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Review

Molecular approaches for the detection and monitoring of microbial communities in bioaerosols: A review

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ABSTRACT

Bioaerosols significantly affect atmospheric processes while they undergo long-range vertical and horizontal transport and influence atmospheric chemistry and physics and climate change. Accumulating evidence suggests that exposure to bioaerosols may cause adverse health effects, including severe disease. Studies of bioaerosols have primarily focused on their chemical composition and largely neglected their biological composition and the negative effects of biological composition on ecosystems and human health. Here, current molecular methods for the identification, quantification, and distribution of bioaerosol agents are reviewed. Modern developments in environmental microbiology technology would be favorable in elucidation of microbial temporal and spatial distribution in the atmosphere at high resolution. In addition, these provide additional supports for growing evidence that microbial diversity or composition in the bioaerosol is an indispensable environmental aspect linking with public health.

Introduction

Microorganisms are ubiquitous in the environment and play key functional roles in nearly all ecosystems (Jaenicke, 2005). Bioaerosols originate from all types of environments, including the atmosphere, soil, freshwater, and oceans, and their dispersal into air is temporally and spatially variable. Airborne dissemination is likely a natural and necessary part of the life cycle of many microorganisms (Morris et al., 2008). Bioaerosols are generally defined as aerosols or particulate matter of microbial, plant or animal origin and includes a wide range of antigenic compounds, microbial toxins, and viruses; the term bioaerosol is often used synonymously with organic dust (Douwes et al., 2003; Peccia and Hernandez, 2006). Bioaerosols are emitted from terrestrial, soil, forest, and desert dust, agricultural and composting activities, urban areas, wetlands, coastal, and marine environments (Gandolfi et al., 2013; Jaenicke, 2005). Modern industrial activities (*e.g.*, waste sorting, organic waste collection, composting, agricultural production, food processing, livestock raising, and

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wastewater treatment systems) also emit large amounts of bioaerosols, resulting in abundant exposure to biological agents (Brodie et al., 2007; Douwes et al., 2003). According to Matthias-Maser et al. (2000), the proportion by volume of biological material among total airborne particulates is 28%, 22%, and 10% in remote continental, populated continental and remote maritime environments, respectively. It has been estimated that 16% to 80% of the mass of primary atmospheric aerosols is from biological sources (Jaenicke, 2005).

The components of bioaerosols range in size. Pollens from anemophilous plants have typical diameters of 17–58 μm, fungal spores are typically 1–30 μm in diameter, bacteria are typically 0.25–8 μm in diameter, and viruses are typically less than 0.3 μm in diameter. Fragments of plants and animals may vary in size. Biological material does not necessarily occur in the air as independent particles. Shaffer and Lighthart (1997) determined that the majority of bacteria at inland sites are associated with particles of aerodynamic diameter greater than 3 μm. Bacteria may occur as agglomerations of cells or may be dispersed into the air on plants or animal fragments, on soil particles, on pollen, or on spores that have become airborne. Bioaerosols are a ubiquitous component of the atmosphere; a large number of these particles are small-sized microorganisms. Airborne bacterial and fungal cells can reach concentrations of ~10^3 and ~10^5 cells/m^3, respectively. Aerosolized bacteria and fungi are present in at altitudes of up to 10–20 km in the troposphere and even altitudes of 20 to 40 km above sea level in the stratosphere (Fahlgren et al., 2011).

Accumulating evidence indicates an important role of bioaerosols in the atmospheric environment (Brodie et al., 2007; Douwes et al., 2003; Georgakopoulos et al., 2009; Peccia et al., 2008). Bioaerosols contribute to atmospheric physical and chemical processes (Fig. 1) (Deguillaume et al., 2008; Jaenicke, 2005). Strong correlations between the variations in atmospheric bacterial community structures over time and the physical and chemical characteristics of air masses have been observed (Fierer et al., 2008; Maron et al., 2005). Ariya and Amyot (2004) suggested that bioaerosols play significant roles in atmospheric chemistry and physics by altering the chemistry of the atmosphere via microbiological degradation, thus modifying the chemical composition of other organic compounds upon collision or contact and driving chemistry at environmental interfaces, such as the air-particle interface. Recent studies have demonstrated that bioaerosols can become attached to ambient particles and have significant climatic effects, acting as cloud condensation nuclei and ice nuclei that can initiate precipitation (Amato et al., 2005; Bauer et al., 2002; Christner et al., 2008; Morris et al., 2008; Sattler et al., 2001). One study determined that approximately 33% of the ice-crystal residues in cloud-condensation nuclei and ice nuclei were biological particles (Pratt et al., 2009).

However, little is known about the composition of atmospheric bioaerosols and how it varies by location or meteorological conditions. Airborne microorganisms are very difficult to assess accurately under field conditions due to factors such as the collection efficiency of the selected sampler (Henningson and Ahlberg, 1994), variations in the robustness of different species of microorganisms, and the difficulty of differentiating strains of the same species (Griffin et al., 2001). The relationship between environmental conditions and bacterial aerial dispersal indicates that microbial compositions could increase the health risk due to pathogens or allergenic components of unclassified environmental bacteria. Bioaerosols may also cause climate change (Brodie et al., 2007). Bioaerosols likely do not survive for long durations due to atmospheric conditions, including wind, moisture, and UV exposure. However, concerns about bioaerosol exposures have increased in recent years because exposure to biological agents in both indoor and outdoor environments has been associated with a wide range of adverse health effects, including respiratory diseases, allergies and even cancer (Douwes et al., 2003; Shelton et al., 2002).

Although modern developments in the fields of microbiology, meteorology, and environmental science have opened up new possibilities for the study of bioaerosols, the field is dominated by a remarkable lack of knowledge and an abundance of speculation. Very few observations have been published comparing the aerial environment with other environments, such as water and soil, and the lack of standard methods, environmental guidelines, and databases complicates the interpretation and comparison of results. Because of this limited microbiological information, the microbial analysis in bioaerosols continues to be explored at high resolution, and new technologies, especially culture-independent approaches, should be exploited for a better understanding of the clinical context of microorganism in bioaerosols. This review provides information on what is currently known about effects of bioaerosols on the public health and describes various sampling and microbial analysis methods to characterize microorganisms.

1. Adverse effects of bioaerosols on human health

Bioaerosol consist of pathogenic or non-pathogenic live or dead bacteria and fungi, viruses, high-molecular-weight allergens, bacterial endotoxins, mycotoxins, peptidoglycans, β(1 → 3)-glycans, pollen, and plant fibers, among other components (Douwes et al., 2003). The best-characterized adverse human health effects of bioaerosol exposure are respiratory symptoms. Lierl and Hornung (2003) observed a strong association between elevated outdoor pollen concentration levels in spring and summer and exacerbations of asthma in children living in the Cincinnati metropolitan area. Ambient fungal spore levels have been correlated with hospital visits for asthmatic symptoms in Canada (Dales et al., 2000). The endotoxin of bacterial bioaerosols has been recognized as an important factor in the etiology of occupational lung diseases including (non-allergic) asthma (Douwes et al., 2003). Isolates of *Escherichia coli*, which are commonly used as an indicator of water quality, were also found in both indoor and outdoor airborne dust in an air quality study conducted in Mexico City (Rosas et al., 1997). This finding suggests that potential pathogenic microorganisms that are fecal-oral pathogens may possibly pose a threat to public health through either airborne or alimentary routes. However, most of these potential pathogens do not cause respiratory diseases in healthy individuals, so inhalation of dust containing them is unlikely to trigger infection (Griffin, 2007; Goudie, 2014).

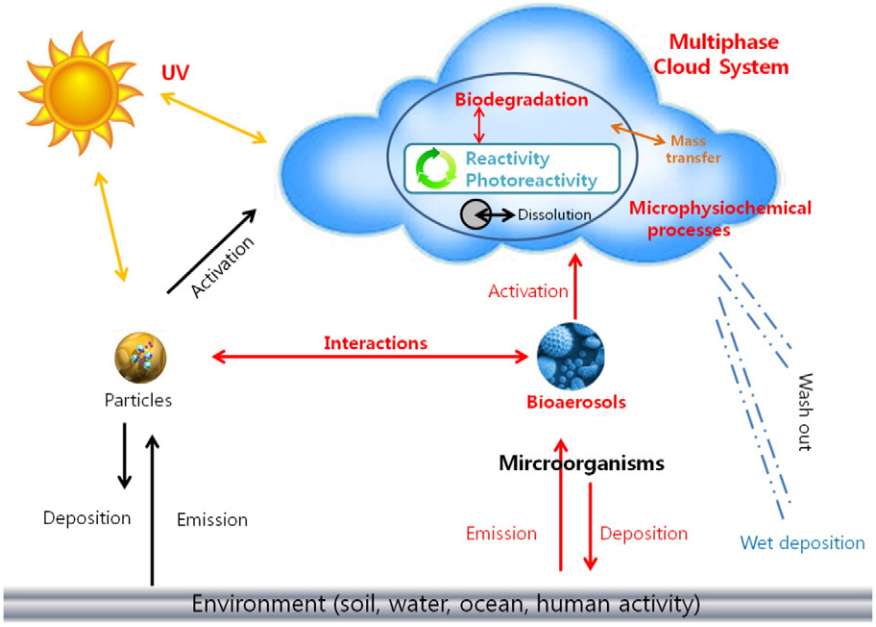

Fig. 1 – Effects of bioaerosol in the atmosphere.

Aeolian dust which is one of the major bioaerosol research areas may originate in many of the world's drylands and have an effect not only on human health but also in downwind environments (Griffin, 2001; Goudie, 2014). They can also transport particulate material, pollutants, and potential pathogens over thousands of km from source. The Sahara, central and eastern Asia, and the Middle East are regarded as the main aeolian dust sources (Griffin, 2001). In some parts of the world, the frequency of aeolian dust occurrences is changing in response to land use and climatic changes, and in such locations the health implications may become more severe (Goudie, 2014). According to previous studies (Griffin, 2007; Goudie, 2014), opportunistic human pathogens, such as *Aspergillus fumigatus*, *Aspergillus niger*, *Staphylococcus gallinarum* and *Gordonia terrae*, have been identified from Aeolian dust bioaerosols. However, there are no reports as yet of human infectious diseases related to long distance dispersal of aeolian dust so far.

Although the adverse effects of bioaerosol exposure on human health are inciting increasing concern, the specific components primarily responsible for these health effects remain unclear. Because microbial species within the same family or genus may differ significantly in pathogenic potential, the identification of bioaerosol allergens and pathogens strictly requires the identification of bacteria, fungi, and viruses at the species or even strain level. In addition, the main mechanisms and roles of biological agents in the development and aggravation of symptoms and diseases have been poorly investigated (Bertolini et al., 2013; Douwes et al., 2003; Cao et al., 2014). Although WHO and US EPA guidelines have established useful methods for identifying microbial hazards, studies of dose–response relationships have been limited, and knowledge about threshold values is lacking (with the exception of a few agents) (Douwes et al., 2003; Gandolfi et al., 2013; Peccia et al., 2008). These knowledge gaps are mainly due to the lack of suitably accurate, quantitative, and comprehensive exposure assessment methods to detect and quantify the presence of microbial pathogens in bioaerosol (Brodie et al., 2007; Douwes et al., 2003; Gandolfi et al., 2013). The assessment and characterization of bioaerosols are important in environmental applications; the level of airborne microorganisms is routinely quantified in terms of the number of culturable microorganisms in a sample. The most significant impacts of bioaerosols are as causative agents of human disease. Increasing international, aerosolized releases of pathogenic bacteria have motivated substantial technological advances in pathogen detection using molecular methods (Kuske, 2006; Peccia et al., 2008; Tringe and Hugenholtz, 2008).

2. Bioaerosol detection and monitoring

2.1. Sampling methods

Current bioaerosol research is primarily focused on the monitoring and control of ambient or target bioaerosols. The effective monitoring of bioaerosols requires efficient collection of microorganisms from the air. An appropriate technique for air sample analysis must also be selected (Alvarez et al., 1995). A wide variety of bioaerosol sampling methods are available, and no standard protocols have been established. A number of sampling devices have been developed for particulate matter (PM) sampling (Gandolfi et al., 2013; Peccia and Hernandez, 2006). These devices have both advantages and disadvantages, and the choice of appropriate sampling method is mainly determined by the objectives of the study (Fahlgren et al., 2011). An accurate sampling site location is also critical for data

analysis. Three principal sampling methods (filtration, impaction, and liquid impingement) are widely used for bioaerosol collection.

Filtration is one of the most widely used atmospheric bioaerosol sampling methods. Filtration involves pumping air through a porous membrane filter to capture bioaerosols. The advantages of this method include suitability for both culture-dependent and -independent studies; low cost and portability; and highly efficient trapping of microorganisms larger than the pore size on the filter surface. Filters composed of cellulose, nylon, polycarbonate, or glass fibers with pores of 0.02 μm are commonly used for collection. The airflow rates of filtration usually range from 300 to 1000 L/min (Griffin, 2007; Peccia and Hernandez, 2006). The efficiency of filters of various pore sizes composed of a variety of materials is typically greater than 95% for particles as small as 0.035 μm in diameter (Lee and Mukund, 2001). Thus, filtration allows the efficient collection of a wide variety of bioaerosols, from bacteria, fungi and pollens to airborne viruses. The bioaerosols that are captured by filters remain viable and may even grow on the filters by absorbing moisture and nutrients from air, such as from organic dust. These bioaerosol components can reside on filters and can be re-suspended in the air due to reverse airflow caused by a temporary reversal of the surrounding pressure or by breakdown and maintenance of the filters. Spore-forming microorganisms may be preferentially recovered, depending on the filtration time and filter size and type. Therefore, new filter materials that are capable of resolving the problems associated with deposited bioaerosols are needed.

Impaction involves the use of an air pump to capture air over the surface of a Petri dish (cassettes and strips are also used) containing nutrient agar. The airflow over the nutrient agar is controlled by slits or holes that are arranged to distribute the airflow evenly over the agar surface and, in some cases, to control particle size ranges. The airflow rates of impactors usually range from 10 to 700 L/min (Griffin, 2007). Spore traps, cascade impactors with glass plates, and MOUDI impactors have all been successfully used to collect fungi and bacteria for subsequent olymerase chain reaction (PCR) analysis (Calderon et al., 2002; Schafer et al., 2003). The benefits of impactors are ease of use, portability, cost, and the ability to assess culturable populations of bacteria and fungi per volume of air. However, the disadvantages of impactors include loss of viability due to impact stress, low sample volumes due to low flow rates, and loss of recovery efficiency due to the failure of microorganisms to adhere to the agar surface (Griffin, 2007).

Bioaerosols can also be captured by impingement of microorganisms in a liquid matrix. A widely used liquid impinger is the AGI-30, which utilizes a low flow rate to bubble air through a liquid matrix. The AGI-30 is a low-cost, efficient method of collecting aerosolized microorganisms for culture-dependent and -independent analyses (Griffin, 2007). Liquid impingers are advantageous for aeromicobiology studies because the liquid matrix can be divided for various analyses, including media culture, direct counts, molecular assays, and cell culture. However, the impinger is constructed of glass and can be easily broken in field studies. The drawbacks of liquid impingement include the low capture rate of some low-flow-rate impingers, high cost, loss of collection fluid to evaporation and violent bubbling, the low capture rate of virus-sized particles, and loss of viability (Agranovski et al., 2004).

2.2. Culture-dependent methods to identify airborne microorganism

Aerobiology studies have traditionally focused on the collection of bacterial cells and the analysis of samples by total count and culture-based techniques. Although some studies have recently applied culture-independent methods, the potential differences in microbial diversity in the atmosphere compared to growth in agar media and the factors that influence the composition of microbial populations (Peccia and Hernandez, 2006). Standardized methods (*e.g.*, ISO methods) are usually considered the reference analytical methods for official controls. Traditional culture methods have typically been established by standard methods to identify microorganisms and microbial hazards. Traditional culture uses selective liquid or solid culture media to grow, isolate, and enumerate the target microorganism and simultaneously prevent the growth of other microorganisms that may be present.

However, conventional culture-dependent methods have some limitations for environmental studies. Total count enumeration methods are laborious, and the identification of microorganisms is problematic. Culture-dependent methods assume that the organisms will grow and produce classic characteristics within a specified period. However, microorganisms that are not culturable under the specific growth conditions imposed in the laboratory remain undetected may induce adverse health effects. The stress of aerosolization and sampling may also result in a loss of culturability. These losses are difficult to assess and may vary within and among species. In addition, culture-dependent methods can take several days to weeks to perform. The majority of cells are believed to be viable but do not form colonies on agar plates (Peccia and Hernandez, 2006), and cell debris, dead microorganisms and microbial components, which may also have toxic and/or allergenic properties, are not detected (Douwes et al., 2003; Griffin, 2007; Tringe and Hugenholtz, 2008). Therefore, rapid, accurate techniques for monitoring airborne microorganisms are absolutely needed to overcome the constraints of traditional culture-based methods.

2.3. Culture-independent methods to identify airborne microorganisms

Culture-independent methods have been used to assess microbial diversity, increasing the specificity of bacterial identification and the sensitivity of environmental studies. Culture-independent-based analyses have recently been applied to various areas of airborne microbiology (Brodie et al., 2007; Hughes et al., 2004; Kuske, 2006; Maron et al., 2005) and have revealed a greater diversity of airborne microorganisms compared to traditional methods such as culture-dependent methods (Brodie et al., 2007; Lee et al., 2009). The sensitivity, specificity and rapidity of molecular techniques have also led to their use for bioaerosol monitoring in the determination of air quality and the detection of airborne pathogens (Alvarez et al., 1995; Bibby et al., 2010; Buttner et al., 2002; Han et al., 2012).

A standard method for the detection of microorganisms in environmental samples is the PCR assay. PCR enables the *in vitro* enzymatic synthesis of specific DNA sequences using thermostable DNA polymerases. PCR uses oligonucleotide primers that are usually 20–30 nucleotides in length and whose sequences are homologous to the ends of the genomic DNA region to be amplified. PCR-based approaches are promising because the organism is detected by amplifying the target rather than the signal and is therefore less susceptible to false positives. A target DNA can be amplified 1-million-fold in less than 1 hr, with a theoretical sensitivity of a single target pathogen (Batt, 2007). PCR is therefore widely used to identify pathogens in water and food, forming the basis for detection systems utilizing nucleic acids. In addition, PCR can be used to enhance the sensitivity of nucleic acid-based assays. PCR-based analysis holds enormous potential for describing the biological fraction of atmospheric and indoor aerosols. The specificity, sensitivity, and reduced processing time of this method are suitable for applications in aerobiological monitoring for the detection of small numbers of target microorganisms. These developments will advance aerosol science and engineering by adding detailed biomass information to the descriptions of PM_{10} and $PM_{2.5}$ organic fractions, tracking infectious and allergenic materials in public health studies, and facilitating the elucidation of the microbial ecology of the atmosphere (Peccia and Hernandez, 2006). Following the experience of aquatic and soil microbial research, the application of these culture-independent methods in air will broadly expand the diversity of microorganisms and other biological material that can be detected, identified, and quantified (Peccia and Hernandez, 2006).

Other molecular tools, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP), are also increasingly applied to perform post-PCR analysis in bioaerosol studies (Georgakopoulos et al., 2009; Lee et al., 2009; Li et al., 2010; Negrin et al., 2007; Nehme et al., 2008). Biochemical assays such as enzyme-linked immunosorbent assay (ELISA) and limulus amebocyte lysate (LAL) have also been applied in bioaerosol studies (Reponen et al., 2011). Until recently, to overcome the limitations of traditional culture-dependent methods, culture-independent methods such as Sanger sequencing, DGGE, and T-RFLP were used to estimate microbial community diversity in most bioaerosol studies (Jeon et al., 2011; Lee et al., 2009, 2010; Maki et al., 2010; Maron et al., 2006). However, the ability of these techniques to investigate microbial community structure in detail is limited. Sanger sequencing scales are poorly in time and cost for the direct sequencing of large genes in a diagnostic setting. T-RFLP may misidentify polymorphisms of the same sequence as novel and tends to return many unknown sequences from samples because only a small percentage of 16S rRNA gene sequences have been recorded in databases. In addition, T-RFLP estimates of diversity can be influenced by sequence composition (Blackwood and Buyer, 2007; Gelsomino et al., 1999; Kisand and Wikner, 2003; Ronaghi, 2001). DGGE is inadequate to evaluate the prevalence and diversity of rare bacteria because the detection limits for specific bacterial groups are fairly high. Consequently, DGGE can easily miss entire rare groups when bacterial primers targeting a wide taxonomic range of bacteria are used (Gelsomino et al., 1999; Hirsch et al., 2010).

More recently, alternative molecular techniques such as fluorescence *in-situ* hybridization (FISH) or flow cytometry (FCM) have been applied to bioaerosol samples for determining viability, because information on microbial abundance and viability is necessary to estimate their influences on public health, downwind ecosystems and atmospheric phenomena (Moter and Göbel, 2000; Wagner et al., 2003; Chen and Li, 2005; Griffin, 2007). FISH enables the detection of not only culturable but also unculturable microorganisms and can therefore help to understand the actual microbial pollution of the environment (Moter and Göbel, 2000; Wagner et al., 2003). As this technique allowing simultaneous visualization, identification, enumeration, and localization of individual microbial cells, FISH is useful for many applications in all fields of microbiology (Bertaux et al., 2007). FCM combined with a fluorescent technique can differentiate between biotic particles and abiotic particles, as well as determine the different physiological status of microbes. Although FCM combined with fluorescent dyes has been widely applied to assess the total concentration and viability of both prokaryotic and eukaryotic microorganisms in aquatic environments, FCM technique has not yet been extensively used to access bioaerosols (Chen and Li, 2005; Liang et al., 2013).

2.4. *Real-time quantitative PCR*

Real-time quantitative PCR (qPCR) has been widely used in microbial ecology to quantify the abundance and expression of taxonomic and functional gene markers in various environmental samples (Smith and Osborn, 2009). Accurate quantification of the target DNA over several orders of magnitude is possible because qPCR permits the quantification of targeted DNA samples by continuous simultaneous detection and quantification of the signal emitted by the fluorescently labeled amplified product during the PCR reaction (Aw and Rose, 2012). qPCR results can be obtained faster, within a few hours, and with less variability compared to PCR-based assays due to the greater sensitivity of fluorescence detection and the elimination of post-PCR detection procedures (Aw and Rose, 2012). In addition, qPCR has greatly increased the speed and sensitivity of PCR-based detection methods (Hanna et al., 2005). Therefore, qPCR is useful for the investigation of gene expression, viral load, pathogen detection, and numerous other applications.

Due to significant improvements in techniques, qPCR is now recognized as a standard method for the identification of water- or food-borne bacterial, viral and protozoan pathogens (Aw and Rose, 2012). qPCR has considerable advantages over conventional PCR-based detection methods, including higher sensitivity and specificity, faster detection, no post-PCR analysis, minimization of bias and contamination during the PCR process, and the capability to provide quantitative results (Aw and Rose, 2012). Dual-labeled fluorescent probes, such as the TaqMan probe, are the most popular for detecting pathogens in environmental samples due to their higher specificity (Aw and Rose, 2012). The advantage of this method is that the target amplicon is verified by the probe, which recognizes internal amplicon sequences. Inclusion of an internal control in each sample to monitor the efficiency of nucleic acid extraction and reverse transcription (for RNA

targets) and the presence of PCR inhibitors is essential for the accuracy of quantitative results, particularly for complex environmental matrices (Aw and Rose, 2012).

An alternative qPCR approach is SYBR green fluorescence. SYBR green fluoresces at low intensity in the absence of dsDNA but at much higher intensity upon binding to dsDNA. When bound to dsDNA, a fluorescent signal is emitted following light excitation. The fluorescence increases after each PCR cycle as the number of amplicons increases. The major advantage of this approach is that SYBR Green can be theoretically used with any primer pair that amplifies only the specific target of interest and generates no other products. Such highly specific PCR products would enable accurate quantification by fluorescence monitoring. However, it is very important to use suitable primer pairs to avoid amplification of non-specific products, which would cause an overestimation of the abundance of the target DNA (Smith and Osborn, 2009). Extensive optimization of the primer concentrations used in SYBR Green qPCR assays may be required to ensure that only the target product is amplified (Smith and Osborn, 2009).

2.5. Microarrays

The extensive development of molecular techniques in the last two decades has greatly facilitated the investigation of microbial communities in environmental samples. Microarrays are powerful molecular tools for parallel, high-throughput detection and quantification of many nucleic acid molecules. Due to the availability of appropriate probe sets, microarrays can be used to detect numerous microbial strains, species, genera or higher clades in a single assay (Bodrossy and Sessitsch, 2004). The major advantage of microarray-based detection is the ability to combine powerful nucleic acid amplification strategies using cross-hybridization with the massive screening capability of microarray technology, which results in high sensitivity, specificity, and throughput. In addition, high-density arrays usually enable reasonable cost, less than US$ 100 per sample (Bodrossy and Sessitsch, 2004). Microarrays can be designed with high-specificity and/or -sensitivity probes against conserved target regions. Therefore, they can be used for both the detection and discovery of genes in various environmental samples (Aw and Rose, 2012).

Although microarrays have successfully been used to analyze gene expression in a large number of environmental studies (Gao et al., 2004; Liu et al., 2003; Mukhopadhyay et al., 2006), there are numerous obstacles to the application of this technique in environmental studies, such as appropriate probe design, periodic updates of the designed probes, coverage of target gene sequences, array specificity, sensitivity and quantitation (Loy et al., 2002; Rhee et al., 2004; Steward et al., 2004; Taroncher-Oldenburg et al., 2003; Tiquia et al., 2004; Wu et al., 2006).

Due to the rapid development of gene databases such as GenBank (http://www.ncbi.nlm.nih.gov/genbank/), high-density phylogenetic and functional micro-arrays can be simultaneously used to understand microbial communities in complex environmental samples (Brodie et al., 2007; He et al., 2007). New microarray-based techniques such as the PhyloChip and GeoChip have recently been used to determine microbial community structure and perform functional gene analyses. The PhyloChip targets the known diversity within the 16S rRNA gene and can simultaneously identify any of thousands of taxa present in an environmental sample (Brodie et al., 2007). The current version (G3) of the PhyloChip allows the simultaneous detection of up to 50,000 bacterial, archaeal and microalgal taxa (Brodie et al., 2007). The GeoChip functional gene array contains approximately 28,000 probes covering approximately 57,000 gene variants of target functional genes assigned to gene categories such as antibiotic resistance, stress, virulence, elemental cycling (C, N, S, and P), metal resistance, organic contaminant degradation, and the diagnostic sequences of soil-borne pathogens (He et al., 2010; Lu et al., 2012). GeoChip technology has been widely used to characterize microbial communities from a variety of environmental samples (He et al., 2010; Lu et al., 2012). Moreover, PhyloChip and GeoChip have been successfully used to monitor bacterial populations during environmental bioremediation, for pathogen detection, and to trace the microbial sources of interesting environmental samples (Brodie et al., 2007; He et al., 2010; Lee et al., 2013; Lu et al., 2012).

2.6. High-throughput sequencing technologies (454 pyrosequencing and Illumina MiSeq, HiSeq)

In the past decade, Sanger sequencing and fluorescence-based electrophoresis methods have been widely used in clinical, environmental, food, and industry applications (Metzker, 2010). Despite technical advances during this era, Sanger sequencing has been association with limitations in many study areas (Metzker, 2010). The rapid development of next-generation sequencing (NGS) technology, such as 454 pyrosequencing and Illumina MiSeq and HiSeq, has enabled a substantial increase in read numbers from PCR amplicons (Bragg et al., 2012; Hamp et al., 2009; Parameswaran et al., 2010; Quince et al., 2011; Wolcott et al., 2009). The revolution in NGS technologies is also reflected in several competing sequencing systems and their rapid advancement (Fig. 2)

Microbial communities in complex environments, including clinical and food science contexts, can now be intensively characterized (Aw and Rose, 2012; Huber et al., 2007; Wolcott et al., 2009). There are two widely applied commercial next-generation DNA sequencing systems: (1) Roche's (454) GS FLX Genome Analyzer, marketed by Roche Applied Sciences; and (2) Illumina's MiSeq and HiSeq sequencers.

As the first high-throughput massive parallel sequencing platform, 454 pyrosequencing uses emulsion PCR of DNA library fragments attached to micro beads. On the new GS FLX Titanium platform, up to one or two million beads, each coated with a clonally amplified DNA molecule, are pyrosequenced in parallel (Metzker, 2010). Continued development of the Roche 454 pyrosequencing platform has increased individual sequence read lengths up to 400–800 bases, and a single run can generate 1 Gb of sequence information as shown in Table 1 and Fig. 2. These longer sequence reads can provide a higher degree of resolution for understanding bacterial community structure (Quince et al., 2011; Wolcott et al., 2009). Minor bacterial populations in environmental samples can also be detected, (Hamp et al., 2009; Parameswaran et al., 2010; Sogin et al., 2006), and no

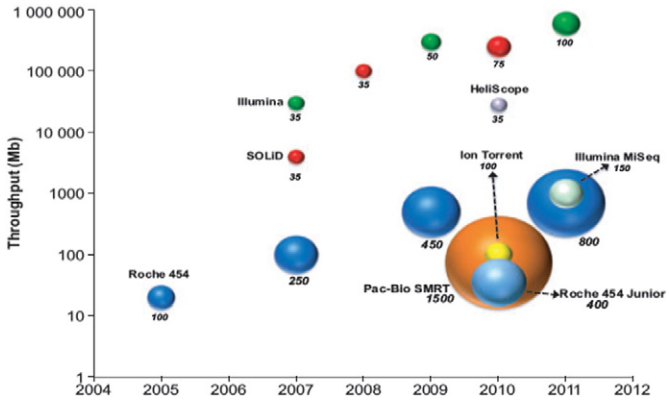

Fig. 2 – Historical development of next-generation sequencing (NGS) technologies. The diameter of each bubble represents the sequencing read length of each platform (Shokralla et al., 2012).

inhibitory substances accumulate during the sequencing process (Ronaghi, 2001). Recent bioaerosol studies (Table 2) have demonstrated that 454 pyrosequencing can successfully determine the diversity, population size, and potential pathogenicity of airborne bacteria based on 16S rRNA amplicons, permitting more definitive phylogenetic classification of individual sequences (Bowers et al., 2011; DeLeon-Rodriguez et al., 2013; Fierer et al., 2012). In addition, because 454 pyrosequencing provides the longest sequence reads, it may be particularly well suited to *de novo* genome assemblies. However, a drawback of this chemistry-based system is inaccuracies in homopolymeric sequence repeats. Insertions are the most common error type in 454 pyrosequencing.

The Solexa/Illumina Genome Analyzer has been widely used in many environmental studies (Bertolini et al., 2013; Degnan and Ochman, 2012; Zhou et al., 2011). This massively parallel high throughput sequencing platform, with throughput in the millions of bases, is similar to the 454 pyrosequencing platform. However, on the Illumina-based platform, clonal DNA clusters are generated by bridge amplification on a glass surface rather than on agarose beads, thus increasing the density and number of DNAs that can be monitored simultaneously (Metzker, 2010). The reversible terminator chemistry of Solexa/Illumina-based sequencing technologies overcomes problems in quantifying the number of bases present in homopolymer repeats (Lu et al., 2012; Quince et al., 2011). Compared to 454 pyrosequencing, the cost of the Solexa/Illumina platform is 10% lower, with higher output (Degnan and Ochman, 2012). Although most previous studies have applied 454 pyrosequencing because of higher throughput on that platform, recent improvements to the MiSeq platform can offer longer read lengths of up to 2 × 250 bases with 8.5 Gb maximum output at relatively low cost. HiSeq sequencing technology has also been upgraded and now provides read lengths of 2 × 150 bases with approximately 200 Gb output. Until recently, the HiSeq platform was the standard approach for whole-genome shotgun sequencing because of its increased read length and output. Now, the MiSeq platform has greater potential for use with 16S rRNA gene sequencing studies because it can provide longer sequence reads, performs at relatively high resolution, and is financially feasible for individual investigators. In addition, a MiSeq-based microbial community analysis protocol was recently developed for the Mothur and QIIME software programs (Caporaso et al., 2011; Kozich et al., 2013). Sequencing of dual-indexed PCR amplicons has also been newly implemented on the MiSeq platform, reducing the number of primer sets required for hundreds of samples (Kozich et al., 2013). MiSeq and HiSeq sequencing have recently been successfully used to characterize microbial communities and to investigate airborne pathogens in bioaerosol studies (Table 2).

2.7. *Metagenomics approaches (16S rRNA amplicon sequencing and whole-genome shotgun sequencing)*

Metagenomic approaches (*i.e.*, 16S rRNA amplicon sequencing and whole-genome shot-gun sequencing) using NGS technologies are appropriate methods to deeply investigate genetic information in mixed environmental microbial communities (Hugenholtz and Tyson, 2008). Metagenomics is the study of microbial communities sampled directly from their natural

Table 1 – Comparison of the performance of next-generation sequencing platforms.

Platform	Template preparation	Read length (bases: bp)	Run time (days)	Output	Cost (per lane)
Roche/454 GS FLX Titanium	Clonal-emPCR	400 bp–450 bp	1–2 day	1 Gb	$1500
Roche/454 GS GLX+	Clonal-emPCR	600 bp–800 bp	1 day	1.7 Gb	$2000
Solexa/Illumina	Clonal Bridge Amplification	75 bp–100 bp	8 day	2.2 Gb	$2000
Illumina HiSeq	Clonal Bridge Amplification	150 bp	8 day	200 Gb	$1500
Illumina MiSeq	Clonal Bridge Amplification	250 bp	1–2 day	8.5 Gb	$1000
ABI SOLID	Clonal-emPCR	75 bp	14 day	300 Gb	$1800
Ion torrent (PGM)	Clonal-emPCR	100 bp	3 hr	1 Gb	$625
PacBio	Single molecule	1000 bp	16 hr	1 Gb	$2000

Table 2 – Summary of the studies of outdoor airborne microbial communities using molecular-based, culture-independent methods and NGS techniques.

Target environment	Sampling method	Sequencing method	References
Agriculture, suburban, forest	Filtration	Pyrosequencing	Bowers et al. (2011)
Agriculture, animal farm	Filtration	Pyrosequencing	Nonnenmann et al. (2010)
African dust	Filtration	Cloning and DGGE	Kellogg and Griffin (2006)
Asian dust	Filtration	DGGE	Maki et al. (2010)
Asian dust	Filtration	Cloning and DGGE	Jeon et al. (2011)
High-elevation site	Filtration	Cloning and Pyrosequencing	Bowers et al. (2009)
High-elevation site	Filtration	Pyrosequencing	Bowers et al. (2012)
Rural	Filtration	Cloning and ARISA	Maron et al. (2005)
Urban, university	Impinger	Cloning	Fierer et al. (2008)
Urban	Filtration	Cloning and Microarray	Desantis et al. (2007)
Urban	Filtration	Cloning and PhyloChip	Brodie et al. (2007)
Urban	Filtration	Cloning	Tringe and Hugenholtz (2008)
Urban	Filtration	Cloning and T-RFLP	Lee et al. (2010)
Urban	Filtration	Pyrosequencing	Franzetti et al. (2011)
Urban	Filtration	Illumina MiSeq	Bertolini et al. (2013)
Urban	Filtration	Illumina HiSeq	Cao et al. (2014)
Upper troposphere	Filtration	Pyrosequencing	DeLeon-Rodriguez et al. (2013)

environment without culturing. This approach can be used to profile taxonomic and functional microbial diversity and composition, and spatial and temporal community changes in response to environmental impacts relevant to human health can be monitored. Therefore, metagenomics approaches are powerful tools for the study of complex microbial communities, particularly investigating overall microbial hazards in bioaerosols. There has been remarkable progress in this field of research due to the advent of low-cost, high-throughput NGS technologies (Hugenholtz and Tyson, 2008). Because approximately 99% of the microorganisms in environments cannot be cultured by current standard methods, metagenomics approaches (as shown in Fig. 3) can open up new opportunities to investigate their community structures, phylogenetic composition, species diversity, metabolic capacity, and functional diversity (Hugenholtz and Tyson, 2008; Tringe et al., 2005). Metagenomics has matured as a new and exciting set of tools making use of advanced sequencing technology. The development of standard techniques will aid in integrating the understanding of microbial communities from various research fields (Tringe et al., 2005; Hugenholtz and Tyson, 2008).

Since the mid-1990s, 16S rRNA gene sequencing has been widely used for the classification of the microbial diversity of complex environmental samples and is one of the first steps in any metagenomics project (Tringe and Hugenholtz, 2008; Shah et al., 2011). The application of 16S rRNA gene sequencing has recently been enhanced by advances in massively parallel DNA sequencing technologies (Fig. 4). NGS technologies, including 454 pyrosequencing and Illumina sequencers, use 16S rRNA amplification primers targeting hypervariable regions (V1–V9). Although the optimal regions for species profiling remain controversial, the best choices of hypervariable regions with the smallest sequencing errors for 454 pyrosequencing (V4–V5) and Illumina MiSeq (V4) have been established. Barcoded 454 pyrosequencing and Illumina MiSeq can produce large volumes of 16S rRNA gene sequences that contain hundreds of thousands of 16S rRNA gene fragments, enabling deep views into hundreds of bacterial communities simultaneously, and have revealed much greater species diversity in many environments (*e.g.*, soil, ocean, air, water, and human). In addition, due to the rapid growth of 16S rRNA gene databases such as SILVA, GreenGenes, and RDP (Ribosomal Database Project), bacterial diversity and populations in various environmental samples can be more easily and rapidly assessed with high taxonomic resolution (Haas et al., 2011; Schloss et al., 2011). Furthermore, 16S rRNA amplicon sequencing has been widely used in primary screening for pathogenic bacterial taxa, leading to the rapid identification of uncultured microorganisms, unique or unusual isolates, and collections of phenotypically identified isolates from environmental and clinical contexts (Chakravorty et al., 2007; Luna et al., 2007; Schmalenberger et al., 2001).

However, 16S rRNA amplicon sequencing using NGS is restricted in its taxonomic resolution and the ability to understand evolution and functional mechanisms at the species level (Shah et al., 2011; Venter et al., 2004). In addition, analysis of 16S rRNA genes is potentially influenced by several artifacts, including chimeric sequences caused by PCR amplification and sequencing errors such as primer mismatches, barcode mismatches, and large homopolymer sequences. Thus, it is absolutely necessary to adopt strategies with less error and bias for analyzing microbial communities based upon 16S rRNA genes from environmental samples.

Alternatively, whole-genome shotgun sequencing of environmental DNA can be used to understand the species composition and diversity of microbial communities. Whole-genome shotgun sequencing is sequences random DNA fragments from various environmental samples (Venter et al., 2004). Organismal DNA is isolated from environmental samples and broken into small fragments that are then sequenced (Fig. 4), usually with NGS technologies such as Illumina HiSeq. As shown in Fig. 4, the fragments are then assembled into larger pieces by identifying overlaps in the sequences using extensively developed assembly programs such as SOAPdenovo, Velvet, CLC assembly, and ALLPATHs-LG. The complete

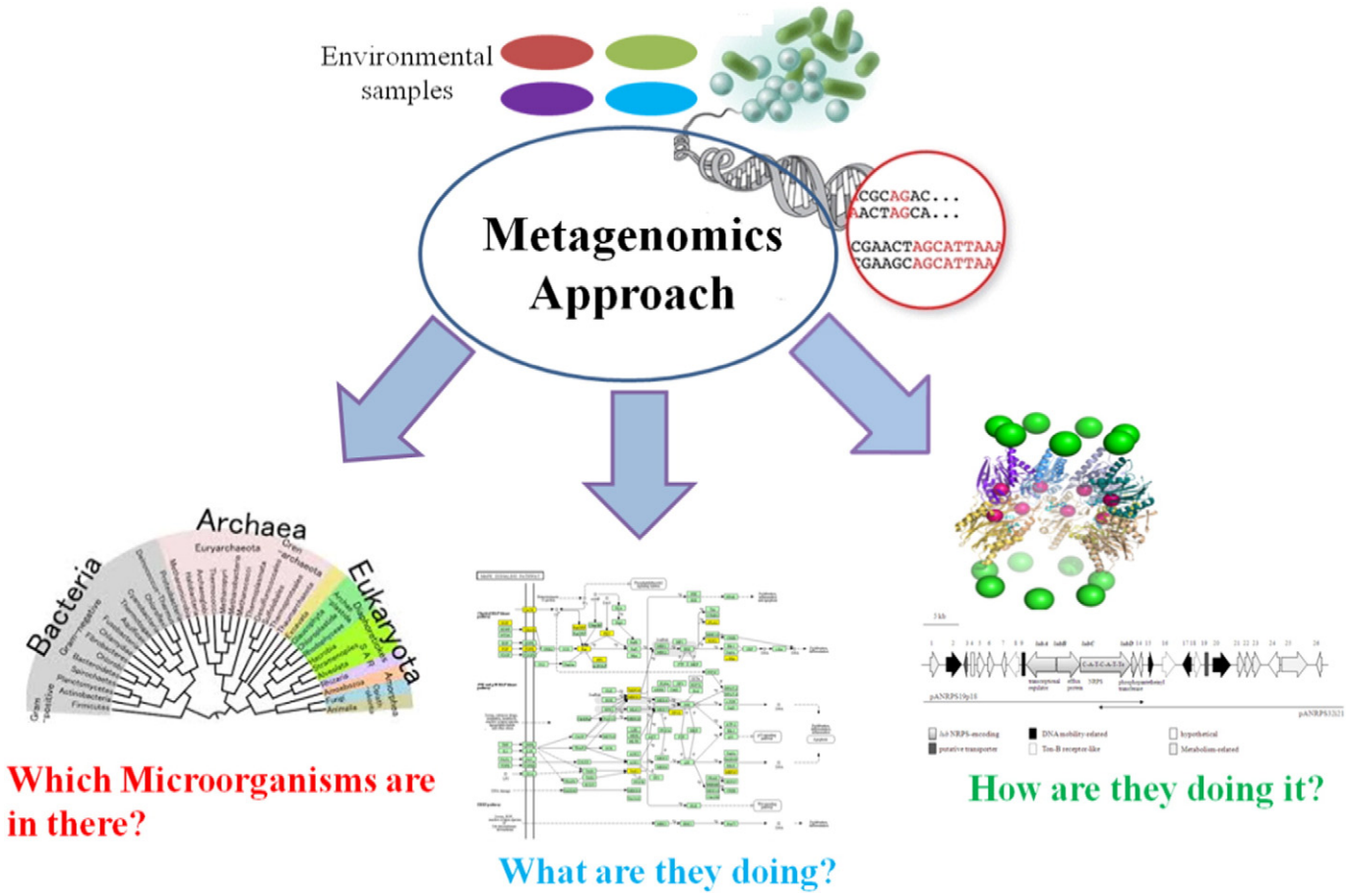

Fig. 3 – Metagenomics approaches are powerful tools for the comprehensive and systematic investigation and classification of environmental samples.

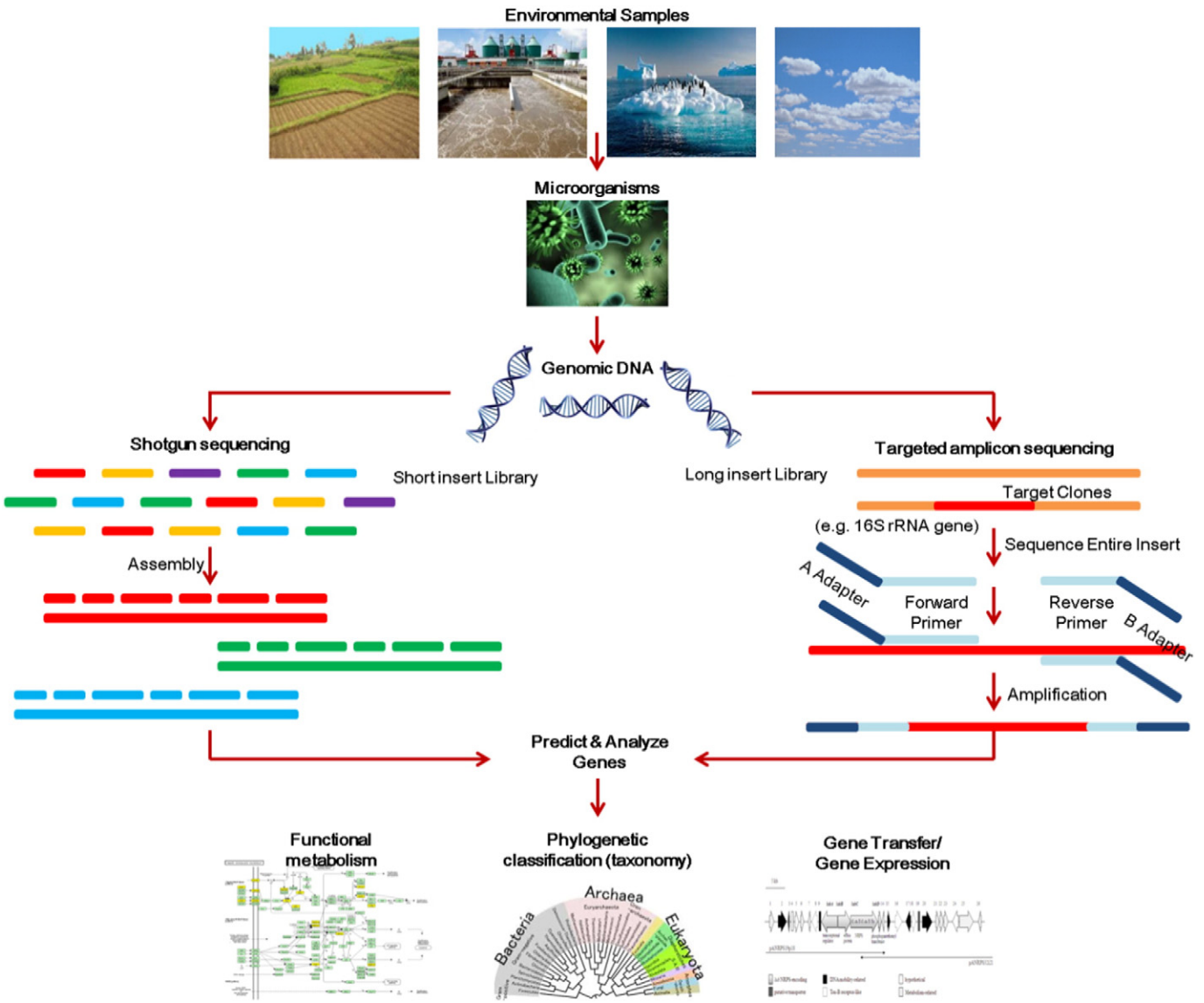

Fig. 4 – Analytical stages and steps for the analysis of metagenomics data from both targeted 16S rRNA amplicon sequencing and whole-genome shotgun sequencing.

genome can be determined by filling the gaps between larger pieces.

This approach has provided a much deeper understanding of some multi-cellular microbes and the environmental context of their evolution compared to that obtained by 16S rRNA amplicon sequencing (Eisen, 2007; Venter et al., 2004). The randomness and extensiveness of whole-genome shotgun sequencing was introduced only a few years ago and has become a suitable method to assess entire microbial community systems such as the human genome, waste water sludge, soil metagenomics, and smog events and for antibiotic resistance gene assays (Cai and Zhang, 2013; Cao et al., 2014; Venter et al., 2001; Zhang et al., 2011). This approach can provide novel and fundamental insights into microbial ecosystems and their impact on the environment (Eisen, 2007; Venter et al., 2004). Metagenomics approaches may be directed at examining microbial compositions and provide relatively unbiased results for not only community structure (species richness and distribution) but also the functional (metabolic) potential of a community (Hugenholtz and Tyson, 2008). Thus, microbial metagenomics is a novel, open field of research, and the application of metagenomics to aerial microbes offers an unconventional approach to explore the great diversity of microbial metabolic capabilities in atmospheric niches. Although whole-genome shotgun sequencing is not subject to the biases and errors inherent to 16S rRNA amplicon sequencing, the relative abundances of entire microorganisms in metagenomics data vary significantly depending on the DNA extraction and sequencing protocols utilized (Hugenholtz and Tyson, 2008; Shah et al., 2011). Furthermore, whole-genome shotgun sequencing still requires very complex analysis processes involving high-resolution assembly, binning, and annotation steps. Despite rapid developments in these processes, assessing their accuracy for real metagenomics data remains challenging due to the lack of references to compare the outputs of each metagenomics study (Thomas et al., 2012). Therefore, a standard database with known reference sequences, such as a 16S rRNA gene database, is urgently needed to comprehensively understand entire microbial community systems.

3. Conclusions

Recent studies of bioaerosols have applied various methodologies with differing scopes and abilities to the elucidation of microbial community structures, depending on the questions being addressed. The most profound impact of bioaerosols is as the causative agent of disease. Recent increases in bioaerosol emissions and concerns about pathogenic bacteria in bioaerosols have motivated substantial biotechnological advances in the molecular methods and approaches used to detect pathogens. Different approaches are used depending on whether the information needed is qualitative or quantitative; specific or general; highly localized or over a broader landscape. The combined powers of selective culture media, multiplexed molecular detection methods, high-throughput sequencing, and new instruments and technologies can improve our capacity to simultaneously detect specific target microorganisms against a complex and variable natural background of atmospheric bacteria/fungi/virus. In addition, these methods can noticeably contribute to our knowledge in terms of the microbial composition, survival and transport of aerosolized microorganisms. Bioaerosol studies are just beginning, and continued new technological advances are absolutely needed to successfully address the currently remaining questions.

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