CHAPTER 4.2

Molecular methods for bioaerosol characterization

Richard C. Summerbell[1,2] Brett J. Green[1,2] Denis Corr[3] and James A. Scott[1,2]

[1]*Dalla Lana School of Public Health, University of Toronto, Canada;*
[2]*Sporometrics Inc., Toronto, Canada;*
[3]*Corr Associates, Hamilton, Ontario, Canada.*

POLYMERASE CHAIN REACTION

Since the work of Maddox (1857), Cunningham (1873), Miquel (1883) and others in the latter part of the 19th century, the detection and classification of bioaerosols has been dominated by methods based on culture and microscopy. The invention of polymerase chain reaction (PCR) in the 1980s transformed all of biology, including the study of bioaerosols. At first, this technique for amplifying genes was primarily used for single species, sampled as tissue specimens or as living cultures (Sontakke *et al.* 2009). Very rapidly, however, scientists realized that by using conservative gene regions as primer areas, they could amplify an indefinitely large diversity of different species from complex mixtures of organisms without *a priori* knowledge of their DNA sequences (Nocker *et al.* 2007). Thus, a very powerful technique for environmental investigation had been made available. By adjusting the primers used, amplification could be targeted at groups of organisms with any level of specificity: classes, orders, families, genera, species or strains/individuals. In cases where there was a strategic advantage in limiting the diversity elucidated from complex environmental samples, specific primers could be targeted at one, several, or multiple species (Haugland *et al.* 2004). These primers could then be used as a battery to investigate single species or to simultaneously look for two to four species at a time, as is made possible in some quantitative 'real time' PCR techniques.

There were numerous practical problems, as with any newly developed technology. Chemicals in environmental materials often inhibited amplification reactions, and new techniques and kits had to be developed for DNA extraction from various substrates, e.g. soil (Schneegurt *et al.* 2003). Conservative primers used on environmental materials often elucidated a far greater range of DNA types than investigators were prepared to deal with. Very time-consuming studies involving the cloning of different DNA amplicons from PCR samples often resulted in sequences that were mostly uninterpretable, belonging to little known organisms such as unclassified soil amoeba species. Nonetheless, thanks to the continual deposition of even uninterpretable sequences in GenBank, a baseline was laid down for future comparison, and possibly for eventual taxonomic resolution of unknown types of organism, depending on funding levels for taxonomic work.

Techniques such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) were developed that allowed improved resolution of estimates of the numbers of prevalent species in natural materials and their relative abundance (Smit *et al.* 1999, Nakatsu and Marsh 2007). Again, with very delicate and time-consuming techniques, individual sequences could be retrieved from DGGE gels and analysed in order to determine the identity of the organisms. In all environmental PCR-based techniques, the possibility of skewing of results by differential affinity of different organisms for primer sequences was difficult to rule out. This potential for bias was parallel to the potential for sampling bias in culture-based studies of microorganisms, and it was repeatedly observed that the similarity between culture and PCR results in environmental samples was not high (Lynch and Thorn 2006). Nonetheless, it was clear that every sequence elucidated by PCR truly identified a DNA type that was present in the sample, as long as procedural contamination was well-controlled, though the organisms elucidated

might be autochthonous or allochthonous in the substrate studied, and might be alive or dead. Indeed, the high ability of PCR to detect dead materials with partially intact DNA diminished its usefulness in some epidemiological studies where only living, disease-causing inoculum was of interest.

Especially initially, there was a strong tendency for such studies based on gene sequences to discover that organisms were misclassified, or that entities thought to be single species or genera were actually multiple taxa, sometimes even very distantly related taxa. Taxonomic work in the last two decades has resolved some of these problems, and moved some remarkably misclassified organisms, such as microsporidia, to the kingdom, class, order, genus, etc., where they belong. In the case of microsporidia, they were misclassified as kingdom Protista but were actually members of kingdom Fungi (Lee *et al.* 2008). All such endeavours have clarified sequence-based environmental analyses and made them more efficient and effective.

In the atmosphere, biogenic particles form a major component of the suspended solid materials that are present (Després *et al.* 2007). These particles include intact biological structures such as fungal spores, pollen grains, microalgae, bacteria, viruses and dormant, desiccated microarthropods and protozoans, as well as partial structures such as broken fragments of fungal spores and hyphae, plant trichomes and fibres, and animal danders. Such particles not only disperse organisms, ranging from ecologically beneficial to epidemic-causing, but also have a significant effect on light dispersion in the atmosphere and in the nucleation of cloud droplets and ice nuclei, essential in the genesis of normal rainfall and other atmospheric precipitation.

A preliminary survey of DNA signatures in fine particulate matter ($PM_{2.5}$, aerodynamic diameter <2.5 µm) in air by Després *et al.* (2007) showed that the majority of sequences that could be obtained were bacterial, particularly α, β, and γ-proteobacteria as well as actinobacteria, followed by fungi (mostly Ascomycota) and plants. One animal sequence was obtained corresponding to a spore-forming protozoan type in the *Alveolata apicomplexa* complex.

Microorganisms, including bacteria, fungi, microalgae and protozoans, often have minute vegetative cells or spores that readily become airborne. For this reason, they are disseminated very readily and, essentially, in many cases may grow anywhere on our planet where conditions for their growth are favourable. Unlike animals and higher plants, they may have very few limitations in geographic range, other than being limited to growing where conditions are supportive. There are notable exceptions for microorganisms that have a specialized relationship with a particular type of range-limited species, whether it be a plant or an animal. For example, specialized parasites, symbionts and decay organisms may only occur in and around the areas where their partners or hosts live. There are also some known, but poorly understood, range limitations in microorganisms, as seen in disease-causing fungi such as the agents of histoplasmosis and blastomycosis (Rippon 1988).

The state of the art with regard to PCR analysis of organism propagules or parts potentially represented in airborne particulates is reviewed below on a group-by-group basis.

An important extension of PCR analysis has been made possible by the use of high density microarrays (Wilson *et al.* 2002, Brodie *et al.* 2007, De Santis *et al.* 2007). In some microarray techniques, arrays are exposed to DNA obtained from environmental samples using the same sorts of amplification, e.g. 16S rDNA, as are used in the preparation of clone libraries. In others, unamplified genomic DNA obtained directly by extracting the samples may be used (Avarre *et al.* 2007). This latter technique, however, is in the very early stages of development.

Yet another level of development lies in making clone libraries of unamplified genomic DNA from environmental samples and then analyzing large numbers of the clones. The clones can be screened for affinity with taxonomically informative genes such as 16S rDNA or functional genes such as cellulases, or can be randomly sub-sampled for sequencing, or even sequenced *in toto* (Handelsman 2004). This collection of approaches, called 'metagenomics,' can be used to explore microbial diversity or to look for particular groups of enzymes or other genetic features of interest. In addition, when large numbers of sequences are obtained in metagenomic studies, cluster analyses of various kinds can be used to analyse the data (Xu 2006, Li *et al.* 2008). This possibility gives rise to a potential for a very detailed characterization of environments, and the statistical distinction of one environment from another in samples (Tringe *et al.* 2005, Dinsdale *et al.* 2008).

Cutting-edge systems for high-throughput sequencing and bioinformatics processing have further revolutionized metagenomic analyses. For example, Dinsdale *et al.* (2008) compared nearly 15 million sequences from 45 microbiomes (bacterial habitats) and 42 viromes (environmental or medical sites where vi-

ruses are present). Currently available technologies like GS FLX Titanium pyrosequencing (Roche Diagnostics) allow the simultaneous sequencing of over 500 Mbp. In a single run the PCR-amplified DNA barcode region can be sequenced to exhaustion in up to 128 community DNA samples with over 10,000 times redundancy. This method is suitable for the metagenomic characterization of low to moderately complex microbial communities. Communities of higher complexity can be sequenced to exhaustion by processing fewer samples simultaneously.

POLLEN, SPORES AND PARTS OF PLANTS

The pollen grains of wind-pollinated (anemophilous) plants are among the most common airborne particulates, and have been intensively studied for decades. The air also includes other plant parts such as pollen grains of insect-pollinated (entomophilous) plants, spores of mosses, ferns and lycopods (clubmosses), and various trichomes (distinctively shaped leaf hairs) and other minute fragments broken away from leaves, stems and decaying wood (Sarna and Govil 1979, Kasprzyk 2004). It is not clear how reliably pollen and trichomes collected from air can be studied using molecular techniques. DNA extraction from such materials often involves distinct processes, since the breakage of heavy and chemically unusual cell walls is usually involved. For example, Zhou *et al.* (2007) picked individual pollen grains from sieved materials and cracked them open between two glass slides prior to doing DNA extraction. Chen *et al.* (2008) developed a protocol for DNA isolation from single pollen grains involving special forceps and alkali/detergent lysis of the walls. Sometimes plant DNA interpreted as deriving from pollen has been detected indirectly; for example, Brodie *et al.* (2007) detected plant chloroplast DNA while DNA microarray probing bacterial-type DNA in urban air (chloroplast DNA is related to cyanobacterial DNA) and stated that this was likely to be from pollen grains. Després *et al.* (2007) detected plant nuclear DNA in urban air samples while using 18S RNA primers intended to amplify animal sequences; these sequences, which included angiosperm, pine, and moss sequences, were only detected in the spring and were interpreted as being from pollen and, in the case of moss, spores. Various studies making bacterial 16S ribosomal DNA clone libraries from collected aerosols have detected plant chloroplast DNA but have not remarked on its probable source (Radosevich *et al.* 2002, Rintala *et al.* 2008). No study has been carried out to determine the degree of correlation between the chloroplast and nuclear plant sequences obtained and the diversity and number of pollen grains and spores detectable by other techniques.

Where trichomes are concerned, there is no published information relating to their molecular detectability from aerosol sources. Molecular studies on these structures are relatively common, although mostly relating to the genomics of their differentiation. For example, there is a trichome harvesting technique for leaves of the genetic model plant *Arabidopsis thaliana* (Marks *et al.*, 2008), designed to facilitate genomic studies. A typical example of a trichome genomic study involving DNA extraction from these thick-walled structures is that of Liu *et al.* (2006) on proteinase inhibitor IIb expression in trichomes of nightshade, *Solanum americanum*. The more data are collected on DNA extraction techniques for these structures, the more accessible they will become as data sources for molecular investigators of bioaerosols.

The minute spores of non-vascular plants such as bryophytes, ferns and lycopods are capable of long-distance airborne dispersal and thus regularly make up a small but significant component of total airborne particulates (Stoneburner *et al.* 1992, Kasprzyk 2004, Sundberg 2005). About 4% of total pollen belongs to entomophilous vascular plant taxa such as willows, maples, elders (*Sambucus*), herbaceous plantain (*Plantago*), and *Tilia* spp. (basswood, linden, lime). Long range dispersal, however, is inhibited in that pollen grains of these species are often fused together into relatively heavy groups by the sticky material known as pollenkitt (Kasprzyk 2004).

Generally, PCR-based plant identification involves sequencing one or both of the two recognized identification barcode regions, the nuclear ITS region and the chloroplast *trnH-psbA* intergenic spacer (Kress *et al.* 2005).

FUNGI

Fungal spores and fragments are among the most common components of total bioaerosols, and are also among the most intensively studied materials in terms of molecular detection. This level of study can be partly ascribed to the high levels of interest in indoor fungal proliferation and fungal aerosol levels in 'sick' and healthy buildings. Most molecular techniques for studying airborne fungi were developed for indoor materials; however, these can readily be adapted for outdoor materials as well. In many cases,

the use of outdoor samples as controls is common in the study of indoor aeromycota.

The identification of indoor fungi by means of diagnostic sequences has been a well-established and commonly used technique since the late 1990s (Haugland *et al.* 2004). Though initially developed for identifying *in vitro* cultures, many approaches can readily be modified to analyse the fungal contents of air samples collected using filter membranes, cyclone samplers or jet-to-plate impactors. Equally, these methods can be used to analyse vacuum-collected dusts. In an early example, Haugland and Heckman (1998) introduced specific primers for the important indoor fungus *Stachybotrys chartarum*. This study was shortly followed by the first of a series of species-specific quantitative PCR (qPCR) studies for indoor fungi, based on use of the TaqMan® fluorogenic probe system combined with the ABI Prism® Model 7700 Sequence Detector (Haugland *et al.* 1999). In this study, *S. chartarum* was again the object of interest; qPCR counts of *S. chartarum* conidia were found to be highly comparable to counts obtained with a haemocytometer. The method was further developed by Roe *et al.* (2001) for direct quantitative analysis of *S. chartarum* in household dust samples.

This TaqMan-based qPCR methodology was extended over subsequent years into a broad ranging methodology encompassing many major indoor air fungal groups (Haugland *et al.*, 2004) and a variety of applications. In conjunction, key studies considered how best to extract DNA for qPCR and related PCR-based analyses of indoor fungi (Williams *et al.* 2001, Haugland *et al.* 2002, Kabir *et al.* 2003). Meklin *et al.* (2004) employed qPCR to evaluate indoor dust from the presence of 82 mould species or species complexes, including members of the genera *Aspergillus, Cladosporium, Penicillium, Trichoderma* and *Ulocladium,* in addition to *Stachybotrys* and the closely related *Memnoniella.* Comparisons between techniques showed that culturing underestimated numbers of key *Aspergillus* species by two to three orders of magnitude. "Mouldy homes" could be distinguished from putatively uncontaminated "reference homes" using mould-specific qPCR (MSQPCR)-based quantification. An online information page about the now widely used technology developed by R.A. Haugland, S.J. Vesper and other members of the US Environmental Protection Agency (US-EPA) group can be found at <http://www.epa.gov/nerlcwww/moldtech.htm>. Currently, 116 primer/probe combinations have been described that target 130 species. Commercial use of these primer sequences for fungal detection requires a licensing agreement with US-EPA. Similar restrictions do not, however, apply to the use of these sequences for non-commercial, research purposes.

The results of MSQPCR have been used to calculate a ratio of species associated with water damage to those arising from outdoor sources (e.g. phylloplane moulds), unrelated to indoor dampness. This ratio, the Environmental Relative Moldiness Index (ERMI), provides a single value between -10 and +20, that describes the potential of an indoor growth source. Although this approach is aesthetically appealing for its simplicity, apparent objectivity and ease of use, the reductive nature of the approach excludes subtle features of the dataset that may otherwise lead an experienced investigator to a different conclusion. Until more data are available to compare ERMI with the results of building inspections carried out by experienced assessors, the technique must be considered investigational.

More medically oriented environmental studies developed TaqMan qPCR for *Aspergillus fumigatus* conidia in filtered air samples (McDevitt *et al.* 2004, Goebes *et al.* 2007). More recently, the accuracy of qPCR for *A. fumigatus* detection in hospitals has been stringently tested by comparison with green fluorescent protein (GFP)-expressing conidia of this species (McDevitt *et al.* 2005).

In conjunction with other modern techniques such as a quantitative protein translation assay for trichothecene toxicity, qPCR was used in an evaluation of *Stachybotrys* from a house where a case of idiopathic pulmonary haemosiderosis had occurred (Vesper *et al.* 2000). However, haemocytometer counts of the relatively large and conspicuous *Stachybotrys* conidia were relied upon on for quantitation in the data used in the subsequent analysis. A later, much more detailed qPCR analysis of homes where pulmonary haemosiderosis had occurred showed that *S. chartarum* was part of a group of species, also including *A. fumigatus* and several other *Aspergillus* spp., that was significantly elevated in quantity in dust samples in affected homes (Vesper *et al.* 2004). Species abundant in affected homes tended to be haemolytic in *in vitro* testing, whereas the common species associated with reference homes were generally not haemolytic. Another significant application of the qPCR technique was to sensitively monitor *Aspergillus* contamination during hospital renovation (Morrison *et al.* 2004) and related infection control applications.

Relatively recent developments have included detailed studies of the fungal contents of dust from various sources, including studies optimizing qPCR to

overcome chemical PCR inhibitors in dust (Keswani *et al.* 2005, Vesper *et al.* 2005). Multi-species qPCR has been applied in comparison of outdoor with indoor air (Meklin *et al.* 2007) and in analysing both fungi and bacteria in building components such as chipboard, paper materials and insulation (Pietarinen *et al.* 2008). These methods have been combined with cloning and sequencing in the analysis of indoor dust to reveal a high prevalence of taxa of the Malasseziales (Ustilaginomycotina), the lipophilic basidiomycetous yeast inhabiting human skin (Pitkäranta *et al.* 2008).

In outdoor air studies, qPCR-based techniques have been used to detect airborne plant pathogens such as *Monilinia fructicola*, the cause of brown rot of stone fruits (Luo *et al.* 2007) and *Fusarium circinatum*, the cause of pitch pine canker (Schweigkofler *et al.* 2004). Spore levels of toxin-producing fungi in airborne grain dust have also been monitored using qPCR (Halstensen *et al.* 2006). In surveys involving large numbers of fungal taxa, cloned libraries have been made from bulk amplification of small subunit ribosomal DNA. The results, however, suggest skewing of the data: a small-subunit library study conducted by Fierer *et al.* (2008) using air samples from Boulder, Colorado, found that fungal sequences from the order Hypocreales (*Fusarium, Trichoderma* and relatives) were overwhelmingly predominant (90% + of sequences) in five libraries derived from aerosol samples. It can readily be seen in conventional air sampling that members of this order never show predominance on this scale; members of the Class Dothideomycetes (*Cladosporium, Alternaria*) are consistently predominant in outdoor air in temperate areas for most of the year (Gregory 1973). Dothideomycetous fungi have, however, spores with resistant, melanized cell walls and may withstand some DNA extraction methodologies even where bead-beating is used to rupture cells, as was done by Fierer *et al.* (2008). In the small number of fungal ribosomal internal transcribed spacer (ITS) sequences obtained by Després *et al.* (2007), *Cladosporium* and Basidiomycota sequences were found, consistent with conventional studies, but no Hypocrealean sequences.

Although qPCR offers major advantages over traditional approaches in the detection and enumeration of indoor fungi, a number of factors influence its performance. Because qPCR directly detects a target gene sequence, strains with variant sequences in either of the primer or probe regions may be missed (false negatives). Conversely, unintended taxa may inadvertently be detected based on their homology to the primer/probe sequences used (false positives). Validation of the analytical specificity of the primer/probe sequences is greatly hampered by the prevailing lack of knowledge of fungal biodiversity, with an estimated 90% of species yet to be discovered (Hawksworth 2006).

A second limitation of qPCR-based techniques relates to the ability to predict biomass by the number of copies of the target sequence enumerated. In the simplest case in a haploid, uninucleate fungal cell, it is possible to establish a standard curve correlating total biomass to the number of copies of a given gene. The difficulty is that this assumption is not transitive to all cell-types of a given taxon, e.g. monokaryotic vs. dikaryotic hyphae, haploid vs. diploid or polyploid cells, nuclear vs. mitochondrial targets, nor is it directly transferable to other gene targets, which may occur in multiple copies (whose total number is species- or strain-specific) within a single genome copy, e.g. ribosomal subrepeat.

Lastly, qPCR-based methods cannot distinguish viable from non-viable material, or cell-bound from cell-free DNA. However, the condition of cellular material may be relevant to the interpretation of the result, e.g. in the risk assessment of reservoirs of potential agents of nosocomial infection. A recent elegant study by Vesper *et al.* (2008) used propidium monoazide to inactivate DNA from dead cells prior to using qPCR to quantify viable conidia. This approach and similar methods may hold promise in tailoring qPCR-based methods to the detection of cell-bound DNA.

Microarray studies have been outfitted to detect fungal DNA signatures in filtered aerosols from outdoor air. This entails loading the arrays with a wide variety of fungal DNA probe sequences suitable for detecting a reasonable proportion of the known biodiversity. Using such a technique, De Santis *et al.* (2005) found that propagules of the fungal phylum Ascomycota (inclusive of most common mould spores of outdoor air) and Basidiomycota (mainly represented by airborne spores of mushrooms and related fungi) were abundant in outdoor air from southern England. They could only be elucidated efficiently, however, when the filter-collected aerosol sample was subjected to long bead-beating times to release DNA from cells with thick walls. This was not especially inconvenient, however, since similarly long beating times were also required for some bacterial types.

BACTERIA

The organisms that have been most extensively studied in terms of molecular detection in the air are the

bacteria. Bacteria are very difficult to identify from their phenotypic characters and, moreover, unculturable bacteria are common; therefore, identification with 16S ribosomal DNA sequences has been the gold standard since the early 1990s (Wilson *et al.* 1990). The application of this identification standard to aerosol samples, e.g. to bacteria on air filters, has developed only in recent years. The development of this area of work has been detailed in a review by Peccia and Hernandez (2006). Techniques used to study bacterial occurrence and diversity in outdoor air have been specifically reviewed by Kuske (2006).

Alvarez *et al.* (1995) developed basic collection and DNA purification techniques for spiked *E. coli* in samples from air, and also developed some dilution procedures for dealing with chemical inhibitors of PCR in the samples. Stark *et al.* (1998) developed a practical application for the technique for monitoring *Mycoplasma hypopneumoniae* levels in commercial swine houses, and refined the techniques for extracting DNA from filter samples. Later, species- or genus-specific techniques were developed for *Legionella* spp. in factory and office air, and *Mycobacterium* spp. above whirlpool facilities in public swimming baths (Pascual *et al.* 2001, Schafer *et al.* 2003). More recent developments on this theme have featured quantitative PCR techniques, to measure, for example, *Staphylococcus* species levels in poultry house air (Oppliger *et al.* 2008), and *Mycobacterium tuberculosis* levels in health care facilities (Chen and Li 2005). *Streptomyces* levels in household dust have also been studied using qPCR (Rintala and Nevalainen 2006). An *et al.* (2006) compared the use of specific primers for *E. coli* with universal primers for total bacterial load in a qPCR study of particulates from laboratory-generated air samples. Rinsoz *et al.* (2008) used three sets of broadly targeted primers to contrast qPCR results with those of epifluorescence microscopy and culture in air samples used to detect total bacterial, staphylococcal and total Gram-negative bacterial loads in poultry houses and wastewater treatment plants.

The sampling and detailed identification of a broad range of bacterial types in air samples began in the current decade, with the publication of studies that featured cloned 16S libraries (Radosevich *et al.* 2002, Maron *et al.* 2005, Després *et al.*, 2007) overlapping in time with studies featuring the use of microarrays (Wilson *et al.* 2002, Brodie *et al.* 2007). Cloned library-based studies were also made of dust as a 'natural' sediment from air (Rintala *et al.* 2008). A very distinctive array of bacteria was obtained by Birenzvige *et al.* (2003) in small-scale cloned libraries from air samples taken in an urban subway (underground railroad), while another unique perspective was afforded by a cloned 16S library study of bacteria in biogas emitted by anaerobic waste digestors and landfill sites (Moletta *et al.* 2007). The bacterial diversity of the air of swine confinement houses was studied using a 16S cloned library by Nehme *et al.* (2008). An ecologically important extension of the cloned library technique was made by Fierer *et al.* (2008), who compared bacterial biodiversity from samples taken within a single geographic area with that of samples from widely separated geographic areas.

De Santis *et al.* (2007) studied samples of urban aerosol, subsurface soil and subsurface water, and found that high-density microarrays detected more bacterial biodiversity than 16S cloned libraries. Some bacterial phyla such as Nitrospira were only detected on the arrays, signalling that there may have been some bias in the amplifications used to generate the cloned libraries. On the other hand, cloned libraries detected novel bacterial types, something that is not possible with microarrays, where detected species must always match pre-selected oligonucleotide targets linked to the arrays. De Santis *et al.* (2005) found that the bacterial types detected by microarrays also depended on how vigorous cell disruption was in DNA extraction procedures from the aerosol samples. Gentle extractions based on short bead-beating times favoured detection of Mycoplasmatales and Burkholderiales, whereas high bead-beating times favoured Vibrionales, Clostridiales, and Bacillales. This is not surprising, since the former group contains many taxa with notably thin cell walls and the latter group contains endospore-formers and other bacterial types generating thick-walled cells. As mentioned above, fungi detected in the same study tended to be best detected with the long bead-beating times used in vigorous disruption. Various potential biases influencing results of cloned library and microarray studies for complex microbial communities have been reviewed by Avarre *et al.* (2007).

An overview of the diversity of types in air samples was obtained in some studies by means of multi-species DNA profiling techniques such as automated-ribosomal intergenic spacer analysis, A-RISA (Maron *et al.* 2005), single-strand conformational polymorphism (SSCP) profiling (Moletta *et al.* 2007), fluorescent heteroduplex profiling (Merrill *et al.* 2003), DGGE (Nehme *et al.* 2008) and terminal restriction fragment length polymorphism analyses, T-RFLP (Després *et al.* 2007). Such techniques tend to generate one characteristically sized DNA band per species present in

a multi-species sample; the analysis thus gives a fingerprint of the entire collection of DNA-extracted, successfully amplified organisms present. Working entirely with the culturable fraction of air samples, Hernlem and Ravva (2007) used flow cytometry to separate viable bacterial cells from other particulates mixed with them in a cyclone-sampled outdoor air sample, and then identified cultures using 16S rDNA sequencing.

VIRUSES

Genetic material, DNA or RNA, makes up a much larger component of viruses than it does in other microorganisms, and the physical and chemical shielding that walls this genetic material off from the outside environment is often easily removed. Viruses thus tend to be very accessible to molecular technologies based on the study of nucleic acids. RNA-based viruses are more difficult to work with than DNA-based viruses because of the chemically labile nature of RNA, and the need to make complementary DNA (cDNA) copies of the genetic material prior to conventional analyses.

The PCR-based study of viruses in air began with the detection of varicella zoster virus (VZV) and cytomegalovirus (CMV, also known as human herpes virus 5, HHV-5) in health care settings (Sawyer *et al.* 1994, McCluskey *et al.* 1996, Suzuki *et al.* 2004). These are both relatively robust DNA viruses in the herpesviruses group. RNA viruses in indoor office air, specifically rhinoviruses causing common cold, were tackled by Myatt *et al.* (2004). These authors used special lysis buffer with carrier RNA to rinse filters, and then converted the captured viral RNA to cDNA with the reverse transcriptase enzyme. A qPCR technique for aerosols of another important RNA virus, influenza virus, was developed by Blachere *et al.* (2007) using the NIOSH miniature cyclone samplers.

Veterinary scientists have taken a leading role in aerosolized virus studies, particular in studies of the recently emerged porcine reproductive and respiratory syndrome virus, PRRSV. Quantitative reverse-transcriptase PCR has been the gold standard in these studies, coupled with impinger air sampling by Hermann *et al.* (2007) and Micro-Tek centrifugal sampling into minimal viral medium by Cho *et al.* (2007).

Several relatively recent crises have also strengthened molecular biological investigation of viruses in aerosols. For example, filters and real-time PCR (rt-PCR) have been coupled with multiplexed immunoassays in an automated pathogen detection system (APDS) designed to monitor air for viruses potentially disseminated by bioterrorists (Hindson *et al.* 2005). The same system was also set up to detect bacteria and toxins. The severe acute respiratory syndrome (SARS) outbreak resulted in PCR-based studies on aerial dissemination of the virus (Xiao *et al.* 2004, Booth *et al.* 2005). Concern about avian influenza led to development of specific rtPCR techniques (Payungporn *et al.* 2004), but there has been very little work done specifically on testing aerosols for these viruses.

Aerosol methods have been developed in studies on viruses in human breath. Techniques have ranged from breathing directly into PCR reaction tubes for detection of HHV-6 (Kelley *et al.* 1994) to use of individual mask samplers for cold viruses (Huynh *et al.* 2008) and of Teflon filters for quantitative PCR of influenza virus coupled with employment of an optical particle counter to measure exhaled particle concentrations (Fabian *et al.* 2008). Some of these techniques aimed at specific types of virus may suggest ways of improving the methods used for studying the overall virus load of aerosol samples.

More general studies have also been conducted, such as that of Sigari *et al.* (2006) based on PCR detection of a number of viruses - especially enteroviruses and reovirus - from aerosols around a sewage treatment facility. The most generalized detection of viruses, however, lies in the metagenomics studies mentioned above, particularly the study of Dinsdale *et al.* (2008). In this study, viral DNA only, not RNA, was isolated from the smallest-size fraction of a serially filtered environmental sample, and included a wide variety of viruses, phages and prophages. This was carried out for 42 distinct sites including habitats like soil, hypersaline water, marine water, fresh water, microbialites (stromatolites, thrombolites), terrestrial animal guts and surfaces, corals and mosquitoes. With suitable filtering, the techniques would be readily adaptable for air as well as for RNA viruses in the airborne particulate material.

MALDI-MS TECHNIQUES

Matrix-assisted laser desorption/ionization (MALDI) is a mass spectrometry (MS) technique that desorbs thermolabile, non-volatile organic compounds including protein, peptide, and glycoprotein biomarkers in addition to oligosaccharides, oligonucleotides and large organic molecules (Claydon *et al.* 1996, Fenselau and Demirev 2001). Unlike other conventional ionization methods that register biomarker

ions in a narrow m/z (mass to charge ratio) range, MALDI is a 'softer' methodology in which sample molecules fragment and ionize following bombardment with a laser light. This method allows the analysis of large biomolecules that were not possible to detect using previous MS methods.

Samples are prepared by pre-mixing with a highly absorbing matrix that enables the transfer of laser energy into excitation energy (Domin *et al.* 1999). This process creates gas phase ions from the surface of the mixture; these ions are then pulsed into a vacuum chamber flight tube (Claydon *et al.* 1996). Positively or negatively ionized biomolecules can be generated and these can be analyzed with a time-of-flight (TOF) mass spectrometer equipped with an ion mirror that deflects ions by means of an electric field. Ion mirrors increase the ion flight path and thereby increase the resolving power of the method to measure masses from 2-40 kDa. For a typical MALDI-TOF MS analysis, mass spectra are expressed as a series of peaks that correspond to individual peptides. Each mass spectrum that is acquired using MALDI-TOF MS corresponds to a molecular fragment that has been released from the cell surface during laser desorption (Edwards-Jones 2000) The development of MALDI-TOF MS has provided a unique method for identifying individual microorganisms by rapidly producing representative spectra (Cain *et al.* 1994, Claydon *et al.* 1996, Domin *et al.* 1999, Fenselau and Demirev, 2001) and these can subsequently be used to discriminate between microorganisms within minutes of sample acquisition.

MALDI-TOF MS has been developed as a rapid method to analyze various biomolecules from both Gram-positive and Gram-negative bacterial cell extracts (Claydon *et al.* 1996, Welham *et al.* 1998, Claydon 2000). This technique has also been performed on whole cell extracts and intact cells (Krishnamurthy and Ross 1996, Welham *et al.* 1998, Lynn *et al.* 1999, Lay 2001, Edwards-Jones *et al.* 2000). The detection limits of MALDI-TOF MS for bacterial material are as low as 1×10^3 cells. Bacteria in vegetative growth (without spores) usually contain 20-60 peaks in the mass range 1-25 kDa. Characteristic mass spectrum peaks have been characterized for bacteria belonging to the Enterobacteriaceae (Lynn *et al.* 1999), *Mycobacterium tuberculosis* (Hettick *et al.* 2004) and *Staphylococcus aureus* (Edwards-Jones *et al.* 2000), but mass ranges may extend up to 40kDa (Welham *et al.* 1998). Larger mass ranges may improve the diagnostic discrimination of analyses performed on bacterial isolates. In these studies, Gram-negative bacteria from the Enterobacteriaceae (Welham *et al.* 1998, Claydon 2000) produced a range of characteristic biomarkers that could be used for identification. However, the presence of more than one bacterial species in a sample was shown to alter the mass spectral profiles, owing to different growth rates and production of interference-competitive metabolites by the bacteria (Cain *et al.* 1994). The identification of species-specific biomarkers is required to successfully differentiate between bacteria in a sample where mixed populations occur, such as normally unsterile body tissues (e.g. lung or epidermal tissue), contaminated water sources or air samples. Identification using specific biomarkers in heterogeneous samples has recently been achieved in the detection of *Escherichia coli* (Siegrist *et al.* 2007) and *Bacillus* spores (Pribil *et al.* 2005).

MS-based identification of microorganisms has mainly focused on pathogenic bacteria, and comparatively little has been published on MS-based strategies for fungal identification. The applications of physicochemical identification of fungi using MALDI-TOF-MS have recently been realized. The fungal kingdom includes diverse lineages of saprobic heterotrophs that are important biohazards in food-processing, agriculture and health. Traditional methods of identification involve subculturing inoculum onto nutrient media and identifying the resulting culture semi-subjectively according to the determinations of a trained microbiologist. In the clinical setting, this has proved laborious, time-consuming, and, with difficult species, error-prone. Quite often, cultures fail to develop observable phenotypic structures required for subjective classification. The advent of immunodiagnostic and molecular techniques has greatly improved diagnostic intervals for a few important pathogens such as *Cryptococcus neoformans* and *Coccidioides posadasii*. Welham *et al.* (2000) demonstrated the feasibility of identifying a variety of fungi via rapid MALDI-TOF MS-based procedures. Although fungi were shown to produce less complex spectra than bacteria, the results of analysis could still be used for discrimination. Unlike traditional methods, morphologically undistinguished hyphae and other simple structures could be used to make up sample preparations that could still be successfully identified (Hettick *et al.* 2008a,b). This capacity for rapid identification has generated much research attention for the further optimization of MALDI-TOF MS as a diagnostic method for fungal pathogens in the clinical setting (Hettick *et al.* 2008a,b).

For fungi, quality control studies have demonstrated that reproducible mass spectra are obtainable

with various *Aspergillus* spp. when mass spectrometric matrices such as α-cyano-4-hydroxycinnamic acid and sinaptic acid are used (Li *et al.* 2000). The spectral profiles of these species are thought to be glycoprotein-based since much of the fungal cell wall consists of polysaccharides. However, smaller concentrations of proteins, lipids and polyphosphates are also present (Welham *et al.* 2000). These structural characteristics produce distinctive spectra in the mass range 2-13 kDa. Such spectral profiles have been used successfully in the food processing and agriculture industries to distinguish aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *A. parasiticus* (Li *et al.* 2000). Medically important *Aspergillus* spp. implicated in invasive disease have also been shown to have highly reproducible mass spectral fingerprints (Hettick *et al.* 2008a). Similar results have been shown for *Penicillium* spp. contaminating fruit surfaces (Li *et al.* 2000). The specificity of MALDI-TOF MS has stimulated interest in developing the methodology as a diagnostic tool for various fields including the identification of crop contaminants and the diagnosis of invasive fungal disease.

Reproducibility has been demonstrated for a range of sampling and culture conditions for bacteria and fungi. However, maintaining constant culture conditions is pivotal for the production of reproducible spectral profiles (Parisi *et al.* 2008). Variations in growth conditions, nutrient medium and growth time can strongly influence spectral reproducibility (Parisi *et al.* 2008). Standardization of sample preparation methods will enable the future development of algorithms that can be used to discriminate species in 'unknown' samples. This has been demonstrated successfully with the identification of various pathogenic bacteria (Hsieh *et al.* 2007) such as *Mycobacterium tuberculosis* (Hettick *et al.* 2006) and various *E. coli* types (Arnold and Reilly 1998, Bright *et al.* 2002). Although fungal cells at different stages of their lifecycle have been shown to have a similar mass spectra (Hettick *et al.* 2008a,b), a good statistical analysis of spectral stability over the life cycle has yet to be made for any fungus.

Other factors that require standardization include the solvent extraction system and mass spectrometric matrix used. These items may influence the quality of the mass spectral profiles and may significantly affect the quality of the data obtained. To date, there is no universal method recognized for preparing microorganisms for MALDI-TOF MS analysis. Some laboratories prepare samples with extensive washing steps to remove contaminants arising from the growth medium, whereas others use a variety of different matrices and solvents (Domin *et al.* 1999). Matrices such as 2-(4-hydroxyphenylazo)benzoic acid and 2-mercaptobenzothiazole have been shown to yield the best spectral profiles and the best mass range of tested matrices (Domin *et al.* 1999).

Proteins may be released and made available for analysis by physically disrupting fungal or bacterial spores using sonication. This process, in addition to the use of an acid solvent, further lyses the microbial components and releases intracellular proteins that yield additional mass spectra. Intracellular lysates have been successfully used to discriminate between various bacteria (Cain *et al.* 1994) and fungi (Hettick *et al.* 2008a,b). In contrast, the presence of melanin has been shown to interfere with MALDI-TOF MS analysis. It has been hypothesized that the negative ions associated with melanin suppress the ionization that is needed to produce observable mass spectra. Heavily melanized fungi such as *Stachybotrys chartarum*, *Aspergillus niger*, *Alternaria alternata* and *Epicoccum nigrum* produce limited mass spectral profiles that do not enable reliable discrimination.

Although MALDI has emerged as a suitable physicochemical and immunodiagnostic technique for the analysis and identification of environmental microorganisms, the feasibility of this technique for the detection of bioaerosols remains questionable. Species specific biomarkers could be used to discriminate between bioaerosols in air samples; however, few studies have assessed the complex diversity of environmentally ubiquitous bacterial and fungal aerosols.

A very important practical consideration lies in the amounts of material required for successful analysis of airborne particulates. The limit of detection for bacterial and fungal materials in MALDI-TOF MS is 1×10^3 spores. In many environments, target microorganisms that may be specific to those environments (mushroom spores, fungal phytopathogen spores, bacteria) tend to occur in low concentrations relative to ubiquitous airborne taxa. The problems of discriminating between target taxa and common bioaerosols are challenging, requiring the development of complex algorithms to identify biomarker spectral profiles. These environmental surveillance considerations have not been explored to date and represent a significant future workload that will have to be completed before MALDI-TOF MS can be used for the detection of geographically localized bioaerosol sources.

IMMUNOCHEMICAL METHODS

A number of additional diagnostic techniques are available for detecting and quantifying a variety of bioaerosols that are associated with pollution plumes. These alternative techniques range from simple traditional methods, such as direct microscopy, to more advanced methods that utilize emerging quantitative immunodiagnostic technologies. Unlike MALDI-TOF MS and PCR, these methods provide a platform for rapid and reliable quantification of certain bioaerosols originating from the environment.

While direct microscopy has been the traditional method used to identify non-viable airborne particles collected on a filter membrane or adhesive backed tape (Flannigan 1997, Prezant *et al.* 2008), it requires a trained microbiologist to examine deposited particles and reproductive structures and classify and quantify them according to their morphological phenotypic features. This technique has several limitations, such as inter-analyst subjectivity and difficulties associated with the identification of incompletely developed asexual fungal spores and simple amerospores, as well as nondescript fungal particulates like hyphal fragments and submicron particulate material derived from fractured cells (Green *et al.* 2006b). Nonetheless, this direct microscopy is widely used in the fungal exposure assessment and indoor environmental remediation fields.

The detection of viable bacteria and fungi in bioaerosols using culture techniques mentioned in Chapters 1.1 and 4.1 is an even more widely utilized method (Flannigan 1997, Prezant *et al.* 2008). However, it takes several hours or days of exponential growth for development of colonies, from which, if they ultimately produce recognizable reproductive structures such as phialides or spores, a trained microbiologist or technician will be able to identify taxa and incorporate this into quantitation procedures. However, successful growth and the emergence of a colony are dependent on the nutrient medium selected, the incubation conditions and the outcome of inter-colony competition. Successful identification then depends on the professional skills of the technician (Prezant *et al.* 2008).

Viable and non-viable methods of direct identification offer an inexpensive alternative to MALDI-TOF and PCR techniques, are widely utilized in the exposure assessment community and are often regulated by professional organizations such as AIHA and ASTM (Prezant *et al.* 2008). Non-viable methods in particular would provide a relatively inexpensive alternative for the identification and quantification of bioaerosol sources that have unique morphology and that are localized within regions expelling pollutants.

The advent of immunological detection technologies has, however, enabled the quantification of various microorganisms without the need for subjective visual assessment of phenotypic features (Trout *et al.* 2004, Schmechel *et al.* 2008). These techniques provide quantification of variously sized particles ranging from whole spores but remain validly interpretable to much smaller detection thresholds that include the measurement ranges of typical submicron biological particulates (Douwes *et al.* 1997, Chew *et al.* 2001, Renstrom 2002, Schmechel *et al.* 2003, Green *et al.* 2005a,b,c, Schmechel *et al.* 2005, Green *et al.* 2006a,b,c, Schmechel *et al.* 2008). The development of monoclonal and polyclonal antibodies (mAbs and pAbs) has been instrumental in achieving assay specificity as well as conferring improved sensitivity (Schmechel *et al.* 2003, 2008). Development of mAbs and pAbs has also enabled the formulation of many detection techniques such as enzyme linked immunosorbent assay, ELISA (Trout *et al.* 2004, Schmechel *et al.* 2008), flow cytometry (Rydjord *et al.* 2007), direct and indirect immunostaining (Popp *et al.* 1988, Reijula *et al.* 1991, Takahashi *et al.* 1993, Takahashi and Nilsson 1995, Portnoy *et al.* 1998, Schmechel *et al.* 2003), western blot (Barnes *et al.* 1993, Cruz *et al.* 1997, Bisht *et al.* 2004), and the halogen immunoassay, HIA (Mitakakis *et al.* 2001, Poulos *et al.* 2002, Green *et al.* 2003, Mitakakis *et al.* 2003, Green *et al.* 2006a,c).

ELISAs in combination with mAbs or pAbs have been widely utilized in exposure assessment studies to quantify various microbial pathogens, bioaerosols, and aeroallergens (Schmechel *et al.* 2008). The method is designed for quantifying various biomolecules such as proteins, peptides, antibodies and hormones. In an ELISA, antigens are immobilized on a solid protein-binding surface, and the antigen is then complexed with an antibody conjugated with an enzyme. Detection is accomplished by incubating the enzyme complex with a substrate that produces a quantifiable product. The quantity of antigen in each sample is then determined by reference to a standard curve of controls.

To date, the development of specific mAbs has not kept up with the diverse spectrum of microorganisms in the outdoor environment (Schmechel *et al.* 2003, Trout *et al.* 2004, Schmechel *et al.* 2008). Many developed mAbs also remain uncharacterized and the extent of cross-reactivity with other common

bioaerosols is often overlooked. Schmechel and colleagues (Schmechel *et al.* 2003, 2008) recently identified the limitations associated with using uncharacterized antibodies. Prior to this study, an anti-*Alternaria* pAb was used in a multi-centre study to determine the concentration of *A. alternata* in homes throughout the United States (Salo *et al.* 2006). *Alternaria* is an important aeroallergen within USA and personal exposure to its spores has been associated with respiratory morbidity (Licorish *et al.* 1985, O'Hollaren *et al.* 1991, Delfino *et al.* 1997, Downs *et al.* 2001, Andersson *et al.* 2003). This commercially available pAb was not studied for cross-reactions prior to its use. In the Schmechel *et al.* (2008) study, it was shown that the pAb cross-reacted with up to 30 different fungi all belonging to the order Pleosporales, which also contains *Alternaria*. These data demonstrated a number of limitations associated with the detection of specific bioaerosols using antibody-based immunoassays, but also highlighted the care that is required when selecting antibodies for the quantification of bioaerosols. Another factor that requires consideration before utilizing mAb- or pAb-based ELISAs is the differential expression of antigens in different stages of the life cycle of the organism. Relatively recently it was shown that the allergen Alt a 1, which was believed to be a spore-based allergen, was actually expressed in considerably higher concentrations following spore germination (Mitakakis *et al.* 2001, Green *et al.* 2003). Based on these findings, it is critical that all samples following collection are immediately processed to avoid any initiation of germination, as this may result in misleadingly amplified results. These considerations are extremely important when interpreting data derived from microbiologically based ELISAs. However, if the necessary steps are taken, ELISAs can offer a sensitive methodology capable of elucidating submicron-sized, morphologically unidentifiable particles that typically elude direct microscopic characterization by a trained analyst.

Following the development of ELISA techniques, Popp *et al.* (1988) developed an indirect immunostaining method that enabled the identification of morphologically nondescript particulates collected on filter membranes. In this method, fungal surface antigens probed with either human antifungal antibodies or mAbs form immune complexes that are immunostained with a fluorescent conjugate. Positive immunostaining results in the fluorescent illumination of the particle that demonstrates the presence of the specific antigen on the particle (Popp *et al.* 1988). Several variations followed the development of this methodology and included a press blotting technique (Takahashi *et al.* 1993, Takahashi and Nilsson 1995) and the HIA (Tovey *et al.* 2000). Of these methods, the HIA has provided some of the most exciting developments including paradigm shifts in the exposure assessment field (De Lucca *et al.* 1999a,b, Poulos *et al.* 1999, De Lucca *et al.* 2000, Razmovski *et al.* 2000, De Lucca and Tovey 2001, Poulos *et al.* 2002, Green *et al.* 2005a,b,c, Green *et al.* 2006a,b, Green *et al.* 2009). Briefly, airborne particulates collected by filtration onto a protein binding membrane are laminated with an optically clear, adhesive-backed coverslip to retain the particles on the membrane. Soluble antigens are extracted from the surface of the particle and immobilized on the protein binding membrane in close proximity to the spore (Green *et al.* 2006b). The extracted native antigens are then indirectly immunostained with either pAb or mAbs or human sera, and the immune complexes are immunostained to form a halo of colour around the particle. This immunostaining technique enables the formation of immune complexes where specific antibodies bind with the respective antigens that are immobilized on the surface of the membrane. Immunostained particles can then be quantified and morphologically identified either by direct microscopy or by dual immunostaining with a mAb or pAb (Green *et al.* 2005a,b,c, 2006a, b, 2009). The HIA has enabled the detection and immunolocalization of aeroallergens derived from cat (De Lucca *et al.* 2000), latex (Poulos *et al.* 2002), dust mite (De Lucca *et al.* 1999a), cockroach (De Lucca *et al.* 1999b), pollen (Razmovski *et al.* 2000) and fungi (Green *et al.* 2005c). Unlike other diagnostic methods, HIA allows the identification of morphologically nondescript particulates that are present in the environment of a patient (Green *et al.* 2005c, Green *et al.* 2006b, Green *et al.* 2009). Depending on the basic research needed to generate specifically characterized mAbs, this technique may become very useful for the detection of bioaerosols locally produced in regions emitting pollutant sources.

In addition, other indirect immunostaining methods utilizing field emission scanning electron microscopy (Sercombe *et al.* 2006) and flow cytometry (Rydjord *et al.* 2007) have recently enabled the rapid detection of microbiological submicron particulate material from air samples. Each of these techniques provides sensitive detection and can quantify picogram quantities of antigen in a sample. The improved detection thresholds and specificity may overcome a number of limitations associated with MALDI TOF-MS and PCR especially as large concentrations of whole

spores are not required for analysis and quantification. The main limitation is that each type of aerosol specifically identified must have its own specific reagent. These techniques are all most easily used in studies involving one bioaerosol type or a few types.

BIOSENSORS

Various other detection methodologies have been recently developed that utilize either immunodiagnostic or PCR techniques in combination with emerging diagnostic technologies. Airborne biosensors, immunosensors, multiplex immunoassays, remote sensing, and pathogen detection technologies are just a few examples that have significantly improved the rapid detection and identification of bioaerosol sources important to homeland security, infection control and occupational exposure.

Biosensors are analytical devices that combine the recognition of biomolecules with electronics for signal measurement (Carlson *et al.* 2000, Rossi *et al.* 2007). For the first time, these advances provide a real-time remote detection platform for the detection and quantification of dispersed biological agents (Ligler *et al.* 1998). Fibre optic biosensors represent the next generation of biosensor technology (King *et al.* 2000). These biosensors enable fibre optic detection of fluorescent signals following the formation of immune complexes in immunoassays. Such biosensors have been successfully deployed in a remote platform for the detection of pathogens, clinical samples, toxins in food samples, pollutants in ground water and aerosolized biological warfare agents (Ligler *et al.* 1998). Recent developments have also improved the portability of fibre optic biosensors. This has lead to the integration of this technology into a remotely controlled, unmanned aerial vehicle (UAV) designed to allow secure, long distance operation of the detection platform. Such methodologies have enabled the remote sensing and rapid detection of very low concentrations of airborne bacterial bioaerosols using simultaneous immunoassays (Dingus *et al.* 2007, Schmale *et al.* 2007).

Antibody-based identification has been exploited in the development of numerous biosensor platforms because of the high sensitivity, specificity and adaptability to field use. Other biosensors have also been developed that enable the detection of microorganisms important in disease diagnosis, pharmaceutical research, agriculture and homeland security. Some of these methodologies include nanoporous silicon biosensors (Rossi *et al.* 2007), quartz crystal microbalance sensory for the detection of influenza (Owen *et al.* 2007), and multianalyte immunoassays based on surface-enhanced Raman spectrometry (McBride *et al.* 2003). Autonomous molecular platforms, such as some autonomous pathogen detection systems that have been developed utilizing orthogonal and multiplexed PCR, are also capable of continually monitoring the environment for biological agents (Hidson *et al.* 2005, Hofstadler *et al.* 2005). The further development of remotely controlled biosensor platforms will have many future applications in the detection of environmental contaminants, in particular pollutants associated with pollution plumes. Again, however, for these techniques to be successful the development of specific antibodies is critical for the specific detection of each target bioaerosol type.

All of these aforementioned methodologies provide an alternative approach to MALDI-TOF MS or PCR techniques for the identification and quantification of plant, algal, bryophyte, fungal and bacterial bioaerosols. The utilization of antibody-based immunoassays enables the rapid and specific detection of many bacterial and fungal species. These methods provide a cost friendly alternative to the other more expensive biochemical and molecular techniques. However, in the interests of this feasibility study, many of the specific organisms have not been fully characterized immunologically, and antibody-based reagents have not been developed. This is especially the case for many anemophilous plant pollen types, fungal basidiospores and algal propagules. Thus, these alternative techniques may currently only be useful for the detection of a small selection of pathogenic bacteria and fungal spores.

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