Baseline and *in Vivo* Total Photoluminescence of Endospore Material Using the Parasitoid Wasp *C. congregata*

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In a laboratory test, total luminescence spectroscopy was used to detect and measure the *in vivo* presence of a biohazard surrogate, endospores of *Bacillus megaterium*, within the parasitoid wasp *C. congregata* (Say) (Hymenoptera:Braconidae). Upon emergence, exposed wasps were allowed to feed on five different concentrations of endospores suspended in 5 mL of honey solution. After 12 h insects were chilled at −80 °C for 5 min to permit capture. Aqueous suspensions were prepared by homogenizing the wasps in 3 mL of deionized water. The total excitation-emission matrix (EEM) was measured for each suspension by using an SLM Series 2 luminescence spectrometer. For wasps exposed to spore concentrations of 3.0×10^2 to 3.0×10^6 colony forming units (CFU)/mL, two intensity maxima were observed. The emission for tryptophan was identified at excitation (Ex) 300 nm and emission (Em) 350 nm. A second emission that resulted from other biological materials including nitroheterocyclic compounds and endospores occurred at Ex 350 nm and Em 420 nm. Changes in the ratio of intensity between the tryptophan and second emission were found to be related to endospore material present in the suspensions at original feeding concentrations of 15 000 to 15 million spores. Intensity ratios for the positive samples equaled 0.9, while the intensity ratios for the control equaled 1.9. One notable difference recorded for the emission spectra was an apparent, but minor, red shift of approximately 5 nm of the second emission when compared against a signature library of pure cultures. Diagnostic information such as this should contribute to methods for the detection and potential identification of biohazard materials with the use of photoluminescence.

Index Headings: **Endospores; Total photoluminescence spectroscopy; Biohazard.**

INTRODUCTION

Bacterial endospores are incredibly hardy life forms that can survive in storage or in the open environment for years, emerging when conditions are appropriate. One species of the genus *Bacillus* has demonstrated viability (e.g., germination and growth) after extraction from the gut of a bee that had been preserved in amber since the Jurassic period.[1] Interest in detecting biohazards for a wide variety of applications ranging from human to environmental health is a primary concern. The toxins associated with some endospores are well known and present a genuine human health risk. In fact, the lethal effects of anthrax (*Bacillus anthracis*) were ascribed to an exotoxin almost a half-century ago. Subsequent work has led to the molecular identification and enzymatic characterization of three proteins that constitute two anthrax toxins, as well as characterization of the relevant gene sequences.[2]

Foraging insects could potentially assimilate endospore material during day-to-day activities and concentrate this material from a point of origin. Studies that utilize many species of insects are being investigated to establish passive detection. Bromenshenk et al.[3] report ongoing research that uses bees to monitor microbial pesticides and their effects on nontarget organisms. In this regard, locally occurring insects could serve as part of an early warning system to indicate the magnitude and direction of a contaminant threat. One advantage of insects as carriers is that they are typically unharmed by the protein toxins that render humans and other vertebrates ill. Therefore, insects that have been conditioned to recognize and forage specific materials could serve as important parts of an overall detection or monitoring system, acting as prime carrier systems responding to scent or pheromone cues. Additionally, insects have demonstrated a learned behavior to retrieve materials, and neurobiological control of insects has been demonstrated by using experiments performed on moths to control insect flight direction with the use of odor modulation.[4,5]

While the use of carrier insects for biohazard monitoring appears promising, some basic issues need to be addressed: (1) the collection of a representative consortium of carrier insects, and (2) confident assays for the presence of endospore material. To accomplish this, we are now conducting research on individuals within local insect communities and their potential as living biohazard carriers to serve as early warning systems. Here, we present results from research focused on the extraction of endospore photoluminescence signatures detected *in vivo* from carrier insects.

Madigan et al.[2] and Holt et al.[6] present standard laboratory methods (e.g., culture methods, microscopy, and plate counts) for identifying bacteria types and concentrations. The limitation of standard methods is that they can be time consuming and may not be expedient enough to quickly detect and quantify a biohazard threat. Additionally, standard methods (e.g., epifluorescence microscopy) are designed for vegetative cells and not typically designed to detect and measure the spectral properties of endospores.[7]

The detection of endospores can be accomplished by using a variety of methods including polymerase chain reaction; however, one successful approach has been the application of spectral photoluminescence, first described by Lewis.[8] Photoluminescence spectroscopy is an optical, analytical chemistry technique also known as total luminescence spectroscopy, or TLS.[9] TLS is described as the simultaneous measurement of excitation, emission, and intensity spectra for a sample of interest.[9,10] This approach permits the recording of a specific luminescence spectral signature in three dimensions. Time, where rel-

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evant to analysis or signature modeling, can also be considered a fourth dimension variable.

Investigations by Dalterio et al.,[11] Bronk and Reinisch,[12] Rosen et al.,[13] and Faris et al.[14] demonstrated both single and multiple excitation and emission photoluminescence of vegetative cells and endospores. Rosen et al.[13] and Pelligrino et al.[15] determined specific wavelength pairs for detecting endospores of *Bacillus spp.* in aqueous suspensions based on terbium-enhanced photoluminescence of dipicolinate acid (dpa). Recently, Fell et al.[16] reported an improvement of the method in the presence of phosphate ions, a major constituent in biological materials. Using TLS, Anderson et al.[17] have measured and catalogued the unenhanced fluorescence of both prokaryotic endospores and eukarotic cysts at a variety of concentrations in nonfluorescent, neutral media such as deionized water and formalin. These measurements are being collected as baseline signatures for matching target-to-library fluorescence spectra. Baseline data will eventually assist the separation of endospore spectra from background constituents including proteins, amino acids, and other biological materials encountered *in vivo*.

MATERIALS AND METHODS

Preparation of Endospores. *Bacillus megaterium* was selected as a biohazard surrogate for this experiment due to its relatively large spore size and known quantum efficiency. Cultures of *B. megaterium* were prepared from stock suspensions. Endospores for each were recovered, filtered, and concentrated by using a 4% solution of formalin. Five concentrations of *B. megaterium* were suspended in deionized water to establish concentration and fluorescence relationships. The same endospore concentrations were prepared with a honey-feeding solution to present to the wasps. All concentrations were prepared by decimal dilution, resulting in the following gradients: 3.0×10^6 colony forming units (CFU)/mL; 3.0×10^5 CFU/mL; 3.0×10^4 CFU/mL; 3.0×10^3 CFU/mL, and 3.0×10^2 CFU/mL.

Preparation of Carrier Insects. For this study, three groups ($n = 20$) of the parasitoid wasp *Cotesia congregata* were reared in a microcosm on the host Manduca sexta L. (Lepidoptera: Sphingidae) as described by Kester and Barbosa.[18] *C. congregata* was selected because (1) it represents genetically pure stocks reared on site, and (2) it is being used in related research in biological contaminants monitoring due to known post-emergence feeding activities. This parasitoid immediately seeks a nectar source for feeding after emergence from a host. The feeding activity of *C. congregata* is followed by a short life cycle that involves mating, egg laying, and eventually death.[18]

Upon emergence, the wasps were exposed to the honey solutions that contained the five concentrations of endospores (see ''Preparation of Endospores'') for 24 h. Nine individuals from each of the five treatment groups were sampled and chilled at −80 °C for 5 min, then homogenized in 3 mL of deionized water with the use of a Tekmar microblender. The resulting suspensions were transferred to sterile mirror cuvets for luminescence spectroscopy.

Endospore Concentrations by Total Luminescence Spectroscopy. Baseline and *in vivo* photoluminescence measurements were collected on endospores of *B. megaterium* to measure sample wavelength excitation, emission, and intensity. All measurements were made by using an AMINCO SLM Series 2 luminescence spectrometer that incorporated a xenon arc lamp as the illumination source and stepper monochromators to control both the excitation and emission wavelengths. The spectrometer is completely computer controlled and was configured to excite from 300 to 600 nm in 10 nm increments. Emission wavebands (300 to 800 nm) were configured to record in 5 nm increments. Endospore material was transferred to mirrored, $10 \times 10 \times 45$ mm quartz cells (cuvets) and baseline fluorescence spectra were measured (with 90° geometry) by recording the excitation (Ex), emission (Em), and intensity for each aqueous suspension. These cells have an internal mirror facing the excitation beam that improves the efficiency of the excitation wavelength by reducing scattering and concentrating available light in the sample.

Second-order diffraction effects were corrected by matching long-pass emission filters. Final measurements were corrected by using instrument software that considers detector quantum efficiency, bandwidth, and transmission factor of the monochromator (SLM). All data were analyzed with the use of Matrix Laboratory (MATLAB) software.

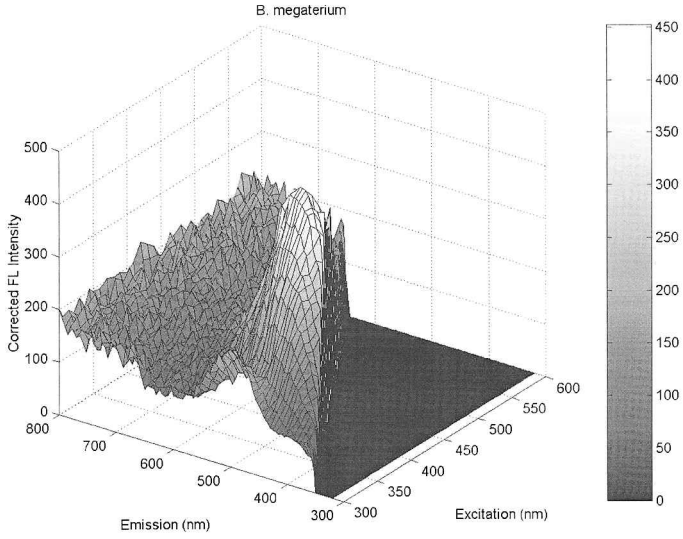
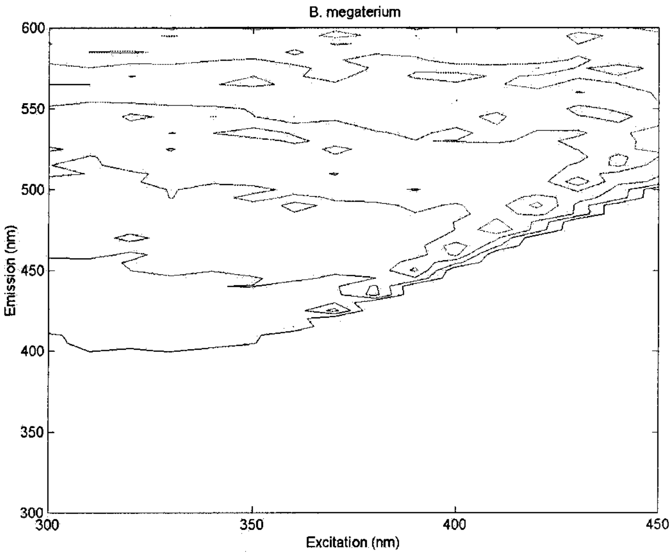
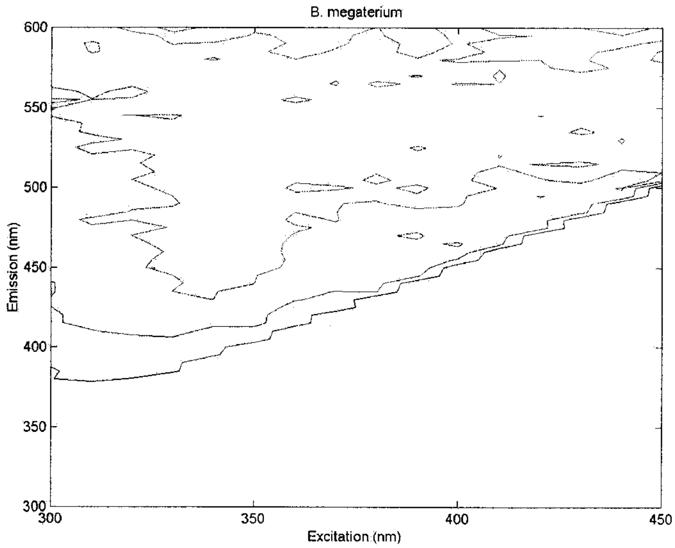


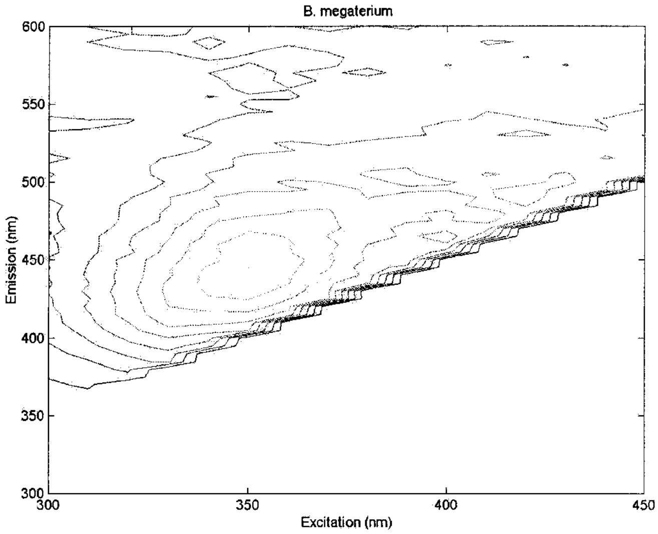
FIG. 1. Total luminescence spectrum for *B. megaterium*.

RESULTS

Baseline Endospore Photoluminescence. The total EEM spectrum for endospores *of B. megaterium* (3.0×10^4 CFU/mL) in deionized water is shown in Fig. 1. The intensity maxima occur at a wavelength pair of Ex 350 nm and Em 450 nm. This spectrum represents baseline three-dimensional measurements for endospores at known concentrations in deionized water. Results of luminescence spectroscopy for concentrations of endospores of *B. megaterium* in deionized water (control) at pH 7.0 and a temperature of 20–23 °C are shown in Figs. 2–5. The contour plots show the total excitation and emission matrices and provide information on the loca-







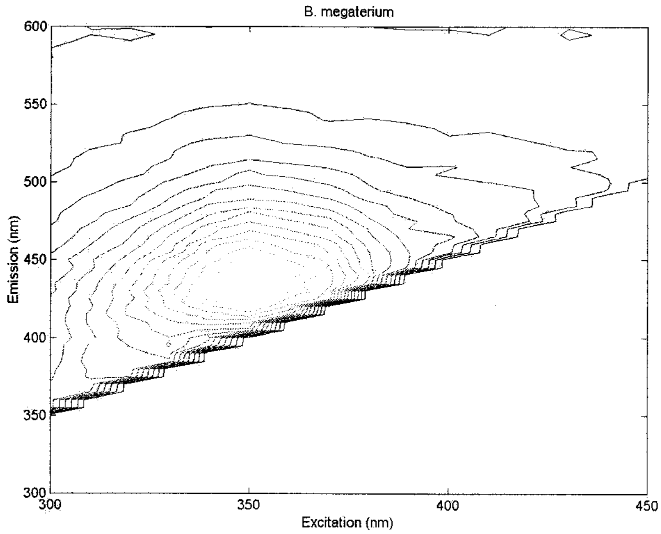


FIG. 2. (Top left) Deionized water blank control.
FIG. 3. (Top right) *B. megaterium* 1500 CFU, fluorescence intensity = 120.
FIG. 4. (Bottom left) *B. megaterium*, 15 000 CFU, fluorescence intensity = 170.
FIG. 5. (Bottom right) *B. megaterieum,* 150 000 CFU, fluorescence intensity = 420.

tion of the sample wavelength excitation and emission intensity maxima. These measurements have been corrected for detector gain and offsets. The recorded emission maxima are primarily a function of the exosporium or protein coating that surrounds the spore case.[2] Other contributions to the signature potentially include a number of bacterial cell constituents that are fluorophores, including nitrogenheterocyclic pteridines, flavins, and pteridine coenzymes.[11]

The centroid of the emission maxima occurred at 435 nm (at Ex 350 nm), and intensity changes were recorded with the sample volume as shown in Fig. 6. These results are comparable to those reported by Bronk and Reinisch[12] for endospores of *B. megaterium* and vegetative cells of *S. epidermidis.*

A relationship between endospore concentration and fluorescence intensity measured at Ex 350 nm is shown in Fig. 7. Similar intensity-volume responses have been documented for endospore material by using extracted dpa. Madigan et al.[2] provide procedural guidelines for microbial optical-volume determinations. Signatures for endospore CFUs at or below 3.0×10^2/mL approached the limits of detection with a marked decay in emission signature approaching that of the deionized water control (see Figs. 2 and 3). Endospore concentrations ranging from 3.0×10^4 to 3.0×10^7 CFU/mL were effectively

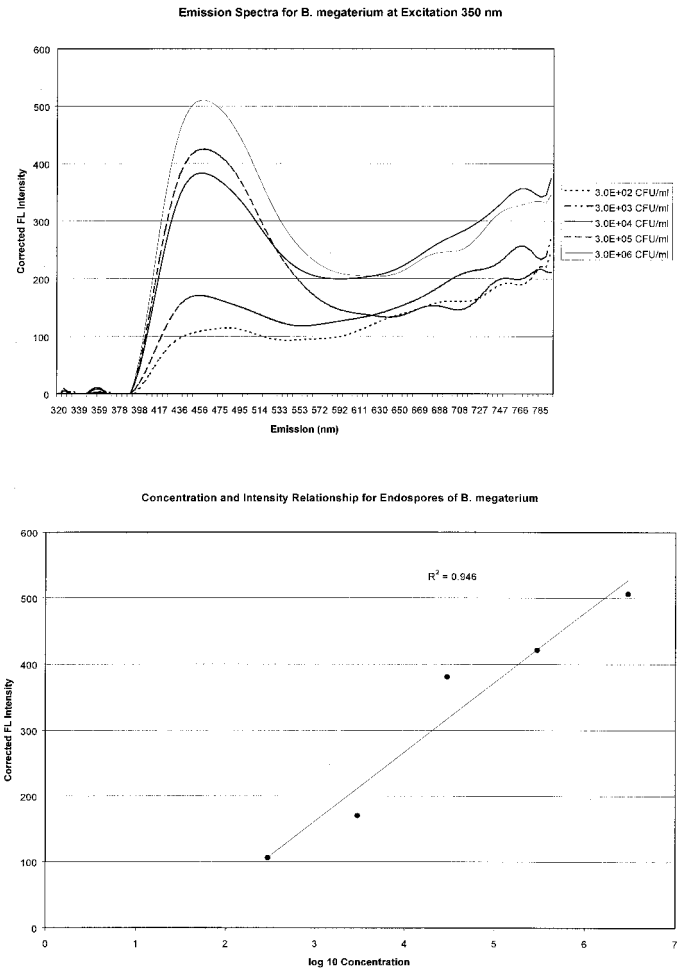


FIG. 6. (Top) Sample luminescence emissions at Ex 350 nm for endospore concentrations of *B. megaterium.*
FIG. 7. (Bottom) Model of endospore concentration and intensity at Ex 350 nm.

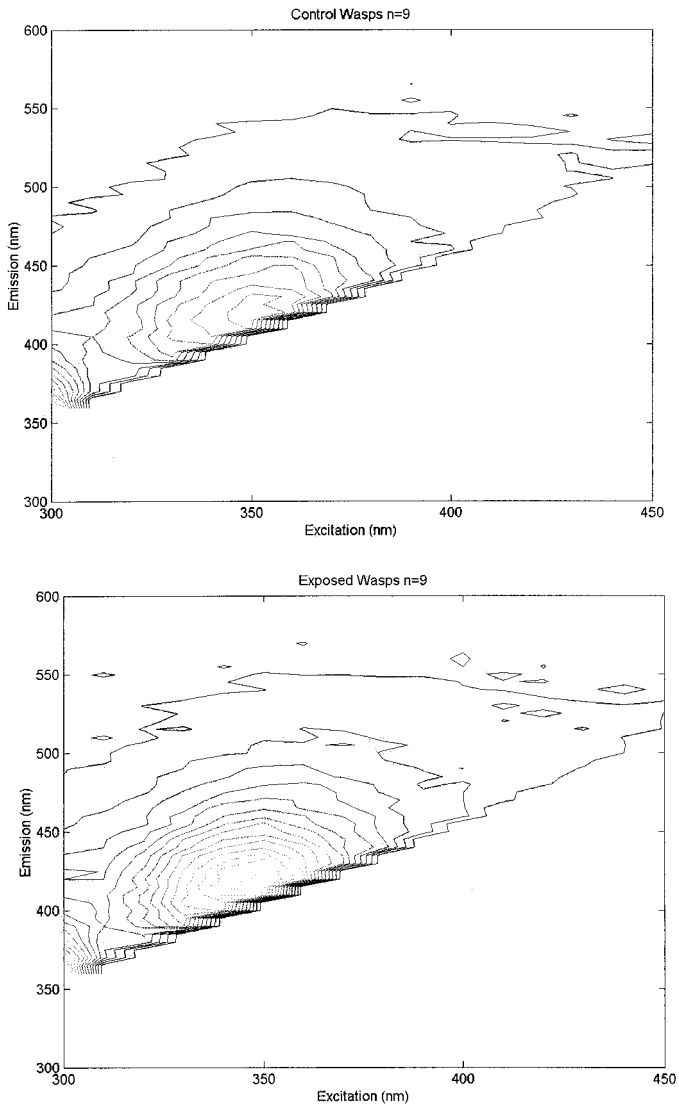


FIG. 8. Contour plots representing total luminescence emission spectra for control—no exposure (top) and exposed (bottom) for *C. congregata.* Sample $n = 9$ individuals.

detected and characterized by TLS, suggesting a concentration/optical density relationship. In experiments, Bronk and Reinisch[12] calculated endospore quantum efficiency (QE) for *B. megaterium* and found sample optical density to be a function of near-total absorption of the excitation wavelength. These findings suggest that, while not changing signature excitation or emission wavelengths, higher spore concentrations exhibit higher absorption maxima and vice versa. This result would translate into an accurate measure of endospore volumes based upon detected emission maxima.

Photoluminescence of Insect Carriers. Total luminescence spectroscopy of insect carriers yielded results that showed that the characterization of the biohazard surrogate (*B. megaterium*) could be detected *in vivo.* Changes in emission spectral characteristics at two excitation wavelengths proved diagnostic in the detection of the presence of endospore material assimilated by the wasp *C. congregata.* Three replicate samples were acquired and all results averaged (Fig. 8). The plots show the primary emission at 350 nm (Ex 300 nm) for the amino acid tryptophan and a second emission that occurred at 420 nm (Ex 350 nm). The second emission appears to be consistent with high quantum yield, aromatic-based proteins, and nucleic acids that would be contained in the tissue suspension plus the endospore material. Photoluminescence increased dramatically in intensity at this wavelength upon exposure of the carrier insects to endospore material.

The intensity changes observed in these measurements show that, when compared to the unexposed control wasps, exposed wasps exhibited near-equal responses in intensity of the tryptophan and second emission (Fig. 9). Ratios of the intensity changes resulted in values of 0.9 (Em 360 exposed/Em 420 exposed) and 1.9 (Em 360 control/Em 420 control). The ratios held constant for exposures at concentrations between 3.0×10^3 and 3.0×10^6 CFU/mL. Endospore concentration levels below 3.0×10^3 CFU/mL resulted in ratios approaching that of unexposed or control wasps (ratio = 2). A relationship was also observed between the fluorescence intensity and endospore concentration measured *in vivo.* A comparison of emissions from unexposed and exposed insects is presented in Fig. 9. This graph shows the intensity relationships between exposed and unexposed homogenized wasp suspensions as a function of the measured emis-

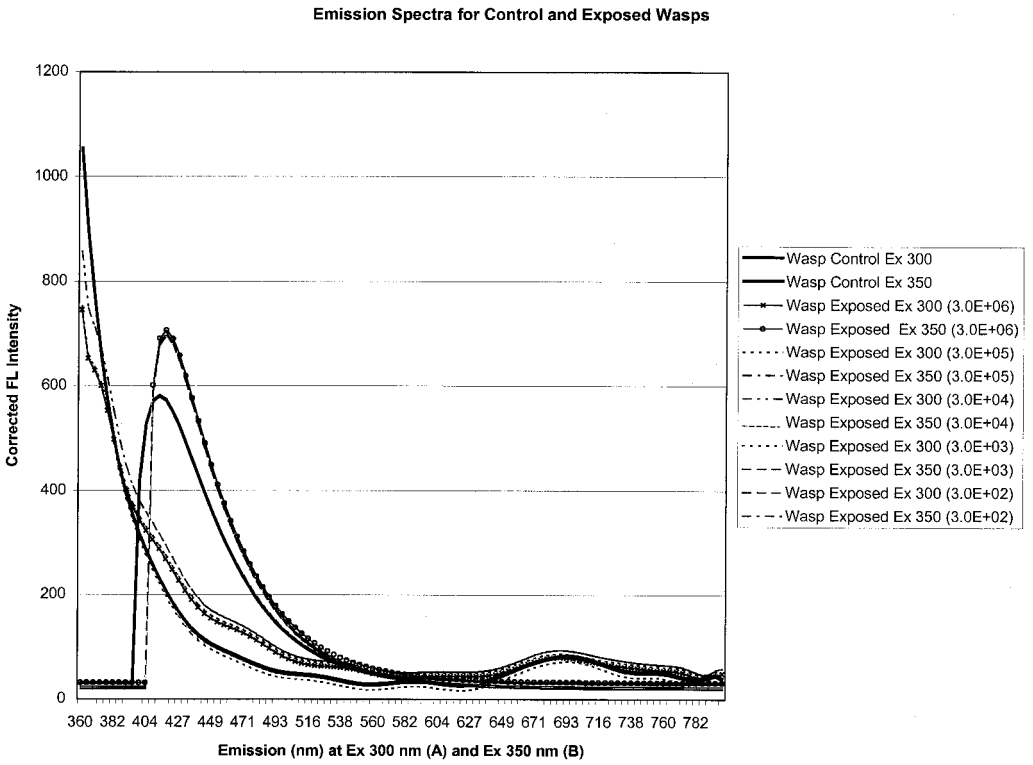


FIG. 9. Graph of Ex 300 nm (A) and Ex 350 nm (B) emission spectra from *C. congregata.* Thick solid lines indicate control measurements. Thin solid lines with symbols indicate highest exposure measurements.

sions. Regression analysis presented in Fig. 10 shows the concentration and fluorescence intensity relationship for both the tryptophan and second emissions. This analysis demonstrates a trend toward a gradual increase in intensity with an increase in exposure (endospore) concentration.

DISCUSSION

For the insect carriers analyzed, a threshold of detection was established. The results showed the presence of endospores occurring at concentrations of less than 3.0×10^3 CFU/mL were below detection limits for this experiment. At lower concentrations, it became apparent that extracting a meaningful signature, while maintaining a high signal-to-noise level, would be difficult without chemically enhancing photoluminescence, as presented by Fell et al.[16]

Two spectral signature characteristics emerged as part of the intrinsic steady-state photoluminescence measured in this experiment. The spectra recorded for exposed and unexposed insects showed both intensity and positional changes of the emission maxima when compared to a baseline of library signatures for pure endospores of *B. megaterium*. The most notable change was the tryptophan emission recorded at Em 360 nm (Ex 300 nm). This signature occurred in both exposed and unexposed homogenized insect suspensions, but was absent in control spectra for pure cultures of endospores. Reductions in intensity maxima were observed for insects exposed to prefeeding concentrations of endospores between 3.0×10^3 to 3.0×10^6 CFU/mL. Also, the exposed insects exhibited reduction in intensity of the tryptophan signature that approached the intensity level measured for the second emission (see Fig. 9).

The second emission exhibited the most dramatic change from unexposed to exposed samples. The region characterized by this signature involved the emission at 420 nm (Ex 350 nm). An increased intensity was observed at this wavelength in wasps that had been exposed to endospore material of concentrations greater than 3.0×10^3 CFU/mL. The use of linear regression to analyze the relationship between intensity and endospore concentration showed that a gradual increase is observed due to spore numbers and potentially higher quantum efficiencies afforded by the increased volume.

CONCLUSION

We found that total luminescence spectroscopy offered an effective way to detect the presence or absence of endospore material *in vivo* by using wasps as insect carriers of the biohazard surrogate *B. megaterium.* An advantage of TLS was the characterization of the excitation-emission spectrum for both pure cultures of endospores and (exposed and unexposed) homogenized insect carriers. This method was successful in the isolation of specific wavelength pairs that could be used to control a smaller, simpler detection system in which carrier organisms are used in monitoring.

The reduction in intensity of tryptophan in the primary emission wavelength and a minor red shift recorded for the secondary emission wavelength for insect suspensions were major deviations from baseline (pure) endospore luminescence measurements. As with any optical technique that involves the detection of biological con-

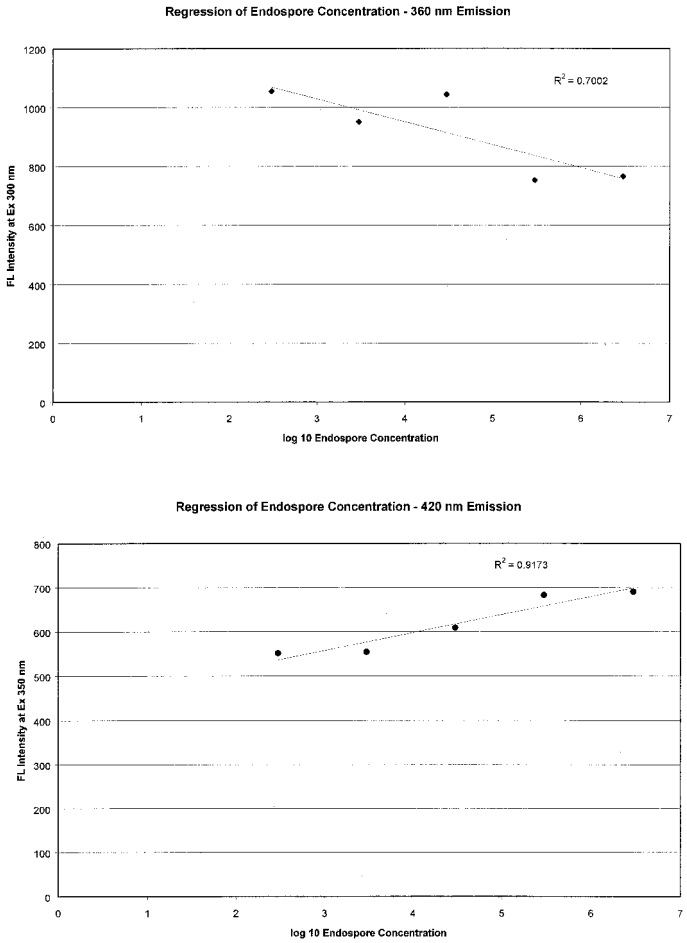


FIG. 10. Regression of endospore concentration on fluorescence intensity.

stituents, more basic analytical chemistry needs to be performed to identify and quantify the materials that cooccur with the material of interest. Additionally, the synergy between known fluorophores and accessory materials should be investigated as well as the potential decay rates of signatures that may constitute a change from those observed in baseline measurements. Finally, enhancement of the *in vivo* photoluminescence of endospore material should be investigated as well as defined target substrate media and fluorescence immunology techniques. All of these analyses are planned for future field investigations that involve other insect consortia.

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