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Feasibility of Detection and Identification of Individual Bioaerosols Using Laser-Induced Breakdown Spectroscopy

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The detection and identification of individual bioaerosols using laser-induced breakdown spectroscopy (LIBS) is investigated using aerosolized *Bacillus* spores. Spores of *Bacillus atrophaeous*, *Bacillus pumilus*, and *Bacillus stearothemophilus* were introduced into an aerosol flow stream in a prescribed manner such that single-particle LIBS detection was realized. *Bacillus* spores were successfully detected based on the presence of the 393.4- and 396.9-nm calcium atomic emission lines. Statistical analyses based on the aerosol number density, the LIBS-based spore sampling frequency, and the distribution of the resulting calcium mass loadings support the conclusion of individual spore detection within single-shot laser-induced plasmas. The average mass loadings were in the range of 2−3 fg of calcium/*Bacillus* spore, which corresponds to a calcium mass percentage of ∼0.5%. While individual spores were detected based on calcium emission, the resulting *Bacillus* spectra were free from CN emission bands, which has implications for the detection of elemental carbon, and LIBS-based detection of single spores based on the presence of magnesium or sodium atomic emission was unsuccessful. Based on the current instrumental setup and analyses, real-time LIBS-based detection and identification of single *Bacillus* spores in ambient (i.e., real life) conditions appears unfeasible.

Laser-induced breakdown spectroscopy (LIBS) is a technology well suited for the analysis of solids, gaseous species, and aerosols, including quantitative measurements of individual aerosol particles. Historically, LIBS has been applied on a statistical basis—spectra from thousands of laser shots are ensemble-averaged and results extracted.[1−5] However, in recent studies, LIBS has been used in conjunction with single-shot analysis based on conditional spectral processing to effectively sample aerosol populations using discrete particle analysis.[6−8] Successful implementation of single-shot LIBS-based aerosol sensing has been reported in several studies of ambient air particulate matter, where particles containing aluminum, calcium, chromium, magnesium, and sodium were detected at analyte masses in the low-femtogram range.[6,9,10] Contemporary issues regarding single-shot LIBS analysis of aerosol particles include the overall analyte sensitivity, precision, and suitable detection algorithms.

An important potential application for LIBS-based aerosol detection is the area of biological materials, including those found dispersed in the ambient air, which are collectively referred to as bioaerosols. With the success of LIBS for aerosol detection and quantitative single-particle analysis, it is natural that researchers have directed their attention to the detection of biological compounds. To this end, several papers have recently appeared addressing the feasibility of LIBS for detection and analysis of bacteria and spores consistent with bioaerosols. Morel et al. investigated the detection of six bacteria (including *Bacillus globigii* as a surrogate for *Bacillus anthracis*) and two pollens using LIBS.[11] The analysis was performed using pressed pellets of nominally pure and homogeneous samples. Morel et al. investigated the classification of the biomaterials based on the relative concentration of trace metal species such as phosphorus, magnesium, calcium, and sodium, which are common to all bacterial spores, as well as CN emission bands. It is noted here that, strictly speaking, LIBS is an elemental analysis tool based on dissociation of the molecular structure into constituent atoms within the laser-induced plasma; hence, detection and differentiation of biological materials based on *direct* analysis of their biochemical composition (e.g., RNA) is precluded. In the Morel study, phosphorus was found to be a useful trace element for analysis. The authors concluded that LIBS will not identify all biological materials, noting that the biological structure is too complex, but rather state that LIBS can be useful as a trigger that warns of signatures that are different from the ambient background. In another study, Samuals et al. examined the LIBS-based analysis of bacterial spores, molds, and pollens using a broadband spectrometer (200−980 nm).[12] As in the above study, biological materials were analyzed as homogeneous layers (dispersed on filter media in this study), but data

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reduction employed principal components analysis. The analysis yielded good grouping between classes of biomaterials (e.g., bacterial spores vs pollens); however, discrimination between the three *Bacillus* spores yielded considerable overlap, with the authors concluding that a more rigorous modeling approach would make additional discrimination feasible. A recent study by Kim and co-workers used LIBS to examine four distinct *Bacillus* types and *Escherichia coli*, performing measurements directly on the bacterial culture plates.[13] LIBS spectra were analyzed primarily by comparing peaks identified as phosphate emission and atomic calcium peaks to an emission peak of an unidentified excited organic species, which provided separation among the five bacterial samples. The authors noted that the experimental results were reasonably reproducible, although no rigorous statistical analysis was performed, and concluded that LIBS is a potential technique for rapid and precise classification of bacteria with minimum sample preparation.

Hybl and co-workers examined a range of bacterial spores, growth media, fungal/mold spores, and pollens using LIBS and principal components analysis.[14] Rather than use pellets or substrate-deposited layers, homogeneous samples were aerosolized in one of two ways: in a microcentrifuge tube by making use of the laser-induced shock wave or aerosolized acoustically by dispersing a dry powder suspension above a loudspeaker. As in the above studies, the biomaterials were found to group well among general classifications, but there was overlap among the similar materials such as the various *Bacillus* samples and between corn smut and oat smut. The authors cautioned that LIBS must be evaluated in a real ambient environment, noting that nearly all of the "signature" elements (i.e., trace elements) are also present in dirt, and further concluded that LIBS will most likely be very useful for detecting bioagents and discriminating them from the natural background. In another recent study, Boyain-Goitia et al. focused on the analysis of single pollen particles by introducing individual pollen particles directly into the laser focal spot on stainless steel sample pins.[15] This approach helped to eliminate contaminants, including potential contaminants that could be released from a substrate material during the ablation process. The authors used both atomic emission from calcium and molecular emission bands, namely, CN and C_2, to assess the ability to discriminate among three different lily pollens and marguerite pollen. Overall, the LIBS trials were reasonably successful in generating distinguishable and reproducible spectra from the bioparticles. However, the authors concluded that LIBS analysis alone would be hard pressed to provide unique identification between pollen and other bioaerosols, let alone to distinguish among species within a particular family of pollen.

A related approach for characterization of the inorganic component of biological materials was reported in a recent paper based on direct injection inductively coupled plasma mass spectroscopy (ICPMS), specifically for the study of three *Bacillus* bacteria, including spores and vegetative cells.[16] As in the above papers, differences in the ratios of trace metals were observed among the three bacteria types, including the elements Mg, P, V, Mn, Co, Cu, Zn, Sr, Ba, and Pb. The authors concluded that the point detection and identification of aerosolized biological materials using an inorganic chemical fingerprint via direct injection ICPMS may be a viable methodology for warfare agents.

In concert, the recent papers discussed above allow several conclusions regarding the unique application of LIBS for classification of biomaterials, in particular bioaerosols. The principal mechanism for discrimination is the identification and exploitation of differences (some subtle, some pronounced) in the atomic emission signals, primarily those emission lines stemming from trace metal species such as calcium, magnesium, sodium, phosphorus, and potassium. This approach necessitates single-particle analysis in any real-world environment, because the presence of concomitant particles will be expected to significantly contribute to these trace elements, as shown for example in published LIBS studies of ambient air.[6,9,10] This is an important distinction from most of the feasibility studies mentioned above, as any collection of large aerosol populations (e.g., ambient air particles captured on filter media) will commingle spectral signatures, making such ensemble analyses baseless. However, single-particle analysis of aerosols is typically characterized by significant shot noise in the resulting spectra, leading to reductions in analyte precision as compared to conventional ensemble-averaged LIBS. With these comments in mind, it is useful to carefully assess the issues of particle detection and shot-to-shot variation in analyte signals relevant to LIBS-based detection of *individual* bioaerosols. Such analyses constitute the goals of the current study.

EXPERIMENTAL METHODS

The experimental system for the current study has been reported in previous studies[17,18] and is briefly described here. For all experiments, a 1064-nm Q-switched Nd:YAG laser (5-Hz repetition rate, 10-ns pulse width, and 275 mJ/pulse) was used to create the plasma using a 50-mm-diameter, 75-mm-focal length UV grade plano-convex lens. The plasma emission was collected on axis with the incident laser beam using a pierced mirror and 75-mm-focal length condensing lens. The plasma emission was subsequently fiber-coupled to a 0.275-m spectrometer (2400 groove/mm grating, 0.12-nm optical resolution), where spectral data were recorded using an intensified CCD detector array. All LIBS data reported in this paper were recorded using a delay of 40 μs with respect to the incident laser pulse and a signal integration time of 60 μs. Similar delays and integration widths were also investigated for the detection of magnesium and sodium.

Bacillus spores: *Bacillus atrophaeous* (formerly *Bacillus subtilis* var. *niger*), *Bacillus pumilus*, and *Bacillus stearothemophilus* (*Geobacillus stearothemophilus*) were purchased (NAMSA, Northwood, OH) as particle suspensions in water with a spore number density of 2.2×10^7 cm^{-3}. The suspensions were stored under refrigeration prior to use.

Bioaerosol flows were generated by the nebulization of the spore suspensions using a pneumatic-type nebulizer as described previously.[17] Prior to use, the spore suspensions were well mixed and then sonified (~5 min) to minimize any agglomeration. The nebulizer was operated with 4 L/min purified dry air, which

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generated a nebulization rate of ~0.12 mL/min. For calibration runs, the nebulization rate was measured for each data point by recording the total mass loss and flow time. The nebulizer output was subsequently mixed with a gaseous coflow stream of 52 L/min purified, dry air. Both the nebulizer air and coflow air streams were dehumidified using an activated alumina drier and HEPA filter prior to use. It is also noted that the activated alumina desiccant effectively removed the carbon dioxide from the compressed air stream. The aerosolized spore number density at the LIBS sample point was controlled by adjusting the spore concentration in suspension via dilution with ultrapurified water. Overall, the aerosol system was designed such that the nebulizer exhausts directly into the center of the coflow air stream, after which the flow enters a tapered mixing/drying section. The coflow geometry shields the aerosols from the chamber walls, which are micropolished to prevent deposition. Following evaporation of the nebulizer droplets, the resulting aerosol stream was characterized by a dilute spore loading (2−5 spores/cm^3), which then flowed through a six-way cross that formed the LIBS sample chamber. The primary LIBS focusing lens was mounted as a window on one side of the six-way cross, resulting in the laser-induced plasma forming directly on the center axis of the aerosol flow stream.

Independent aerosol size and concentration data were recorded using a commercial light-scattering instrument (PMS, Inc., Boulder, CO) with sensitivity over a range from 100-nm to 2.5-μm particle diameter. The instrument sampled 270 cm^3/min, which was drawn directly from the aerosol sample stream ~15 cm downstream from the LIBS focal spot.

Light microscopy was performed using a 100× objective in conjunction with an imaging CCD. Aliquots of the original spore suspensions were dried onto a glass microscope slide, which were subsequently imaged. Spore size measurements were made by manually measuring the size of a number of spores, which were calibrated using a reference standard under the same imaging conditions. Average spore diameters were recorded by measuring the average center-to-center distance of adjacent spores as observed in a wide range of spore clusters.

RESULTS AND DISCUSSION

Aerosol Generation and Single-Particle Sampling. To support the primary goal of the current paper, namely, detection and analysis of individual spores, a number of steps were taken to ensure and validate that single spores were presented to the laser-induced plasma volume. The laser-induced plasma may be considered in the context of a statistical sampling volume for the analysis of aerosols, as detailed in a previous study under similar experimental conditions.[18] The statistical plasma volume defines the expected aerosol sampling rate as modeled using a Poisson sampling probability. Accordingly, the probability of sampling a single particle under dilute loadings is expressed as

$$F = 1 - \exp(-NV_p) \quad (1)$$

where F is the sampling frequency (i.e., particle hit rate), N is the aerosol number density (particles/volume) in the LIBS sample chamber, and V_p is the effective plasma sample volume. The plasma sample volume is considered to be 1.1 mm^3 for the current parameters based on previous measurements.[18]

Table 1. Average Spore Diameters Measured with Visible Light Microscopy

species	spore diameter (μm)
B. atrophaeous	1.06 ± 0.21
B. pumilus	0.94 ± 0.16
B. stearothemophilus	1.39 ± 0.30

To promote single-particle detection, spore suspension concentrations were adjusted to produce aerosolized number densities equal to either 2 or 5 cm^{-3}. Based on eq 1 and the plasma sample volume, the corresponding expected LIBS sampling rates (i.e., spore hit rate) are 0.22 and 0.55%, respectively. Sampling rates less than 1% are considered sufficiently low such that only single-particle sampling events are expected, in that the probability of hitting two aerosol particles simultaneously is negligible.

The aerosol sampling calculations were validated in several ways. First, the resulting particle hit rates were directly observed to scale with particle number density. For all experiments, *Bacillus* spores were detected based on the presence of the 393.4- and 396.9-nm calcium atomic emission lines. Full details of the spectral processing algorithms and particle sampling criteria are given below. *Bacillus atrophaeous* spore suspensions were diluted and nebulized to produce a calculated aerosol concentration of 2 cm^{-3} at the LIBS focal point. A sequence of 8000 laser pulses yielded a total of 10 calcium-based spore hits, for a corresponding particle hit rate of 0.13%. This is in very good agreement with the predicted hit rate of 0.22%, noting that additional hits were triggered but were subsequently rejected due to the lack of pronounced calcium emission signals on both calcium lines, as discussed below. New *B. atrophaeous* spore suspensions were then prepared and nebulized to produce a calculated aerosol concentration of 5 cm^{-3}. A sequence of 12 000 laser pulses yielded a total of 35 calcium-based spore hits, for a corresponding particle hit rate of 0.3%, which is also in very good agreement with the predicted sampling rate of 0.55%. In addition, the measured sample rates scale remarkably well with the predicted spore concentrations (i.e., 0.13/2−0.30/5).

A second set of measurements was performed to independently corroborate the presence of a dilute suspension of individual spores. The optical microscopy provided a direct measurement of the physical spore size. Representative images are presented in Figure 1, and the results of these measurements are summarized in Table 1. These physical spore diameters were then used in conjunction with the commercial light-scattering instrument to verify aerosol loadings and the presence of individual (i.e., nonagglomerated) spores. Detailed measurements were again performed on the *B. atrophaeous* suspensions. The light-scattering instrument provides total particles counts for a given sample time (300 s) and corresponding gas sample rate (270 cm^3/min) for each of eight size channels that together cover a size range from 100 nm to 2.5 μm. The light-scattering instrument was first calibrated using monodisperse silicon oxide particles of 1.02-μm diameter, which correspond closely in size to the measured diameter of the *Bacillus* spores. For the *B. atrophaeous* suspensions, the peak in the measured light-scattering-based size distribution was identical to the peak measured with the silica microspheres, namely, in the size bin corresponding to 0.7−1.0 μm. The exact agreement in equivalent scattering size between the 1-μm silica particles and

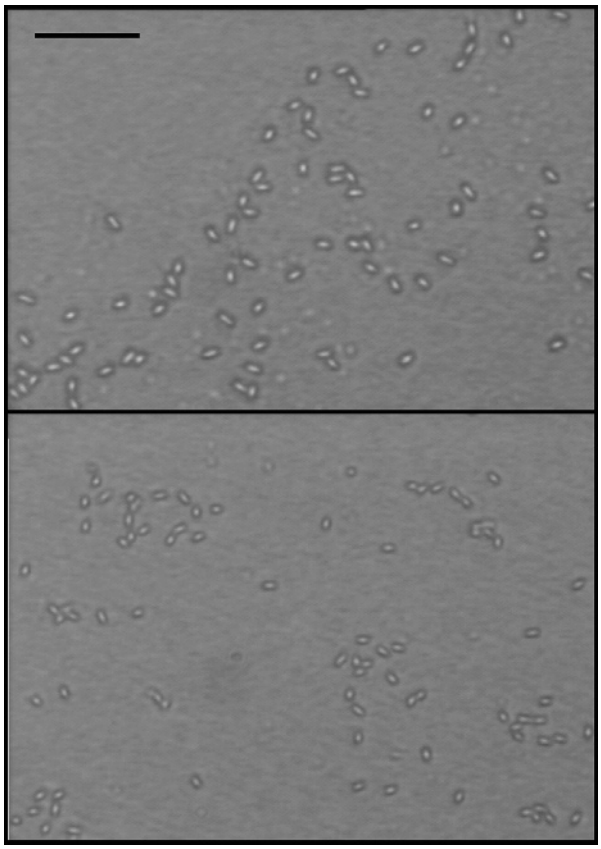

Figure 1. TEM micrographs of *B. atrophaeous* (top) and *B. pumilus* (bottom) spores recorded using 100× objective. Scale bar equal to 10 μm.

the 1-μm *B. atrophaeous* spores is supportive of the presence of *individual* spores in the aerosol stream. Furthermore, nearly 2/3 of the total particle counts corresponding to the aerosolized spores were recorded on the 0.7−1.0-μm size bin, with the remaining 1/3 split about evenly between the 0.5−0.7- and 1.0−2.0-μm bins. No counts were recorded in the 2.0−2.5-μm size bin. Finally, the total aerosol counts recorded with the light-scattering instrument provide an independent measurement of the aerosol number density. The *B. atrophaeous* aerosol loadings based on the light-scattering data were 0.4 and 1.2 cm^{-3} for the two predicted concentrations of 2 and 5 cm^{-3}, respectively. The scaling of the light-scattering number densities agrees very well with the predicted values, while the absolute values are low by a factor of ~4. However, the absolute agreement is considered acceptable, given that the sampled spores must flow through an additional 1-m length of sampling line where particles may be lost to the walls and that the sampling efficiency of the light-scattering instrument is less than unity. In aggregate, the above data validate the presence of a dilute sample stream primarily comprising aerosolized, individual (i.e., nonagglomerated) *Bacillus* spores.

LIBS Analysis. As mentioned above, the key to single-shot LIBS detection of aerosol particles is the identification of a particle "hit" based on the presence of a targeted analyte emission line or lines. Suitable approaches for single-shot detection of ambient air particulates have been reported,[9] while a detailed study of conditional spectral processing for single-particle detection in the context of spectral noise and false hit rates was reported in a recent study by Carranza et al.[8] For the current work, a two-step approach was used. The first step was based on the identification of "candidate" particle hits using a real-time algorithm similar to the approach detailed by Carranza et al. Specifically, the peak-to-base (P/B) ratio was calculated using the calcium emission lines for each recorded spectrum. The P/B ratio was calculated by averaging the intensity over 7 pixels (~0.2 nm) centered about the expected calcium emission line and then dividing by the average intensity of an adjacent baseline region selected to avoid any interfering atomic emission peaks. For the relatively flat background region near the 393.4- and 396.9-nm calcium emission lines, the resulting P/B signal was about unity in the absence of calcium emission. A suitable P/B threshold value was selected by steadily increasing the threshold until a desired number of false hits (i.e., hits in the absence of calcium) was realized. The optimal false hit rate is between 1 and 2 false hits/2000 laser shots, corresponding to a false hit rate of 0.05 and 0.1%, which eliminates a large number of high-noise events while maintaining sufficient sensitivity to actual analyte emission.[8] The false hit rate was set by nebulizing ultrapurified water only, such that no calcium was present in the sample stream. A threshold value was set to 80−85% above the average P/B ratio recorded in the absence of any calcium, which produced the desired false hit rate of ~1/1000 laser shots.

The second step in the identification of single-shot bioaerosol hits involved the separation of false particle hits from actual particle hits. Specifically, the low aerosol loadings used to promote single-particle detection resulted in an actual particle hit rate comparable to the expected false particle hit rate. Therefore, a suitable procedure must be used to separate the resulting spectra. One approach used in previous studies makes use of the presence of two distinct atomic emission lines,[9] such as the case with calcium emission lines at 393.4 and 396.9 nm. The algorithm uses calibration curves for each emission line to calculate the respective analyte response (i.e., equivalent analyte concentration). The spectrum is considered to correspond to an actual particle hit if both equivalent analyte concentrations agree to within some set tolerance, typically a factor of 2. A similar approach was adopted for the current study, but rather than setting an automatic tolerance factor for agreement between analyte responses of the two emission lines, each spectrum identified as a "hit" was manually inspected for the presence of distinct emission peaks corresponding to both emission lines. The actual range of agreement between the two analyte peak responses is discussed in detail below. For this limited study in which a relatively small number of spectra were examined, the aggregate approach for single-particle detection was considered quite satisfactory, providing a robust rapid screening in combination with a final acceptance based on the clearly discernible presence of the analyte emission profile. Clearly, the implementation of any real-time particle detection schemes must employ automated discrimination algorithms. However, the purpose of the present study is a careful evaluation of the particle-to-particle statistics related to LIBS analysis of bioaerosols. It is therefore desirable to include the greatest possible range of particle hits, including those hits approaching the noise limit, while eliminating "hits" corresponding to spectral noise. The conclusion is that these goals are best accomplished by final manual inspection, as development of a robust spectral processing algorithm is not in the scope of this paper.

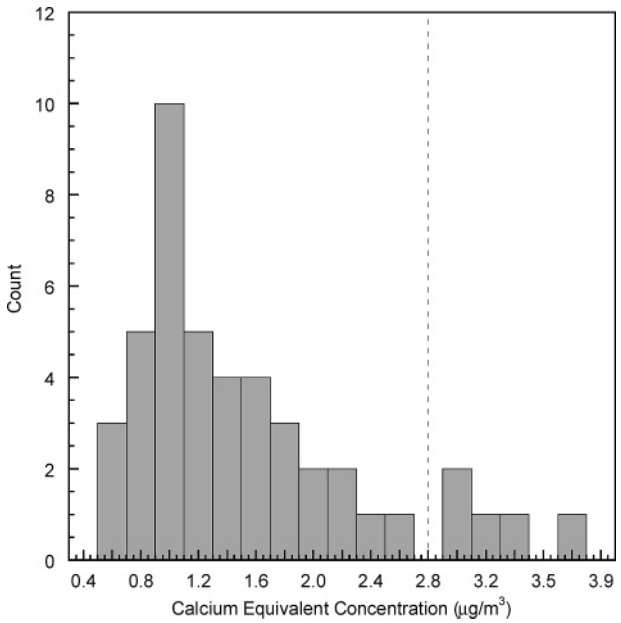

Figure 2. Histogram of the calcium emission response for *B. atrophaeous* spores, represented as the average equivalent calcium concentration, for all 45 identified spore hits.

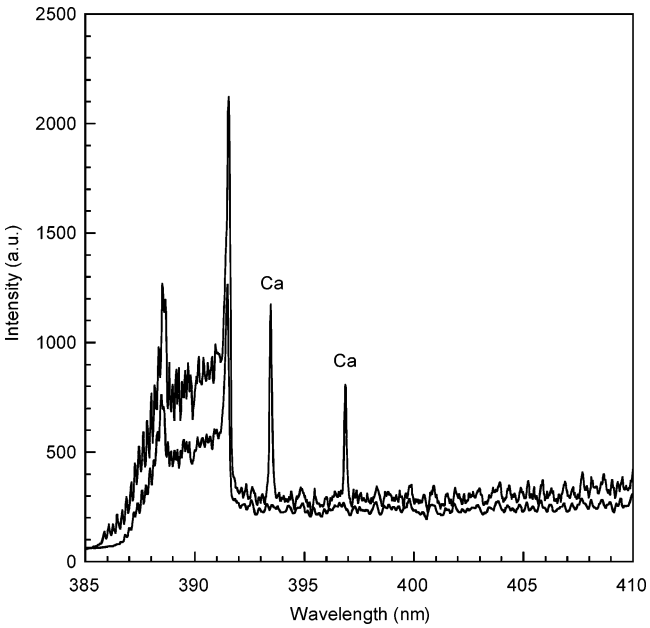

Figure 3. Ensemble-averaged spectrum of 40 identified individual hits of *B. atrophaeous* spores, along with a representative ensemble-averaged spectrum corresponding to the absence of any spores. Spectra have the same intensity scale.

A total of 20 000 laser shots produced 45 confirmed particle hits for the aerosolized *B. atrophaeous* spores, based on the clear presence of the 393.4- and 396.9-nm calcium emission lines. For quantitative analysis, calcium emission calibration curves were prepared by nebulizing solutions of dissolved calcium (SPEX Inc.) to produce a range of known calcium mass concentrations through the LIBS sample chamber. Five-point calibration curves (each point in triplicate) were prepared for each calcium emission line using a range of calcium concentrations between 8.5 and 85 $\mu g/m^3$ in addition to calcium blanks corresponding to the nebulization of ultrapurified water only. The resulting calibration curves were highly linear ($R = 0.992$), although the final calibration curves were fit to a second-order expression ($R = 0.995$) to promote high accuracy near the zero points given the low analyte response of the spores. For each *B. atrophaeous* particle hit, an equivalent calcium concentration was calculated using both the 393.4- and 396.9-nm calcium emission lines and corresponding calibration curves. The two values were then averaged to produce a representative equivalent calcium emission response for each spore. A histogram of the calcium analyte response, represented as the average equivalent concentration, for all 45 identified hits is presented in Figure 2.

Careful examination of the histogram reveals two key features, namely, the presence of a well-defined log-normal-like distribution with a modal value of ~ 1 $\mu g/m^3$ and the presence of a second cluster of five hits in the range of equivalent calcium concentrations between about 2.9 and 3.9 $\mu g/m^3$. These two features may be interpreted in several ways, including that the five hits corresponding to the highest concentrations may be a long tail, although disjointed, of the well-defined distribution or, alternatively, that these five points may in fact be a second mode. It is the latter interpretation that is concluded to be the most likely. Specifically, the well-defined portion of the histogram displays a clear mode and rather sharp overall structure, which as discussed below, is expected for an aerosol population consisting of quite monodisperse bioaerosols, as evidenced by the microscopy analysis. Hence the presence of significantly larger spores (i.e., 2−3 times the mean calcium signal), although possible, is not expected. Second, while much care was taken to promote an aerosol stream of single *Bacillus* particles, is must be considered that a small fraction of the aerosolized spores may consist of clusters of two, three, or more spores. The microscopy revealed the presence of primarily isolated spores, despite the tendency of suspensions to cluster upon evaporation, which is consistent with the conclusion of single-particle detection. However, whether two or three spores are clustered in solution prior to nebulization, or whether two or more isolated spores are entrained in a single nebulizer droplet and consequently joined upon desolvation, it is not unexpected that some two- or three-spore clusters are sampled by the laser-induced plasma. Finally, a single spectrum was recorded with a calcium analyte signal more than 25 times greater than the average signal recorded for the spectra concluded to be single spore hits. This single spectrum was concluded to be either a contaminant particle or unusually large cluster of spores and was excluded from further analysis. With these comments and conclusions in mind, the *B. atrophaeous* data in Figure 2 can be further analyzed.

The 40 single-shot spectra concluded to correspond to individual *B. atrophaeous* spores were subsequently analyzed in a number of ways. The average spectrum of all 40 shots is presented in Figure 3, along with an average of 1000 shots recorded with the nebulization of only ultrapurified water. The spectrum of the *B. atrophaeous* spores reveals the clearly discernible calcium emission lines at 393.4 and 396.9 nm and otherwise is characterized by structure nearly identical to the deionized water spectrum. The band head and emission features at 391.4 and 388.4 nm are attributed to the N_2^+ first negative system,[19] and the absence of

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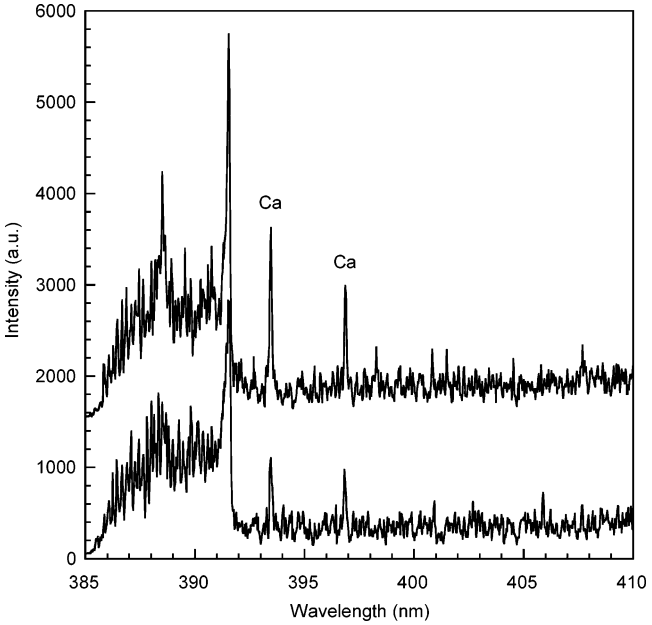

Figure 4. Representative single-shot spectra corresponding to individual hits of *B. atrophaeous* spores. The upper spectrum corresponds to an absolute calcium mass of 4 fg and the lower spectrum an absolute calcium mass of 2 fg. Both spectra have the same intensity scale, with the upper spectrum shifted vertically for clarity.

any emission from the CN violet system (primarily at 388.3 and 387.1 nm) is also noted. The overall spectral quality of the ensemble-averaged data is revealed in the quantitative analysis of the two calcium emission lines. Using the calcium calibration curves discussed above, the 393.4- and 396.9-nm emission lines correspond to an equivalent calcium concentration of 1.09 and 1.06 $\mu g/m^3$, respectively, the difference being statistically insignificant. The average equivalent calcium concentration, based on the two emission lines, is 1.08 $\mu g/m^3$ for the ensemble average of the 40 individual *B. atrophaeous* particle hits.

As presented in previous papers, the equivalent concentration of a single-particle hit is readily converted into an absolute analyte mass by multiplying by the effective laser-induced plasma volume.[7,18] The current experimental conditions have been carefully evaluated in a previous paper, revealing an effective emission-based plasma volume of 2.4 mm^3, noting that the emission-based plasma is larger than the effective plasma sample volume.[18] Using this plasma volume, each of the individual spore hits can be converted to an equivalent absolute calcium mass. For this calculation, the two effective calcium concentrations based on the two emission lines were averaged to calculate the absolute mass. The agreement between these two emission lines is discussed further below in the context of single-shot spectra. For the 40 individual *B. atrophaeous* spores, the average measured calcium mass is 3.1 fg with a relative standard deviation (RSD) of 39.6%. The measured calcium mass ranged from 1.2 to 6.1 fg. To smooth the significant shot noise associated with individual LIBS spectra, the average mass was also calculated directly from the ensemble average of the 40 *B. atrophaeous* hits, which yields an average of 2.6 fg of calcium. Representative single-shot spectra are presented in Figure 4, which correspond to individual calcium masses of 2 and 4 fg. As compared to the ensemble averages of Figure 3, the single-shot spectra are characterized by a marked increase in shot noise, which is to be expected. Using the measured spore diameter of 1.1 μm (see Table 1) and assuming unity density, the measured absolute calcium mass of 2.6 fg (based on the average spectrum) corresponds to a calcium mass fraction of 0.5%, which is in good agreement with the reported range of 1−3% for several *Bacillus* species based on spore digestion and atomic absorption spectroscopy.[20] The overall range of a particular trace metal species among different types of *Bacillus* spores can vary by over 1 order of magnitude.[16] The average absolute calcium mass in the 5 "clusters" described above is 7.7 fg, or nearly 3 times the value of the mean of the 40 identified individual spores. This is consistent with the conclusion of clusters of two or three individual spores for the five spectra designated as such.

A fundamental aspect of the detection of bioaerosols with LIBS remains the ability to quantitatively discriminate bioaerosols first from the widely varied background of concomitant ambient aerosols and, second, from among closely related bioaerosols (e.g., *B. atrophaeous* vs *B. anthracis*). As discussed in the introduction, such discrimination must exploit possible differences in the spectral properties among similar bioaerosol compounds. Because carbon, nitrogen, oxygen, and hydrogen are present in gas-phase species in significant quantities in ambient air, working with these elements is problematic; hence, most attention has turned to the analysis of trace metals. Accordingly, a primary goal of the present study is to provide a better understanding of the single-shot precision that may be expected for bioaerosols with regard to trace metals analysis. While efforts were made in this study to realize single-shot detection of individual *Bacillus* spores, there is no way to independently analyze the calcium content on a spore-to-spore basis. However, with two calcium emission lines present, the degree of variation between the measured equivalent calcium concentration from the 393.4- (0−25 414 cm^{-1}) and 396.9-nm (0−25 192 cm^{-1}) emission lines provides an effective way to assess the precision of the LIBS technique, given that these two lines originate from the same atoms. The relative percent deviation between the analyte responses of these two lines was calculated as the difference in the equivalent calcium concentrations based on the 393.4- and the 396.9-nm emission lines normalized by the average value of the two equivalent concentrations. Hence, perfect agreement in analyte response between the two lines would yield a relative percent deviation of zero, while a difference of a factor of 2 would yield a relative deviation of 66.6%. A probability plot of the distribution of the relative percent deviation of the calcium analyte response for the two calcium emission lines is presented in Figure 5 for the 40 identified individual *B. atrophaeous* spore hits. As expected, the data scatter about zero percent deviation; however, the data are slightly skewed toward a negative percent deviation. Firm conclusions with regard to the skewness are difficult to formulate with the rather limited sample space, but perhaps this trend results from the relatively weaker 396.9-nm emission line being more affected by shot noise adding to this analyte emission peak.

The Figure 5 data provide a solid measure of the expected analyte precision for single-shot analysis. As discussed above, the average spectral data yield excellent analyte agreement between the two lines, even for a sample of 40 spectra. However, for a given single-shot spectrum, the degree of uncertainty can be

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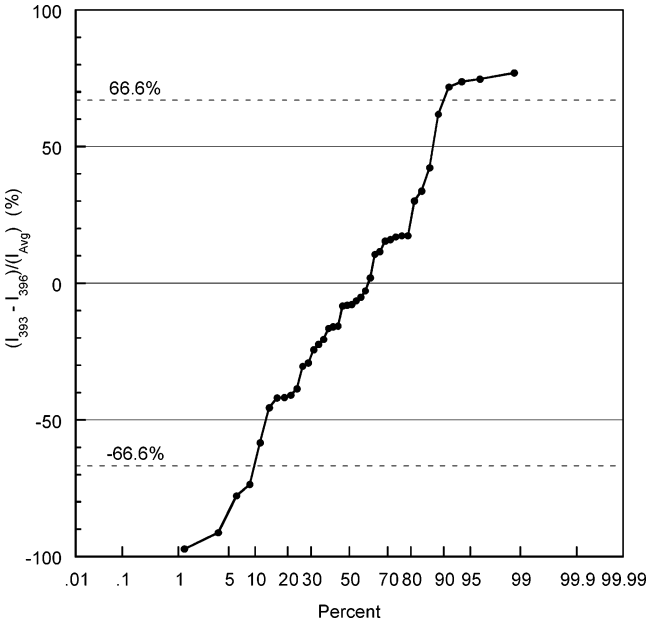

Figure 5. Cumulative probability plot of the distribution in relative percent deviation of the calcium analyte response for the two calcium emission lines based on the spectral analysis of 40 identified individual *B. atrophaeous* spores.

quantified by the agreement in analyte response of the two calcium emission lines. Analysis of the absolute value of the Figure 5 data yields an average deviation of 34.8% for the analyte response of the two calcium emission lines, with a relative standard deviation of 78%. If one considers a factor of 2 in agreement between the analyte responses of the two calcium lines (i.e., 66.6% relative deviation), 32 of the 40 hits are found to fall within this range. It is interesting to note that the current results are remarkably consistent with the first published results of LIBS-based single aerosol analysis.[6] In that study, the single-shot, single-particle analyte response of nominally monodisperse, iron-rich particles was found to vary within a factor of 2 about the mean analyte response based on a single iron emission line. The above data suggest that single *Bacillus* spores may be detected on the basis of calcium emission at overall mass loadings approaching 1 fg, which is consistent with detection limits reported previously for ambient air particles.[9] However, the precision of measuring the calcium response for a single-shot, single-spore measurement must be considered to carry a representative uncertainty on the order of 35%. It is also noted that calcium detection limits and precision in the context of laser-induced plasma emission do not in general reflect an absolute shortage of photons, but rather the competing continuum emission. The detection of larger *Bacillus* clusters (i.e., agglomerates) is expected to yield an enhanced signal-to-noise ratio, although extrapolation of the resulting single-shot precision needs additional research.

The use of additional spectral information is also addressed with respect to molecular emission features. Recall the work of Boyain-Goitia et al., in which CN and C_2 emission bands were analyzed in combination with atomic emission,[15] noting that the lily pollen used in their study were significantly larger than *Bacillus* spores. In the current study, the lack of CO_2 in the sample air stream (i.e., absence of any background CN emission) provided an excellent opportunity to assess the detection of carbon originating from an individual *Bacillus* spore via the CN violet system. No CN emission was observed (see Figure 3) from the spectra corresponding to the *Bacillus* spores, although significant CN emission was observed with plasma formation directly in ambient air, which contains sufficient CO_2 for CN formation.

The above measurements were repeated for aerosolized suspensions of the *B. pumilus* and *B. stearothemophilus* spores. For the *B. pumilus* spores, 25 000 laser shots yielded a total of 6 confirmed spore hits. The average spectrum of the six hits corresponds to an equivalent calcium concentration of 0.72 and 0.78 $\mu g/m^3$ for the 393.4- and 396.9-nm emission lines, respectively, which are in good agreement. Based on the average of these two values, the average calcium mass was calculated as 1.8 fg. Clearly the smaller *B. pumilus* spores yield a smaller calcium mass as compared to the *B. atrophaeous* spores, which is consistent with the reduced single-particle sampling rate (i.e., 6/25 000) as the absolute calcium mass values approach the calcium-based single-spore detection limit. In addition, the single-spore calcium masses were found to scale nicely with the particle size. Specifically, the volume ratio of the *B. atrophaeous* to the *B. pumilus* spores is equal to (1.057/0.935),[3] which yields a value of 1.44. The ratio of average calcium mass for these two spores is equal to 1.43, in near-exact agreement with the overall spore mass ratio assuming equal spore density. The *B. stearothemophilus* spores were analyzed in less detail, with 8000 laser shots recorded at the lower aerosolized spore concentration. Only two single spectra were determined to correspond to actual *B. stearothemophilus* spore hits, which yielded single-spore calcium masses of 2.8 and 2.9 fg. While no detailed conclusions can be made based on two individual spores hits, the *B. stearothemophilus* results do confirm the detection of individual spores of this third *Bacillus* spore type, while also yielding calcium masses consistent with their somewhat larger particle size, as observed in Table 1.

A fundamental factor in the ability to discern among various bioaerosols is based on the presence of multiple trace metal species, such as magnesium, potassium, and sodium in addition to calcium. It is the exploitation of differences in the relative ratios of such species that would form the basis of LIBS as a technique for detection and discrimination of bioaerosols, as suggested previously.[11−16] To examine the feasibility of such analysis, the ability to detect magnesium and sodium in the *Bacillus* spores was also investigated. Sodium detection was based on the 589.00- and 589.59-nm doublet, and magnesium detection was based on the 279.55- and 280.27-nm lines. Both of these detection schemes are based on pronounced spectral lines in a laser-induced plasma and have been successfully used to detect ambient air particulate matter and bioaerosols.[6,9,10,14] A total of 54 000 laser shots were analyzed in an attempt to record spore hits based on these two elements. A careful examination of the spectral data revealed not a single spectrum concluded to correspond to an actual spore hit, as evidenced by the presence of magnesium or sodium emission lines. The conclusion is that the single-particle mass loadings of magnesium and sodium of these three *Bacillus* spore types are below the detection limits for single-shot LIBS detection limits under the present conditions. The failure to detect these trace elements is considered significant (if one plans to analyze the ratio of multiple trace metal species) and rather definitive, given the well-controlled laboratory bioaerosol streams free from any

concomitant particles and the additional step of careful manual spectral analysis.

CONCLUSIONS

The overall goals of this paper are to investigate the LIBS-based analysis of individual bioaerosols, namely, *Bacillus* spores, in well-controlled laboratory experiments, as well as to quantify the detectibility of such spores based on trace metal species and the corresponding precision of the analyte signals. The current results enable one to comment on the feasibility of LIBS-based discrimination and identification of bioaerosols such as *Bacillus* spores based on analysis of atomic emission signals, namely, those emission lines stemming from trace metal species such as calcium, magnesium, and sodium. Such an approach necessitates single-shot, single-particle analysis in any real-world environment, because the presence of concomitant particulate matter is expected to significantly contribute to these trace elements. It is concluded that detection and identification of single *Bacillus* spores is not feasible using current implementations of laser-induced breakdown spectroscopy. This conclusion is based on the difficulty of simply detecting a single spore via calcium emission, the inherent degree of uncertainty in a single-shot analyte signal, and the lack of detectable analyte signals corresponding to other expected trace elements. Advanced data processing schemes are not expected to alter these conclusions, in that these inherent limitations are fundamental issues, namely, limits of detection and single-shot analyte precision. The current findings with regard to precision and detection limits are consistent with previously published findings regarding LIBS-based analysis of general aerosol particles.

Ultimately, the differences among bioaerosols such as the *Bacillus* spores in this study are rooted in their molecular structure (i.e., amino acid *sequences*). Furthermore, the very nature of trace metals in *Bacillus* spores and other bacteria is fundamentally different from the role of an amino acid, for example. The latter constitute genetic coding and are therefore exactly defined for a given species regardless of preparation. The former play a more regulatory role; for example, calcium is known to affect the heat resistance or germination of bacterial spores.[21,22] The calcium uptake in *Bacillus* spores has been shown to depend strongly on the calcium concentration in the growth medium, as measured by changes in spore resistance to sterilization methods.[23] With these additional comments in mind, the very concept of an atomic emission "spectral fingerprint" for a particular spore type is questionable outside of a well-controlled set of spores; hence single-spore discrimination based on elemental analysis of laser-induced plasma atomic emission is not well founded.

The use of additional spectral information is also addressed with respect to molecular emission features. In the current study, the lack of CO_2 in the sample air stream (i.e., absence of any background CN emission) provided an excellent opportunity to assess the detection of carbon originating from an individual *Bacillus* spore via the CN violet system. The failure to detect spore-derived carbon via CN emission in a spectral background free of any CN bands is considered significant and precludes such detection in the CN emission-rich spectrum of ambient air. An important implication of the inability to detect carbon from the *Bacillus* spores via CN emission is the loss of a potential means to corroborate trace metal emissions such as from calcium with emission from an organic component of bioaerosols.

While the primary goal of this paper is to address direct LIBS-based analysis of single bioaerosols, this work is also motivated by interest in the role of LIBS as an auxiliary trigger for bioaerosol particles in ambient air. Based on the current results, the most promising analyte lines for such detection correspond to calcium emission, although sodium or magnesium should be detectable in large spore clusters, in consideration of recent measurements.[11,14] Notwithstanding the conclusions presented above on the absolute identification of spores, the use of LIBS to assess rapid changes in calcium-rich aerosols, for example, which could then trigger more sophisticated analyses, must be considered in the general context of LIBS-based analysis of ambient air particulate matter (PM). LIBS has been successfully employed for detection of ambient air PM, including identification of particles rich in calcium, magnesium, and sodium.[6,9,10] More recent measurements using the same instrumentation and methodology described in this study examined calcium- and sodium-rich ambient air PM over a five-week period. For over 500 000 laser shots, the average calcium-based particle hit rate was 0.28%, or slightly more than 4 particles/1500 shots (a 5-min sample period at 5 Hz). However, the natural fluctuations in the calcium-rich particle sample rates over 5-min sample periods revealed a variation of 1 order of magnitude with respect to the mean rate of 0.28%, namely, from as low as 0% to more than 1.7% (26 hits/1500 shots). In view of the diverse particle-rich background of ambient air, the development of algorithms to *rapidly* detect changes in elemental mass loadings using LIBS and subsequently linking such changes to a particular bioaerosol species with a modest degree of confidence also appear inconsistent with the analytic strengths and capabilities of LIBS.

Overall, LIBS is a powerful analytical method that is particularly well suited for the single-shot elemental analysis of metal-rich aerosol particles, notably for real-time analysis of ambient air. Given the great potential of LIBS, however, it is essential that researchers continue to critically assess the technique, including developing novel methodologies to enhance LIBS as an analytical technique and quantifying both the merits and inherent limitations of laser-induced breakdown spectroscopy.

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