfully used acridine orange for determining total number of airborne microorganisms. This should be executed by skilled technicians familiar with the morphologies of airborne microorganisms.

In the context of direct enumeration techniques, either filter-capture or liquid-capture devices can be economically used in future aircraft studies, but because of higher detection limits intrinsic to direct counting techniques, extended sampling times and/or high-volume equipment may be required. While filter-capture devices are not reliable for culturing assays, they have the highest documented capture efficiencies and have been successfully used for both direct microscopy and genetic amplification; however, the advantage to liquid-capture devices is that they provide for samples to be concurrently analyzed by many different methods, including culturing, microscopy, and molecular-based techniques. Culture-based methods can and should be used in conjunction with direct microscopic counts to provide a link to past aircraft air quality studies and current air quality guidelines. Although solid-phase impaction has been used in past studies, liquid-capture (impingement) rather than impaction should be considered as a sampling method of choice due to the lower sampling stresses, the options for concurrent cultivation on different media (from the same sample), and availability for microscopy and genetic amplification. Handling liquid-capture devices generally requires more delicate attention, and surreptitious sampling would be more difficult; the potential for data yield, however, may be worth the tradeoff to operate liquid-capture equipment in lieu of solid-phase impactors.

Culturing Viruses: The need for airborne virus quantitation in commercial aircraft environments has been clearly identified (ASTM Meeting on Aircraft Air Quality, New Orleans, LA, October, 1999). Past aircraft environment studies (DOT, 1989; ATA, 1994) have acknowledged that adequate technology for airborne virus quantitation was not available at the time of execution; this situation has not effectively changed, and a practical quantitative approach using viral culturing techniques is not available. With currently available sampling and analysis methods, airborne virus analysis based on culturing will be quantitatively indeterminate for the foreseeable future (Sattar and Ijaz, 1997). For exposure assessments, both the sampling and analysis of airborne viruses need further development before they can be reliably applied to aircraft environments. However, genetic amplification is widely acknowledged to hold tremendous promise as a tool for quantitative airborne pathogen analysis and may also improve viral detection - provided that the sampling and collection efficiency of virus particles from air can be established and controlled. Regardless of the accuracy of analytical techniques, until aerosol collection efficiency of virus particles is better established, quantitative airborne virus characterization will remain indeterminate.

Genetic Amplification and Airborne Pathogens. Genetic amplification protocols are now well-established, and semi-quantitative PCR has been successful for presence/absence studies of airborne pathogenic viruses and bacteria in clinical and laboratory settings. Semi-quantitative PCR is defined here as the detection of target DNA sequences above a standard-established method detection limit (*e.g.*, > 100 copies of *M. tuberculosis* DNA). The tremendous forensic potential of PCR has been generally agreed upon, and given the ever increasing sizes of genetic databases, both the specificity and detection limits offered by genetic amplification may soon be unrivaled by current techniques (*i.e.*, microscopy and culturing). Several reports acknowledge that there are significant environmental interferences that may impair accurate PCR characterization of microorganisms in environmental samples; some of these interfering agents are undoubtedly present in aircraft cabin air, but may be mitigated during the sampling and analysis process.

Currently, quantitative PCR is not reliable for environmental samples. Quantitative PCR analysis of environmental samples is, however, an area of intense research, and efforts are currently being directed toward quantifying airborne pathogens, specifically *Mycobacteria* (Schaffer, 1999). If quantitative aerosol-based PCR protocols are proven in the peer-reviewed literature, this analytical approach should be used for future aircraft air quality studies. The true time horizon for such developments is unknown, but compared to other developing bioaerosol technologies (*e.g.*, fluorescence imaging), appears promising within reasonable time frames. If quantitative PCR licensing costs were reasonable, economical, large-scale surveys (hundreds of flight segments) could be executed surreptitiously using PCR techniques, as only low-volume filter sampling is required, and compositing samples is intrinsic to the filtration process (overload is avoidable through dilution). Storage techniques for DNA are established and reliable, such that DNA-containing air samples may be archived for future analysis.

DNA sequences for target pathogens must be agreed upon prior to air sampling for any future studies relying on PCR analysis. Using epidemiological-based rationale, a prioritized database of available bacteria and viral primer sequences should be compiled for bioaerosol analysis of aircraft environments. Some bacterial primer sequences may be obtained from the Ribosomal Database Project (Maidak *et al.*, 1999) or from industrial biotechnology sources (e.g. Perkin Elmer Cetus, Genetech, Inc.). PCR primer sequences for virus analysis appear to be available predominantly from the scientific literature.

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LA, 1999. The results from this study are key to demonstrating the validity for practical application and potential for information yield of immunoassays when applied to exposure assessments in aircraft.

Bioaerosol Air Quality Modeling in Aircraft. The environmental situations in aircraft pose a unique environment to which sequential box models and CFD have been applied. The results of past epidemiological studies applied to disease transfer in aircraft have strongly suggested that proximity to pathogen sources (infected passengers) are a key factor to passenger exposure levels on aircraft. Multi-zone models have the capability to address and simulate the near- and far- field effects experienced on aircraft, and account for the various ventilation system configurations in the commercial fleet. Given that HEPA and other filtration systems have been added to some modern commercial aircraft, and that one-pass disinfection systems have been proposed, existing models should be modified to simulate exposure to airborne pathogens in different airliner cabin configurations and operating modes (i.e. recirculation rates, fresh air exchange rates). Given the potential power of these modeling tools, and the growing interface between environmental microbiology and aerosol physics, bioaerosol modeling of aircraft environments should now be considered a valid and conservative approach for bioaerosol exposure assessments of airline passengers.

In general, the interpretation of contaminant concentration data, weather it pertains to chemicals or biological agents, depends on the specific model of room air mixing that is assumed appropriate. The recent applications of sequential box models and CFD to simulate airborne contaminant concentrations, oxygen levels, and RH in aircraft environments, has demonstrated the utility of these tools for economic evaluation of non-microbiological human exposures (Ryan et al, 1988; Arnold et al, 1999; Baker, et al, 1999). Further, indoor airborne contaminant modeling has been successfully extended to include pathogenic bacteria and their response to *in situ* disinfection

systems, ventilation dilution, and filtration. With appropriate modifications, these models may be extended to study the distribution and abundance of airborne pathogens and in aircraft cabins.