Seasonal Variability in Bacterial and Fungal Diversity of the Near-Surface Atmosphere

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ABSTRACT: Bacteria and fungi are ubiquitous throughout the Earth’s lower atmosphere where they often represent an important component of atmospheric aerosols with the potential to impact human health and atmospheric dynamics. However, the diversity, composition, and spatiotemporal dynamics of these airborne microbes remain poorly understood. We performed a comprehensive analysis of airborne microbes across two aerosol size fractions at urban and rural sites in the Colorado Front Range over a 14-month period. Coarse (PM_{10-2.5}) and fine (PM_{2.5}) particulate matter samples were collected at weekly intervals with both bacterial and fungal diversity assessed via high-throughput sequencing. The diversity and composition of the airborne communities varied across the sites, between the two size fractions, and over time. Bacteria were the dominant type of bioaerosol particles, we analyzed the bacterial communities in greater detail using a bacterial-specific 16S rRNA gene sequencing approach. Overall, bacterial taxonomic richness and the relative abundances of specific bacterial taxa exhibited significant patterns of seasonality. Likewise, airborne bacterial communities varied significantly between sites and across aerosol size fractions. Source-tracking analyses indicate that soils and leaves represented important sources of bacteria to the near-surface atmosphere across all locations with cow fecal bacteria also representing an important source of bioaerosols at the more rural sites during early fall and early spring. Together, these data suggest that a complex set of environmental factors, including changes in atmospheric conditions and shifts in the relative importance of available microbial sources, act to control the composition of microbial bioaerosols in rural and urban environments.

INTRODUCTION

Bacteria and fungi are abundant in the near-surface atmosphere with thousands to millions of cells per cubic meter of air. These microbial communities are also highly diverse with hundreds of taxa identified in collected aerosol samples. A subset of these cells may be plant or animal pathogens or serve as triggers for allergies and allergenic asthma in humans. There is also increasing evidence that microbial cells may have important influences on atmospheric conditions. For example, the smallest cells can remain aloft in the atmosphere for extended periods of time, sometimes reaching the upper troposphere where they may reflect or absorb incoming sunlight, impact cloud formation, and alter cloudwater chemistry. Despite accumulating evidence that microbes often represent a major proportion of total aerosol particles, both in the near-surface atmosphere and in the upper troposphere, the temporal and spatial variability in airborne microbial diversity has received little attention compared to the large body of research focused on the physical and chemical characteristics of atmospheric aerosols.

One reason that the diversity of airborne microbes has remained understudied (despite many decades of research on the topic) is that only a small fraction of the total diversity of airborne bacteria and fungi can be characterized using culture-based techniques or by direct microscopy. With recent advances in high-throughput sequencing, we can gain unprecedented insight into the diversity and composition of airborne bacteria and fungi. Such work has yielded novel insight into the types of microbes found in the atmosphere and how airborne microbial communities change across time and space, including changes in composition across land-use types.
seasons, \(^{2,23,24}\) and storm events.\(^{9}\) By direct comparison of airborne microbial communities with those found in other microbial habitats, these molecular approaches have also been used to determine the sources of microbes found in the atmosphere. The dominant sources of bacteria to the atmosphere can include environments as varied as soils, water bodies, leaf surfaces, and, in some cases, animal feces,\(^{3,5,9,25}\) with the relative importance of these different sources varying as a function of altitude, season, and location.\(^{2}\) Despite this body of research, our understanding of microbial dynamics in the atmosphere has been hindered, in part, by the paucity of long-term, high-resolution data sets characterizing how airborne microbial communities vary in time and how such variability may be related to changes in atmospheric conditions. There are numerous studies that have used more traditional microbiological techniques (including culture-based analyses or direct microscopy) to quantify how individual bacterial or fungal taxa change in abundance over time,\(^{1,2,6,2,7}\) but as these techniques likely miss a large proportion of the bacterial or fungal diversity in the atmosphere,\(^{2,8}\) the temporal dynamics of whole communities remain understudied. As has been demonstrated in a range of systems, including the human gut,\(^{28,29}\) freshwater systems,\(^{30,31}\) and ocean waters,\(^{32,33}\) sequenc- ing-based analyses of microbial communities across high-resolution time series can provide unique insight into microbial dynamics and the factors structuring microbial communities.\(^{9}\) To our knowledge, the study described here represents the most comprehensive temporal analysis of microbial communities in the near-surface atmosphere to date. We used high-throughput sequencing to characterize both bacterial and fungal communities in the coarse (PM\(_{10-2.5}\)) and fine (PM\(_{2.5}\)) aerosol size fractions above rural and urban landscapes in Colorado, USA, with aerosol samples collected every sixth day for 14 months. This unique data set allowed us to address the following questions: Do microbial communities vary in composition between urban and rural locations? Does microbial diversity and community composition differ across the aerosol size fractions? Is the temporal variability in microbial communities predictable and attributable to changes in atmospheric conditions? What are dominant sources of microbes to the near-surface atmosphere and how does the relative importance of these sources change across seasons?\(^{10}\)

## METHODS

### Sampling Locations and Aerosol Collection

Aerosol monitoring was performed at two locations in Denver and two locations in Greeley, CO. Denver is the largest urban city in the Colorado Front Range (population = 610,345)\(^{35}\) while Greeley, CO, is a smaller city (population = 92,625)\(^{34}\) in a rural location surrounded by agricultural land-uses (including feedlots and cultivated fields) with these monitoring sites having been described previously.\(^{35}\) The Denver monitoring sites included a traffic-influenced residential site in the industrial suburb of Commerce City (ALS) and a residential urban-background site located approximately 5 km west of downtown Denver (EDI). Greeley monitoring sites were both classified as rural–residential, with one located closer to the city center (MAP) and the other located in the suburban fringe (MCA). Two open air cattle feedlots with a combined capacity of 167,000 cows are located approximately 11 km south and 18 km southeast of Greeley.\(^{32}\) Denver sites were located 11.1 km apart from one another, and the Greeley sites were 4.5 km apart.

Medium-volume (50 L min\(^{-1}\)) dichotomous aerosol samplers were located on the roofs of elementary schools at all monitoring sites. Samples were collected every sixth day over 24-h sampling periods spanning 14 months starting in March 2010 as part of the Colorado Cooperative Urban–Rural Sources and Health (CCRUISH) study. Aerosol samplers collected both coarse and fine filter samples with a 50 L min\(^{-1}\) PM\(_{10}\) inlet\(^{34,35}\) and \(\mu m\) cut-point virtual impactor\(^{34}\) with approximately 30 m\(^3\) of air filtered per sampling event.\(^{34}\) All microbial analyses were conducted on 47 mm quartz aerosol collection filters (Tissuquartz, Pall Corp.). Field blanks were collected concomitantly with particle samples, and all quartz filters were baked for at least 12 h at 500 °C prior to use.

### DNA Extraction and PCR Amplification

DNA was extracted from filter punches (1.1 or 2.2 cm\(^2\) filter area) using the PowerSoil\(^{36}\) well DNA isolation kit (MoBio Laboratories, Carlsbad, CA). Filter punches were aseptically loaded into individual wells of the bead beating plate, lysis buffer was added to the wells, and the plates were heated to 65 °C for 10 min followed by 20 min of shaking in the MoBio\(^{36}\) well plate shaker (MoBio Laboratories, Carlsbad, CA). The remaining steps in each extraction were performed according to the manufacturer’s instructions. In total, we extracted DNA from 326 samples (four sites with approximately 40 time points per site with both coarse (PM\(_{10-2.5}\)) and fine (PM\(_{2.5}\)) fractions analyzed per site/sampling time).

We used a multidomain sequencing-based approach to characterize the biological diversity and determine the relative abundances of bacteria, plant pollen, and fungi on the collected filter samples. A portion of the small-subunit rRNA was amplified using a multidomain PCR primer set (515f, 1391r)\(^{2,37}\) that was designed to capture a portion of the small-subunit rRNA gene from Archaea, Bacteria, and Eukarya (including plants and fungi).\(^{40}\) PCR cycling conditions follow those described previously.\(^{40}\) Amplicons were pooled at equal concentrations, and PCR cleanup was performed on the pooled DNA using the UltraClean PCR clean up kit (MoBio Laboratories, Carlsbad, CA). Pyrosequencing of the multidomain PCR products was performed on a 454 Life Sciences Genome Sequencer at the Brigham Young University DNA sequencing facility (Provo, UT) using the GS FLX+ system and the Titanium chemistry.

To characterize the airborne bacteria in greater detail, we used the approach described previously\(^{31}\) that involves amplifying a region of the 16S rRNA gene with primers specific to bacteria and archaea, then sequencing the amplicons on the Illumina HiSeq platform. Briefly, the V4 region of the 16S rRNA gene was amplified with primers 515f/806r that included the Illumina flowcell adapter sequences. The reverse primer also contained a 12-bp barcode, facilitating multiplexed sequencing of partial 16S rRNA genes. PCR amplicons were pooled at equimolar concentrations and PCR cleanup was performed on this pool using the UltraClean PCR clean up kit (MoBio Laboratories, Carlsbad, CA). Sequencing was performed on an Illumina HiSeq2000 sequencer at the Genome Institute at Washington University (St. Louis, MO).

### Sequence Analyses

Multidomain pyrosequences were processed using the QIME pipeline.\(^{42}\) Briefly, raw sequences were assigned to specific samples by identification of the sample specific barcode. Quality filtering of multidomain FLX+ pyrosequence reads was performed in a similar fashion to earlier 454 sequencing projects.\(^{3,4,20,40}\) Sequences were then assigned to phylotypes using UCLUST reference based
Figure 1. Domain level taxonomic distributions (A) and the dominant bacterial phyla (B) over a 14-month time course from aerosol samples collected in Denver and Greeley, CO, further separated by size fraction (coarse PM\textsubscript{10−2.5}, fine PM\textsubscript{2.5}).
**RESULTS**

**General Characteristics of the Collected Bioaerosols.** Bacteria dominated the sequences obtained from the collected bioaerosols, making up nearly 70% of all multidomain pyrosequence reads. Fungi made up approximately 21% of the pyrosequence reads followed by plants (both chloroplast reads and reads assigned to the Viridiplantae), which accounted for 9% of all reads (Figure 1A). Archaeal 16S rRNA genes are also amplified using this multidomain sequencing approach, however only a few archaeal sequences were observed (Figure 1A). Ascomycota was the dominant fungal phylum; making up on average 78% of the fungal sequences while the Basidiomycota represented the remaining fungal sequences (21% of all fungal sequences) (Supporting Information Figure S2). For further details on the specific types of fungi identified in these samples, see Supporting Information Table S1. Because the majority of the reads were classified as belonging to bacteria, we analyzed the bacterial communities at an increased sequencing depth using a bacterial-specific Illumina sequencing approach on all collected air samples. With nearly 10 million quality bacterial sequences, we found that the airborne bacterial communities were highly diverse and variable with the number of phylotypes per sample ranging from 29–690 across all 326 air samples (phylotypes are defined as those sequences sharing ≥97% sequence similarity). The dominant bacterial phyla included the Actinobacteria (22% of all reads), Bacteroidetes (9.7%), Firmicutes (28.2%), and Proteobacteria (where the α-, β-, and γ- subgroups accounted for 11.8%, 7.8%, and 15.0% of all reads, respectively) (Figure 1B). Further details on the specific bacterial taxa identified from these samples are provided in Table S1.

**Geographic Variability.** When comparing the relative abundances of each domain level group (bacteria, fungi, and plants) across the two cities, only the bacteria exhibited significant differences in relative abundances when grouped by city, making up a larger fraction of the Greeley bioaerosols (72% on average) than the Denver bioaerosols (67% on average) (Figure 1A) (P = 0.04). Bacterial richness was significantly higher in the Greeley samples compared to the Denver samples, as determined by comparing the average number of phylotypes per city (266 phylotypes per sample in the Greeley data set versus 205 phylotypes from the Denver data set, P = 0.005). Likewise, the Denver and Greeley airborne bacterial communities were significantly distinct from one another when all samples collected from each city were compared (PERMANOVA P < 0.0001, Supporting Information Table S2). The bacterial orders most responsible for these differences included the Pseudomonadales, Sphingobacteriales, Rhizobiales, Rhodospirillales and the Burkholderiales, which were all significantly more abundant in the Denver samples than in Greeley.

Figure 2. Bacterial orders that were found to have significantly different relative abundances across either cities or aerosol size fractions (ANOVA overall P < 0.05). All sample taxa significantly differed across city and size fraction. The listed bacterial taxa are members of the Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria, and error bars represent 1 standard error of the mean. Superscripts above taxa indicate phylum and/or subphylum: A Actinobacteria; B Bacteroidetes; Fi, Firmicutes; αP, α Proteobacteria; βP, β Proteobacteria; and γP, γ Proteobacteria.
the Greeley samples ($P < 0.05$ in all cases). In contrast, sequences assigned to the *Actinobacteridae*, *Bacteroidales*, *Lactobacillales*, and *Clostridiales* groups were significantly more abundant in the Greeley air samples than in the Denver samples ($P < 0.05$ in all cases) (Figure 2 and Supporting Information Figure S3).

**Differences between Size Fractions.** Samples from Denver and Greeley were further divided into coarse (PM$_{10-2.5}$) and fine (PM$_{2.5}$) aerosol size fractions to determine if aerosol size selection influences microbial community structure. Bacterial to fungal ratios were not influenced by size fraction in Greeley ($P > 0.05$); however, these ratios did differ significantly between Denver coarse and fine samples ($P = 0.04$), where fungi were relatively more abundant in the Denver coarse mode (26% fungi) compared to the Denver fine mode (18% fungi, $P = 0.001$). In contrast, plant sequences (likely from pollen) were more abundant in the Denver fine mode samples than the Denver coarse samples ($P = 0.02$ Denver fine = 10.2% and Denver coarse = 5.9%). The coarse aerosol size fraction in both Denver and Greeley had significantly higher levels of bacterial taxonomic richness than the fine size fraction ($P < 0.001$ for both comparisons), and the coarse and fine bacterial communities were also distinct from one another as determined by the weighted UniFrac pairwise distances (PERMANOVA, $P < 0.001$ for Denver coarse vs fine and $P = 0.02$ for Greeley coarse vs fine). The bacterial orders most responsible for the compositional differences between size fractions included the *Clostridiales* and *Pseudomonadales*, which were observed at significantly higher abundances in the coarse samples ($p < 0.05$). However the relative abundances of these bacterial groups varied less between size fractions than they did between the two cities (Figure 2).

**Temporal Variation in Airborne Communities.** The composition of the bioaerosols collected from Denver and Greeley varied significantly over the 14-month sampling period. Bacteria were typically the dominant domain-level taxon throughout the study period; however, fungal abundances peaked in early spring to late summer, a pattern that was most apparent in the coarse size fraction (Figure 1A). Total bacterial diversity also varied over time, as the number of bacterial taxa per sample peaked in the late summer to early fall, gradually declining to minimum diversity levels during the winter to spring months (Supporting Information Figure S4). The community-level variability was partially driven by the relative abundances of *Actinobacteridae* and the *Pseudomonadales* that displayed roughly opposing patterns, with the *Actinobacteridae* reaching maximum abundances in the late summer and the *Pseudomonadales* reaching their highest levels during midsummer.

The relative abundances of these groups were fit to a sine/cosine function to determine if the observed abundance patterns varied predictably with season. The *Actinobacteridae* exhibited significant seasonal variability using this model in the Denver coarse, Greeley coarse, and Greeley fine samples ($P < 0.05$) (Figure 3A), as did the *Pseudomonadales* in the Denver coarse, Greeley coarse, and Greeley fine sample types ($P < 0.05$) (Figure 3B). As might be expected, these two bacterial taxa were also correlated with shifts in temperature over time (Supporting Information Table S4). To further examine the effect of time on airborne bacterial communities, we used a distance-decay approach to determine if samples collected closer together in time harbored bacterial communities that were more similar in composition compared to samples collected further apart in time. The Denver coarse, Greeley coarse, and Greeley fine sample types each displayed significant time-decay relationships suggesting a gradual decrease in
Figure 4. Relative importance of different environments as potential sources of bacteria to the samples collected from Denver and Greeley in the two size fractions. Bacterial taxa indicative of each of the three sources: soils, leaf surfaces, and cow feces are described in Supporting Information Table S3. Colors represent the three dominant sources: soils = red, leaf-surfaces = green, and cow fecal material = orange. Numbers represent the summed size fractions. Bacterial taxa indicative of each of the three sources: soils, leaf surfaces, and cow feces are described in Supporting Information Table S3. However, given that human and dog feces were not significant bacterial sources (accounting for <1% of sequences) and were therefore not included in Figure 4. Soils and leaves appeared to be a consistent and dominant source of bacteria to the near-surface atmosphere over the majority of the sampling period at both cities. However, the airborne communities in Greeley also appeared to be influenced by cow fecal communities during the early fall and early spring months (Figure 4). Cow feces were only represented by a single indicator group, the Ruminococcaceae family (Supporting Information Table S3). However, given that human and dog fecal samples were included in our current analyses, we can be reasonably certain that the appearance of this cow fecal indicator in the Greeley samples does signify a cow fecal signature in the near-surface atmosphere of Greeley, CO. Possible sources of cow fecal bacteria include two large cattle feedlots located near Greeley, as manure has been shown to be the dominant particle type emitted as PM$_{10}$ from cattle feedlots. 

### DISCUSSION

On the basis of the relative abundance of sequence reads (which is not necessarily proportional to biomass concentrations owing to differences in gene copy numbers and cell sizes), bacteria were the dominant domain level taxon with occasional spikes in fungal and plant taxa over time (Figure 1A). This finding is consistent with the settling velocities of cells from these broad taxonomic groups. For example, bacteria are typically the smallest and should therefore be expected to have longer atmospheric residence times than fungal spores and pollen grains, which may in part explain the high bacterial contribution to the bioaerosols collected here (Figure 1A). These results are also consistent with a recent study examining the microbial taxa of the upper troposphere, where bacteria outnumbered fungi by an order of magnitude. However, by binning major taxonomic groups into size classes to compare atmospheric residence times, we are assuming that cells are freely suspended in the atmosphere, and it is unclear if this assumption is valid or if the majority of cells are attached to larger particles or cell aggregates. The spike in fungal abundances during the late spring to early summer roughly coincides with the beginning of the North American monsoon season in Colorado, when thunderstorms are prevalent. Convective instability indicative of an incoming thunderstorm has been shown to stimulate the emission of highly concentrated fungal plumes, suggesting that fungal spores, bacteria, and other smaller bioaerosols likely ride the upward moving air currents to an altitude where long-range transport and dispersal are favorable. Furthermore Elbert et al. observed that Ascomycota concentrations in the near surface atmosphere increase during and immediately after rainstorms, with two other studies showing that total bioaerosol concentrations (most likely bacteria and fungi) also increase during rain events. These fungal spikes likely impact those people suffering from allergies and asthma, as direct links between thunderstorm activity and childhood asthma and allergy hospital admissions have been made previously. 

The major bacterial phyla identified in this data set (Figure 1B, Supporting Information Table S1) were similar to the taxa identified in a number of other recent air surveys; however the communities were not panmictic. The diversity and community composition of the airborne bacterial communities (Figures 2 and Supporting Information Table S2) differed significantly between the two cities; a small rural city surrounded by agricultural land-uses (Greeley) versus the far larger and far more urbanized city (Denver). This finding is consistent with previous work showing that urban and rural locations may often harbor distinct bacterial communities, due in part, to shifts in the relative contribution of bacterial source environments across distinct land-use types. Aerosol size fraction also influenced the diversity and composition of the airborne bacterial communities. The coarse size fraction (PM$_{10-2.5}$) had levels of bacterial diversity that...
were roughly 80% higher than the diversity observed in the fine size fraction (PM$_{10-2.5}$). While similar studies of airborne bacterial diversity across size fractions do not currently exist, our results are comparable to the diversity patterns observed in aquatic habitats. For example, particle associated bacterial communities derived from rivers, coastal regions, and the open ocean are typically more diverse than their free-living planktonic counterparts. Attachment of airborne cells to the coarse-mode aerosol particles may indirectly increase airborne bacterial diversity as the larger particles may confer protection from harsh atmospheric conditions (such as UV exposure) and may even supply carbon and energy sources for particle-associated cells. Furthermore, the coarse aerosol mode has greater spatial heterogeneity and temporal variability in the PM$_{10-2.5}$ mass concentrations and inorganic composition as compared to PM$_{2.5}$ (fine).

Although it has previously been reported that airborne microbial communities can be highly variable over short time scales, we found that differences in community structure were larger between seasons than within seasons (Supporting Information Table S2), similar to findings reported previously. The observed seasonality could be driven by changes in meteorological conditions, changes in the contributions of individual source environments, or some combination of the two factors. The only meteorological variable found to be significantly correlated with airborne bacterial community composition was temperature (Supporting Information Table S4), which is consistent with earlier studies. However, this does not necessarily imply that air temperature exerts a direct control on airborne microbial communities, rather this pattern could be related to seasonality, a conclusion supported by the fact that the fall and spring air samples harbored distinct communities (PERMANOVA pairwise comparison, $P < 0.001$) but similar mean temperatures.

A more parsimonious explanation for the temporal variability observed in microbial community structure is that seasonal shifts in the relative importance of different source environment contributions, not shifts in individual meteorological parameters, are driving the observed temporal patterns. This is supported by the source tracking analysis which indicates that leaf sources dominated in the spring and early summer months (when deciduous plants would have leaves) with leaf sources dropping in importance in winter and fall months after plant senescence (Figure 4). Interestingly, the seasonal variability is more evident in Greeley than in Denver (Figure 4), which may be a consequence of the land area surrounding Greeley being predominately used for agricultural purposes, where seasonality in plowing, crop cultivation, and livestock operations (see below) may contribute to differences in bacterial sources. Also, the seasonal pattern in soil-derived bacteria roughly follows the temporal pattern in crustal element sources identified from elemental analysis of the same filters with soil-derived bacteria peaking in abundance during late summer and fall when dust loads are probably highest due to low soil moisture levels and increased temperatures.

We also investigated the decay in airborne bacterial community composition over time and found a significant time-decay relationship (Supporting Information Figure S5). This indicates that, on average, the airborne bacterial communities become less similar to one another with more elapsed time between samples. This highlights that samples do not necessarily need to be collected very frequently (daily or weekly) to capture broad intra-annual dynamics in airborne microbial communities—a finding that may be important for bioaerosol monitoring and the design of studies linking airborne bacteria to human health outcomes. The seasonal patterns observed in this data set are likely driving the significant time-decay patterns observed here, with similar time-decay patterns having been documented in an interannual study of marine bacterial communities but not in other commonly studied microbial habitats (including streams, human skin, and the human gut). Together these results suggest that atmospheric communities, like many marine bacterial communities, exhibit fairly predictable variability in composition over time with seasonality driving gradual shifts in the relative abundances of individual bacterial taxa over time.

Using an indicator taxa approach, we identified the taxa indicative of likely microbial source habitats and, then, determined their relative importance to the bacterial communities of the near-surface atmosphere. As might be expected, soils and leaf-surfaces appeared to be a steady and abundant source of bacteria to the atmosphere. However, cow feces were also a significant source of airborne bacteria in the Greeley air samples, where two large cattle feedlots are located in reasonably close proximity to the Greeley sampling locations (<20 km). While only a single cow fecal indicator was identified in our analysis, this bacterial family, the Ruminococcaceae, made up a significant fraction of the Greeley airborne communities during the months of September, October, March, and April (Figure 4). This bacterial family has been shown to be a dominant member of cow gut bacterial communities in a number of other recent studies but is represented in much lower proportions in the gut communities of humans and dogs, making this group a potentially useful indicator of cow fecal contamination. The livestock industry currently monitors a number of nonbiological indicators such as ammonia, volatile organic compounds, and particulate mass concentrations; however these pollution indices could be significantly improved with the addition of a microbial component to monitor biological contamination events. Furthermore, these results may have important implications for a number of cattle industry practices including lagoon turnover, manure scraping at feedlots, and the application of lagoon fluids on surfaces, all of which may contribute to the emission of cow fecal bacteria to the near-surface atmosphere.

This study did not examine what impacts (if any) these bioaerosols may have on human health or the health of other animals and plants. However, as the baseline variability in the types of microbes found in the atmosphere is just beginning to be assessed, it is currently difficult to establish linkages between airborne microbes and health outcomes. This long-term temporal analysis of microbial bioaerosol communities demonstrates how microbial analyses can be used to identify disturbances to this environment, which may be characteristic of microbial contamination events that are important to human and environmental health. More generally, this work and the increasing body of research on airborne microbes highlights that assessments of air quality would benefit by including culture-independent molecular surveys of microbial diversity to complement the physical and chemical analyses currently used in most air quality studies.
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(33) Annual estimates of the resident population for incorporated places over 100,000, Table 1. U.S. Census Bureau, accessed December 27, 2010, 2009 ed.


