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RAPID COUNTING OF LIQUID-BORNE MICROORGANISMS BY LIGHT SCATTERING SPECTROMETRY

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Abstract: Fast and sensitive techniques are needed to determine microorganism presence in liquid samples. In this research, the feasibility of using light scattering spectrometry for enumerating the biological particles in liquid samples was investigated. A particle size spectrometer was used to count six commonly found microbial species suspended in liquid with and without microbiological stains applied: *Pseudomonas fluorescens*, *Micrococcus* spp. vegetative cells and *Bacillus subtilis* var. *niger* endospores were stained with Acridine Orange and Crystal Violet, while *Cladosporium cladosporioides*, *Penicillium melinii* and *Aspergillus versicolor* fungi were stained with Acridine Orange and Lactophenol Cotton Blue. The counts obtained with the spectrometer were compared with those obtained with a phase-contrast microscope. It was found that the spectrometer counted about 32% of non-stained *B. subtilis* endospores and this percentage increased to almost 90% for stained endospores. Among the investigated species of fungi, the counting efficiency of *P. melinii* was the only one significantly affected by the application of the stain Lactophenol Cotton Blue: the fraction of counted fungal spores increased from 64% (non-stained spores) to about 100% (stained spores). The observed difference in counting efficiency may serve as a basis for differentiating biological from non-biological particles in liquid samples.

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INTRODUCTION

Some airborne microorganisms, especially pathogenic or allergenic ones, are known to cause a wide range of respiratory and other health disorders in people present in residential and occupational environments [6, 17, 19, 24, 25, 28, 29, 31, 32, 34]. When evaluating exposure to airborne microorganisms, air samples are often collected into a liquid medium. The concentration of microorganisms in liquid samples is then assessed by microscopy analysis or by cultivation. However, microscopic counting is a time and labor-consuming process [3, 18]. The cultivation method only enumerates the culturable microorganisms in a sample and is often slow (requiring from one to several days). In addition, the cultivation method has been noted to underestimate the true quantity of microorganisms in a sample [11]. The determination of only the culturable microorganism

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concentration in air samples is often not sufficient, because immune systems can respond to the antigens of both culturable and non-culturable microorganisms. Therefore, determination of the total microbial particle count is also desirable. Thus, fast and sensitive techniques are needed to analyze the microorganism concentrations in liquid samples. Fast determination of the total biological particle count in a liquid sample may yield important information about the microorganism dynamics in the medium of origin (e.g., air): a large or sudden increase in the concentration of biological particles indicates proximity to a particle source. In case of an intentional release of biological particles, e.g., in a biowarfare attack, such information could serve as a trigger for activating other bioaerosol sensing systems. Therefore, there is a need to explore new or to modify existing techniques in an effort to develop quick and sensitive methods for determining microorganism concentrations in liquid samples.

Several non-culture methods can be used for the rapid enumeration of microorganisms in liquid samples. Polymerase chain reaction (PCR) is used to amplify small quantities of genetic material to determine the presence of microorganisms. The PCR method is extremely sensitive, but requires special expertise and expensive equipment [12]. Real-time PCR has been applied for the enumeration of microbial particles from air samples [1]. Other instrumental techniques, e.g., chromatography, impedimetry [16], dielectrophoresis [3], have also been used to detect the microorganisms in liquid. However, most of these methods require relatively high concentrations of microorganisms for reliable detection. Henningson *et al.* [13] found that, the Coulter Counter gave higher precision and larger yield compared to microscopy when estimating total microorganism number in a liquid sample. However, the Coulter Counter does not differentiate between biological and non-biological propagules. Day *et al.* [7] demonstrated that flow cytometry could be used to differentiate late-blight pathogen *Phytophthora infestans* from certain other airborne biological particles.

This research investigates the feasibility of using light scattering spectrometry for rapid counting of microorganisms suspended in liquid. In light scattering spectrometry, the particles pass through a sensing zone illuminated by a laser light. Due to the difference in reflective index between the particles and the carrier medium, the light is reflected off the particles and reflection intensity is correlated with particle size. Light scattering technology is relatively inexpensive, and spectrometers employing this technique have been routinely used to monitor non-biological particles in liquids [15]. The light scattering technique has been efficiently used to count and size microorganisms in the airborne state [23, 26]. This method, however, has not been applied to enumerate biological particles in a liquid.

Microbial cells have a significant water content and therefore may have a reflective index similar to that of water which makes the water-borne microorganisms less "visible" to the spectrometer's sensors. Thus, only a fraction of the suspended microorganisms would be counted. In light or epifluorescence microscopy, various stains are applied to the microorganisms to enhance the contrast between the microorganisms and the background, and to facilitate the differentiation of biological particles from the non-biological. A similar effect could be expected for liquid-borne microorganisms that are stained and then counted with the light scattering spectrometer. Furthermore, it could be expected that stained liquid-borne microorganisms could be enumerated more accurately, and that this phenomenon could serve as a basis for differentiating biological and non-biological particles in a liquid sample. Thus, the main goal of this research was to explore the accuracy of light scattering spectrometry when counting liquid-borne microorganisms, and also to investigate whether the counting efficiency is affected by the use of microbiological stains.

MATERIALS AND METHODS

Liquid-borne particle spectrometer. In this research we used a Liquid Optical Particle Counter (LOPC) "Liquilaz S05" (Particle Measuring Systems Inc., Boulder, Colorado) as the liquid-borne particle spectrometer. This instrument, which samples liquids at 20 mL/min, is equipped with a sensor measuring solid non-biological particles from 0.5–20 µm in size, and is capable of counting from $0–2 \cdot 10^4$ particles/mL. The instrument determines the size of the particles and enumerates them in 16 separate size ranges.

Biological particles. The tests were performed with six microorganisms: *Cladosporium cladosporioides*, *Penicillium melinii* and *Aspergillus versicolor* fungal spores, as well as bacterial cells of *Pseudomonas fluorescens* and *Micrococcus* spp., and bacterial endospores of *Bacillus subtilis* var. *niger* (BG). These microorganisms are commonly found in ambient air [9]. We have included BG endospores in several of our previous studies [2, 20, 22, 33] because they are used by the Armed Forces in testing new biosensors for their ability to respond to particles of biological threat, such as airborne *B. anthracis* spores.

Cladosporium, *Aspergillus* and *Penicillium* spp. have been shown to cause allergic and inflammation reactions and to produce mycotoxins which can cause serious health outcomes [4, 5, 8, 10, 14, 21, 28, 35].

C. cladosporioides, *P. melinii* and *A. versicolor* fungal strains have previously been isolated from a mouldy building. Prior to their use in the experiments, they were cultured in Petri dishes with Malt Extract Agar (MEA) (Becton Dickinson Microbiology Systems, Sparks, Maryland, USA), and were then incubated at 25ºC for seven days. The spores were separated from the hyphae by using a method described by Schmechel *et al.* [30], where 0.5 µm diameter glass beads are added to the Petri dish to separate spores from the hyphae, while leaving

most of the hyphae intact. The glass beads with the separated spores were then transferred to sterile deionized filtered water (5 Stage Milli-Q Plus System, Millipore Corp., Bedford, Massachusetts, USA) and vortexed for 1 min. with a vortex touch mixer (Model 231, Fisher Scientific Company, Pittsburgh, Pennsylvania, USA). Once the glass beads had settled, spores suspended in water were separated and used in the experiments. Examination of the spore suspension was conducted under a phase-contrast microscope (Model Labophot 2A, Nikon, Tokyo, Japan).

The *B. subtilis* endospores were received from the US Army Edgewood Laboratories (courtesy of Agnes Akiyemi and Dr. Edward Stuebing, Edgewood Chemical and Biological Research, Center, Aberdeen Proving Ground, Maryland, USA). These spores were washed twice with sterile deionized filtered water by centrifugation at 7,000 rpm for 10 min. *P. fluorescens* (ATCC 13525) cultures were obtained from the American Type Culture Collection (Rockville, Maryland, USA). The *Micrococcus* spp. was isolated from a previously collected air sample and its genus was confirmed using API Staph tests (bioMérieux Inc., Durham, North Carolina, USA). The two latter species were cultured in Petri plates containing Trypticase Soy Agar (TSA) (Becton Dickinson Microbiology Systems, Sparks, Maryland, USA) and were incubated at 30ºC for 18 hours. The cells were then washed off with sterile deionized water. The cells in the obtained suspensions were washed three times by centrifugation at 7,000 rpm for 10 min. Upon their preparation, all microorganisms were suspended in 10 mL of sterile deionized water and their concentrations were determined under a phase-contrast microscope at magnification 400× using a hematocytometer (Petroff-Hausser Counter, Hausser Scientific Partnership, Horsham, Pennsylvania, USA). The microscope-derived microorganism concentrations served as the primary standard when evaluating the performance of the light-scattering spectrometer.

Staining of the biological particles. To potentially increase the reflective index difference between the microorganisms and water, we stained the microorganisms with microbiological stains. The *P. fluorescens*, *Micrococcus* spp. vegetative cells and *B. subtilis* var. *niger* endospores were stained with Acridine Orange (AO) (Sigma Chemical CO, St. Louis, Missouri, USA) and Crystal Violet (CV) (Difco Laboratories, Detroit, Michigan, USA), while the *C. cladosporioides*, *P. melinii* and *A. versicolor* fungal spores were stained with Acridine Orange and Lactophenol Cotton Blue (LCB) (Becton Dickinson Microbiology Systems, Cockeysville, Maryland, USA). For each microorganism, the water-based stain suspension was added to a microbial suspension for 20 min. After staining, the microorganisms were removed from the suspension using centrifugation (7,000 rpm for 10 min.) and were then resuspended in 10 mL of sterile deionized filtered water. The concentrations of stained microorganisms in the suspensions were determined under the microscope using the procedures described above.

Counting the microorganisms with a light-scattering spectrometer. When counting the microorganisms using the light-scattering spectrometer, suspensions of both stained and non-stained microorganisms were diluted from 10^1–10^5 times and each dilution was counted separately. From the counted dilutions, only those that registered between 5,000–15,000 counts (middle of the spectrometer's sensitivity range) were used for data analysis. The obtained counts (Number/mL), were then converted to the microorganism concentration in their initial suspension (i.e., without dilution). The concentrations obtained by counting with the spectrometer, $C_{counter}$, were compared with those obtained using the microscope, $C_{microscope}$. The ratio of the two values, which is an indicator of the counting accuracy, is presented as the Fraction of Counted Microorganisms, η:

$$\eta = C_{counter} / C_{microscope} \qquad (1).$$

The Fraction of Counted Microorganisms served as the primary parameter in determining the optical spectrometer's accuracy when counting the liquid-borne microorganisms. Each measurement was performed three times.

All particles, especially biological ones, are not single-sized, but have a certain size distribution. From the size distribution one can determine the mean (average) size of the particles and also the median (middle value) size. Thus, when a light-scattering spectrometer (the "Liquilaz" Liquid Optical Particle Counter in our experiments) counts the particles, the instrument also determines the size distribution of those particles, i.e., it registers the particles in appropriate size bins. Any changes in the microorganism size distribution due to the application of stains would indicate that the microorganisms' light-scattering properties have also changed. These changes could be used for differentiating biological particles from non-biological ones in a liquid sample. Therefore, we not only counted (enumerated) the stained and non-stained microorganisms using the light-scattering spectrometry, but also determined their size distributions. The size distributions were determined three times for each solution.

Enumeration of microorganisms when both biological and non-biological particle are present in a liquid. We also enumerated the microbial particles when both biological and non-biological particles were present in a liquid. For this purpose, we used Polystyrene Latex particles (PSL) (Bangs Laboratories, Fishers, Indiana, USA) of 0.83 µm diameter as the non-biological particles and *B. subtilis* var. *niger* endospores as the biological particles. In this experiment, 5 mL of sterile deionized filtered water containing a known concentration of PSL particles was mixed with 5 mL of the same purified water containing an unknown concentration of the microorganisms, and the new suspension was analyzed with the

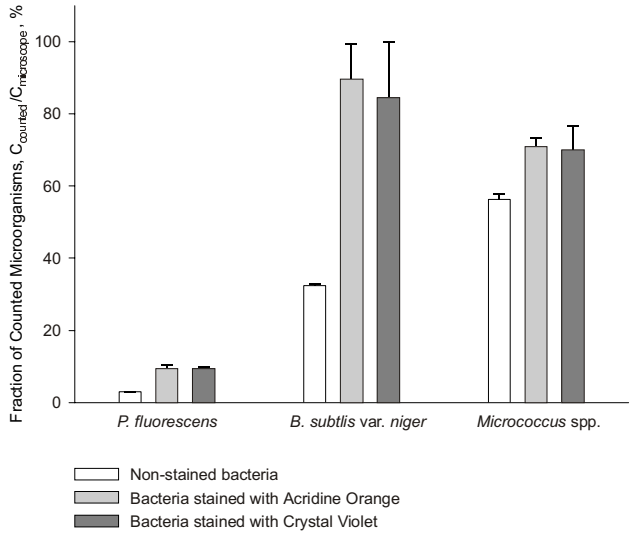

Figure 1. Fractions of three different species of bacteria counted by a light scattering spectrometer with and without stains applied.

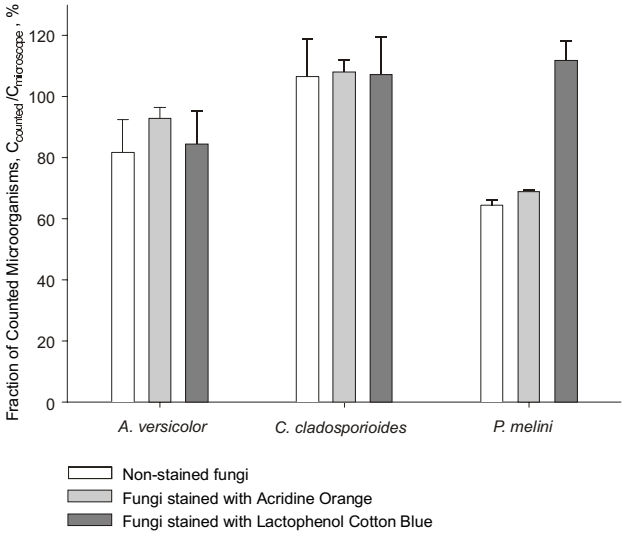

Figure 2. Fractions of three different species of fungi counted by a light scattering spectrometer with and without stains applied.

light-scattering spectrometer. During the analysis, a certain number, $N_{non\text{-}stained}$, was obtained. This suspension was then stained with Acridine Orange for 20 min. After the staining, the endospores and PSL particles were removed from the suspension using centrifugation. The particles were then resuspended in 10 mL of purified water and were counted with the optical particle size spectrometer. During the measurement, a certain number, $N_{stained}$, was obtained. The number of BG endospores in the suspension, N_{BG}, was determined by the following formula:

$$N_{BG} = \frac{N_{stained} - N_{non-stained}}{\eta_{stained} - \eta_{non-stained}}, \quad (2)$$

where $\eta_{non\text{-}stained}$ is the fraction of microorganisms counted when no staining is applied, and $\eta_{stained}$ is the fraction of microorganisms counted when a certain stain is applied. These measurements were repeated three times.

Data analysis. The data analysis was performed using analysis of variance (ANOVA), available as an add-in to Microsoft Excel 2000. *P* values of < 0.05 were considered significant.

RESULTS AND DISCUSSION

Enumeration of microorganisms with the light-scattering spectrometer. The primary goal of this research was to determine the accuracy of the light-scattering spectrometry when counting water-borne biological particles, and to investigate the application of microbiological stains as a means for improving the counting accuracy.

Figure 3 presents the size distributions of three bacterial species used in the tests. These size distributions were measured for both non-stained and stained microorganisms. In this Figure, the ordinate presents the number of microorganisms registered in a specific size range. To avoid artifacts due to differences in the width of the size ranges, the registered number of particles (ΔN) is divided by the difference in logarithm values of the upper (d_u) and lower (d_l) particle diameters of a particular size range. To facilitate the comparison of the size distributions obtained with stained and non-stained microorganisms, the peak of each size distribution was normalized to unity. The peak of *P. fluorescens* bacteria was registered at 0.5 µm, which is the smallest size that the LOPC can register, and the median measured diameter was 0.53 µm. The mean size of the same bacteria in the airborne state measured with a light scattering spectrometer was 0.6 µm [22]. Thus, it is likely that due to a small difference between the reflective indices of water and bacteria the laser light is minimally reflected which causes the LOPC to "miss" the bacteria. The size distribution of *P. fluorescens* bacteria stained with Crystal Violet (CV) was essentially unchanged, although slightly more particles larger than 0.7 µm were registered. The size distribution of *P. fluorescens* stained with Acridine Orange (AO), however, indicated several times more particles larger than 0.7 µm than for non-stained bacteria. The median diameter of *P. fluorescens* stained with AO increased to 0.68 µm from the 0.53 µm median diameter for non-stained bacteria. The actual median diameters of liquid-borne bacteria, as determined by light scattering, are likely to be smaller than the values indicated above because this spectrometer only registers particles larger than about 0.5 µm. However, the observed increase in median size suggests that application of stains increases the difference between the reflective indices of *P. fluorescens* bacteria and water, thus intensifying the light scattering.

The size distribution of *B. subtilis* var. *niger* endospores remained essentially the same for non-stained and stained spores. The median measured diameter of the spores was 0.73 µm in all three cases. The size distribution of the third bacterium used in the tests,

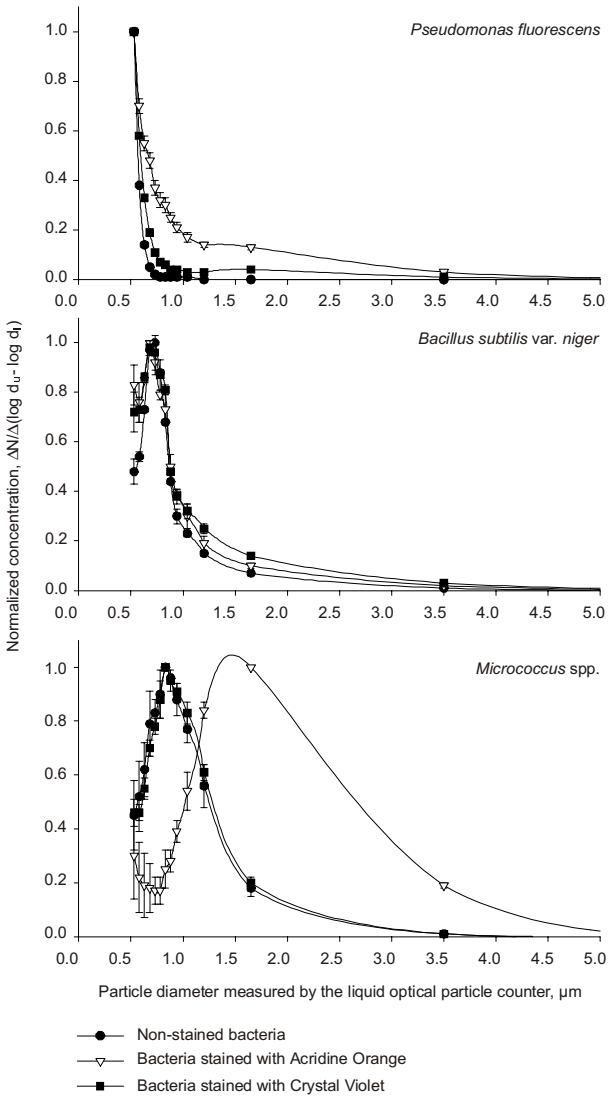

Figure 3. Size distribution of three different bacteria determined by a light scattering spectrometer with and without microbiological stains applied.

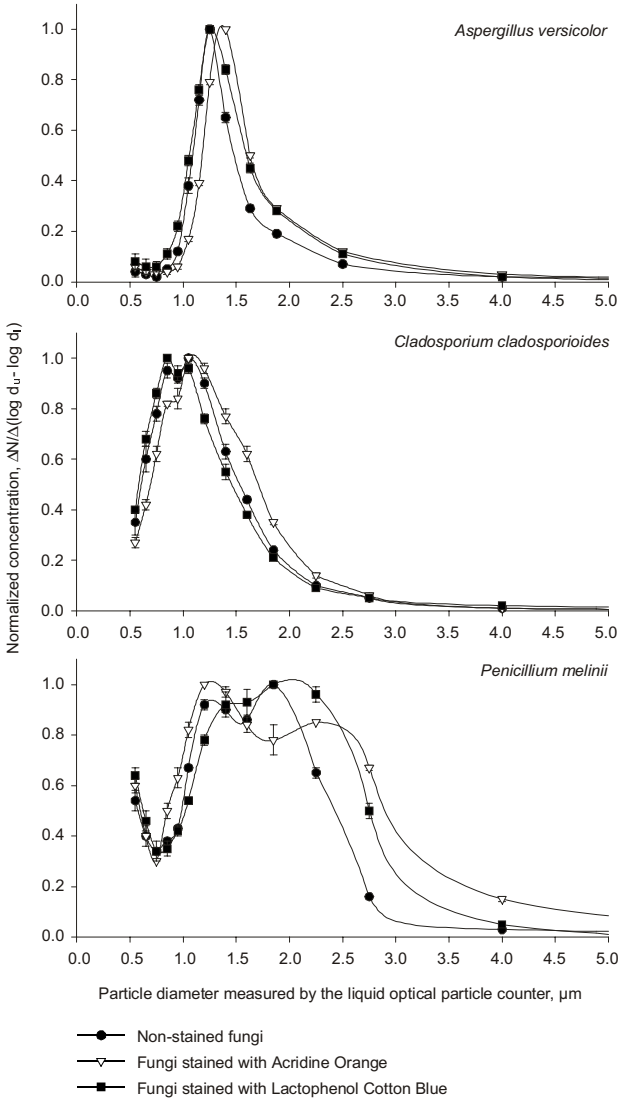

Figure 4. Size distribution of three different fungi determined by a light scattering spectrometer with and without microbiological stains applied.

Micrococcus spp., significantly changed when stained with AO. Its median size increased almost twice: from 0.8 μm (non-stained bacteria) to about 1.5 μm (bacteria stained with AO).

These results show that application of microbiological stains may significantly affect the light-scattering intensity of some bacteria. This increase in intensity causes a shift in median bacterial diameter, as registered by a light-scattering spectrometry.

Figure 1 compares the concentrations of bacteria in liquid, as determined by the light-scattering spectrometer, with the bacterial concentrations determined under a microscope. The comparison was performed for both stained and non-stained bacteria. In this Figure, the ordinate shows the Fraction of Counted Microorganisms, η. For easier comparison, η is presented not as a dimensionless ratio (as in Equations 1 and 2) but as a percentage value. As seen in this figure, only about 3% of non-stained *P. fluorescens* bacteria were counted by the spectrometer, while more than 9% (three-fold increase) of the stained bacteria were registered by the light-scattering spectrometer. This difference was statistically significant with $p < 0.0001$. There was no statistical difference in counts of *P. fluorescens* bacteria when stained with AO versus CV ($p > 0.05$). The spectrometer counted about 32% of non-stained *B. subtilis* endospores compared to the count obtained by the microscopic counting technique ($\eta_{non\text{-}stained} = 0.32$). This fraction increased to about 89% (2.5-fold increase) when the endospores were stained with AO, i.e., $\eta_{stained} = 0.89$. This difference was statistically significant ($p < 0.01$). However, there was no statistically significant difference in the count of *B. subtilis* endospores stained with AO versus CV ($p > 0.05$). Among the three bacterial species tested, *Micrococcus* spp. resulted in the largest fraction counted of non-stained bacteria: 56%. This fraction increased to 70% ($p < 0.05$) when the microorganisms were stained and then counted. As in the previous two cases, there was no statistically

significant difference when counting the microorganisms stained with AO versus CV ($p > 0.05$). The obtained results show that stained bacteria scatter more light than their non-stained counterparts and, thus, the light-scattering spectrometer registers significantly more stained bacteria compared to the non-stained ones.

Figure 4 shows the size distributions of three fungal species used in the tests. These size distributions were measured for both non-stained and stained micro-organisms. Similar to Figure 3, the ordinate shows the normalized number of microorganisms registered in specific size bins. The measured median diameter of non-stained *A. versicolor* was 1.25 µm, and increased to about 1.4 µm when this fungus was stained with Lactophenol Cotton Blue (LCB). The median size of *A. versicolor* stained with Crystal Violet (CV) was about 1.3 µm. The median diameter of non-stained *C. cladosporioides* was 0.95 µm and did not change when this fungus was stained with LCB. The median diameter slightly increased (to about 1.1 µm), however, when *C. cladosporioides* was stained with Acridine Orange (AO). The median diameter of *P. melinii* was about 1.6 µm and increased to about 2 µm when this fungus was stained with AO and LCB.

The aerodynamic diameter of the fungal spores investigated in this research ranges from 1.8–3.1 µm [27]. Thus, similar to results obtained with bacteria, it seems that the difference between the reflective indices of non-stained fungal spores and water is small which results in low light scattering intensity of the LOPC. Therefore, the diameters of water-borne fungal spores registered by the LOPC are smaller than those measured for spores in the airborne state. Application of microbiological stains causes only a relatively small increase in light scattering intensity by the fungal spores and, therefore, their diameters measured by the LOPC change only slightly.

Figure 2 compares the concentrations of fungal spores in the liquid, as determined by the light-scattering spectrometer, with the fungal concentrations determined under a microscope. The comparison was performed for both stained and non-stained fungal spores. As seen in this Figure, the instrument counted 82% of non-stained *A. versicolor*. This fraction increased to 92% and 84%, when this fungus was stained with AO and LCB, respectively. Either change was not statistically significant ($p > 0.05$). Figure 2 shows that about 100% of non-stained *C. cladosporioides* were enumerated by the spectrometer. About the same number ($p > 0.05$) of these fungi was enumerated by the spectrometer when they were stained with AO or LCB. The high fraction of counted *C. cladosporioides* spores, both with and without staining, can be explained by the dark pigmentation of these spores. Oval *C. cladosporioides* spores have a brown-olive to brownish-black hue and such dark objects scatter enough light to be registered by the spectrometer. As seen in Figure 2, about 64% of non-stained *P. melinii* were counted by the LOPC. The counted fraction increased to 69% ($p < 0.05$) when this fungus was stained with AO. When *P. melinii* was stained with LCB, the spectrometer counted more particles than were observed under the microscope, i.e., the fraction of counted fungi exceeded 100%. This discrepancy may be due to the optical particle spectrometer registering some of the small propagules (they became more "visible" after staining), which were not counted under the microscope. Therefore, the spectrometer registered more *P. melinii* propagules stained with LCB compared to the number of registered non-stained spores. However, since the size distribution is normalized to unity, this increase in not very distinct in Figure 4. The counted fraction of *P. melinii* stained with LCB was statistically different from that of the non-stained fungus ($p < 0.001$).

The obtained results show that among the three investigated species of fungi, only *P. melinii* became more "visible" to the light-scattering spectrometer when it was stained, i.e., the fraction of counted particles increased 1.5 times when compared to non-stained microorganisms. Apparently, the changes in light-scattering properties of fungi depend on the species investigated. Similar to this result, Day *et al.* [7] observed that the addition of fluorescent brightener enabled differentiation between *P. infestans* sporangia and powdery mildew conidia when using flow cytometry.

Enumeration of microorganisms when both biological and non-biological particles are present in a liquid. When both biological and non-biological particles are present in a liquid, both of these particles are counted by a light-scattering spectrometer, if their particle sizes are within the size measurement range of this instrument. In this case, the obtained count would overestimate the concentration of biological particles present. However, Figures 1–4 show that more biological particles of certain species are counted by a light-scattering spectrometer once these microorganisms are stained. Thus, we hypothesized that this observation could be used as the basis for differentiating the concentration of biological particles from that of non-biological particles, when both types are simultaneously present in a liquid sample.

This assumption was tested using 0.83 µm diameter PSL particles and *B. subtilis* var. *niger* endospores employing the procedures described above. As determined earlier, $\eta_{non\text{-}stained} = 0.32$ and $\eta_{stained} = 0.89$ for *Bacillus subtilis* var. *niger*. During the experiments, $N_{stained}$ was measured as $10 \cdot 10^7 \pm 0.6 \cdot 10^7$ and $N_{non\text{-}stained}$ as $6 \cdot 10^7 \pm 0.4 \cdot 10^7$. Thus, using Eq. 2, the number of BG spores in the suspension, N_{BG}, was estimated to be $7 \cdot 10^7 \pm 0.4 \cdot 10^7$ per mL. The number of BG spores obtained by counting under the microscope, $N_{microscope}$ was about $7.3 \cdot 10^7 \pm 1.0 \cdot 10^7$ per mL. The difference between N_{BG} and $N_{microscope}$ was not statistically significant. Thus, this result shows that the suggested method for differentiating biological and non-biological particles is feasible if the fractions of counted microorganisms applied, with and without staining, are known beforehand. This can be achieved by performing experiments and creating a database of counted fractions for various microorganisms.

CONCLUSIONS

The use of light-scattering spectrometry for counting biological particles suspended in liquid samples may become a fast and reliable sample analysis method. The performed experiments have shown that the accuracy for determining the concentration of biological particles depends significantly on the microorganism species investigated. For some bacteria, the fraction of counted particles can be as low as a few percent. However, once the fraction of a specific microorganism counted by the light scattering spectrometer is known, this technique could be used for fast analysis of environmental samples or laboratory suspensions containing known species.

This research has shown that the light-scattering properties of microorganisms suspended in water may be varied by the use of microbiological stains. Thus, the fraction of microorganisms counted by a light scattering spectrometer depends on whether microbiological stains are applied. The actual increase in count depends on the microorganism species investigated and the microbiological stain used. It may also depend on the lowest (threshold) particle size detectable by the light-scattering spectrometer. Our experiments indicate that the difference in particle count between stained and non-stained microorganisms may be used as a basis for differentiating biological and non-biological particles in liquid samples. This approach is encouraging for developing a fast analysis method for liquid samples.

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