IN SITU DETECTION OF DEAD/LIVE BACTERIA BY MEANS OF FLUORESCENCE SPECTROSCOPY

An Undergraduate Research Scholars Thesis

by

MATTHEW WALCK

Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Peter Rentzepis

May 2017

Major: Electrical Engineering

TABLE OF CONTENTS

ABSTRACT

In Situ Detection of Dead/Live Bacteria by Means of Fluorescence Spectroscopy

Matthew Walck
Department of Electrical Engineering
Texas A&M University

Research Advisor: Dr. Peter Rentzepis
Department of Electrical Engineering
Texas A&M University

Bacterial infections cause thousands of deaths every year. A method for fast, in situ detection of bacteria and confirmation of their live/dead status would be invaluable. Excitation-emission matrices (EEMs) and synchronous fluorescence spectroscopy were used to study both individual bacterial components and whole bacteria and their reaction to ultraviolet radiation. Principle Component Analysis (PCA) was used to identify and plot the differences between the spectra. The results from these experiments have led to the development of a method of detecting bacteria and determining their viability. The results from this research will allow better prevention of infection and treatment of wounds.

ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Peter Rentzepis, for his patience and continual willingness to provide insight and advice. His guidance was invaluable in the completion of this research. He sets a great example as a researcher and mentor, and I have learned a lot from him.

I'd also like to thank Umang Goswami for his advice and expertise, as well as his assistance in obtaining bacterial cultures. Finally, I would also like to thank Omar Ashour, for providing several of the Matlab programs used in this research.

CHAPTER I

INTRODUCTION

Bacterial infections are responsible for loss of life in the United states. It is estimated that there are over two million infections, causing twenty-three thousand deaths, each year from antibiotic resistant bacteria alone[1]. There are nearly one million healthcare-associated infections (HAIs) in the US every year, resulting in over 70,000 deaths[2]. Many of these infections could be prevented by a method of rapidly detecting bacteria and determining their viability. However, current methods for determining the live/dead status of bacteria, such as bacterial cultures, are often slow, involving incubation times measured in hours[3]. The time cost of these methods makes infeasible the large-scale detection and elimination of bacteria in hospital and clinic settings, increasing the risk of human infection.

Principle Component Analysis, PCA, has been shown to be useful for bacterial identification [4]. PCA is a statistical algorithm that allows a multidimensional dataset to be reduced in dimension[5]. It accomplishes this by organizing the dataset so that its dimensions are arranged by the amount of the data's variance contained in each dimension. This allows the data to be visualized in fewer dimensions than the original dataset without a loss of valuable information. Excitation-emission matrices are useful tools in fluorescence spectroscopy due to their ability to display all the spectroscopic information three dimensionally[6]. EEMs are taken by combining multiple individual fluorescence spectra, using varying excitation wavelengths, that are plotted in three dimensions where one axis is the excitation wavelength, another is the emission wavelength, and the third is the intensity of the emitted light. When used in conjunction with

multivariate methods, like PCA, they can "identify various independent fluorescence groups in the fluorescence spectra" [7]. Synchronous fluorescence spectroscopy differs from conventional fluorescence spectroscopy in that, in synchronous spectroscopy, the excitation wavelength is scanned at the same rate and time as the emission wavelength, while, in conventional fluorescence spectroscopy, the excitation wavelength remains constant and only the emission wavelength is scanned [8]. Synchronous spectroscopy is useful for multicomponent analysis for samples that cannot be resolved via conventional fluorescence spectroscopy[8] and has been used to identify a number of bacteria[9].

The objective of this study is to develop a method for fast, on site detection of bacteria and immediate determination of the mortality status of the in-situ bacteria via various spectroscopic techniques. A handheld system that consists of a spectrometer and a UV source has been designed as a part of this study. This system would also be capable of bacterial detection in areas where conventional detection methods would not be feasible, such as in the field away from capable medical facilities, and thus allow for more effective treatment of bacterial infections and other contaminations. In addition to detection, the system would have the ability to destroy the pathogens via its UV source before they can infect human hosts. This would be especially invaluable in hospital and clinical settings because it would help to drastically reduce the number of preventable HAIs. The designed system could be used to scan emergency rooms and medical equipment quickly to ensure that they are not contaminated with viable pathogens, thus lowering the probability of a patient or staff member contracting an infection.

CHAPTER II

METHODS

Materials

The two bacteria studied in this research were *Escherichia coli* MG1655 and *Bacillus thuringiensis.* The *E. coli* were grown in Luria Bertani and the resulting suspension was centrifuged at 4000 rpm. The pellet was resuspended in 9% saline solution and centrifuged to remove the growth media and ensure purity of sample. The *Bacillus* suspension was prepared by rehydrating the *Bacillus* spores with saline. Samples were prepared to have a OD around .5A at 271nm. The bacteria were stored at 4°C until their use.

This research employed the usage of two spectrometers. Absorption spectroscopy was performed using a Shimadzu UV160U UV-visible recording spectrophotometer. This study utilized the absorption spectra of bacterial suspensions to determine their concentrations. Fluorescence spectroscopy was performed using a Shimadzu RF-5301PC spectrofluorophotometer.

Ultraviolet irradiation of the various samples was achieved using an Oriel mercury arc lamp. The light incident on the sample had an intensity of between $3\frac{mW}{cm^2}$ and $9\frac{mW}{cm^2}$, depending on the experiment being performed. To measure the effectiveness of the UV light at different power levels, bacteria were cultured in petri-dishes after specific time periods for the different powers and these cultures were used to see when the bacteria were dead.

Bacterial Components

Most of the fluorescence in the spectra of bacteria comes from two amino acids: tryptophan and tyrosine. Both amino acids absorb and emit UV light. The peak excitation of tryptophan occurs

at 285nm and the peak emission occurs at 350nm, whereas tyrosine's peak excitation occurs at 280nm and peak emission occurs at 300nm. Since these two amino acids make up such a great portion of the fluorescence spectra of bacteria, they are worthy of individual examination.

Emission and Synchronous Spectra

The excitation spectra and emission spectra of tryptophan and tyrosine were recorded and used to determine the proper $\Delta\lambda$ for performing synchronous spectroscopy. The proper $\Delta\lambda$ is employed to isolate and distinguish components of the spectrum. This $\Delta\lambda$ can be found by the equation $\Delta\lambda = \lambda_{ex} - \lambda_{em}$, where λ_{ex} is the maxima of the excitation spectrum of the sample, λ_{em} is the maxima of the emission spectrum, and $\Delta\lambda$ is the separation between excitation and emission used for synchronous spectroscopy. For tyrosine, $\Delta\lambda$ was found to be 20nm; for tryptophan, $\Delta\lambda$ was found to be 65nm. Using these two $\Delta\lambda$s for synchronous spectroscopy will allow the isolation of tyrosine and tryptophan, respectively.

Suspensions of tryptophan and tyrosine were made with concentrations such that their peak fluorescence prior to irradiation was around 80-100. These samples were then irradiated with UV light in small time intervals starting with 30 seconds and moving up to several minutes. After each irradiation, the emission spectra and synchronous spectra for each were taken.

Excitation Emission Matrices

Suspensions of tryptophan and tyrosine were made and an EEM encompassing 220 nm to 500 nm emission and 220nm to 340nm excitation was taken of each. Each solution was then irradiated with UV light at $8\,\frac{mW}{cm^2}$ for 15 minutes and the EEM was taken again.

Principle Component Analysis

Ten emission spectra, with excitation wavelengths of 280nm and 285nm, respectively, were taken of suspensions of tyrosine and tryptophan. The solutions were then irradiated with UV light, and 10 more emission spectra of each were taken. PCA was performed on the emission spectra from the samples, both together and separate. This procedure was repeated for a solution containing a mixture of tyrosine and tryptophan.

Bacteria

Emission and Synchronous Spectra

Suspensions of *E. coli* and *Bacillus* were made and their fluorescence spectra taken with an excitation wavelength of 285nm. Then, synchronous spectra were taken with $\Delta\lambda$ of 20nm and 65nm, corresponding to the $\Delta\lambda$ chosen for tyrosine and tryptophan, were taken of the suspension. The samples were then irradiated for small intervals of time ranging from 30 seconds to a few minutes, and the spectra recorded after each irradiation.

Excitation Emission Matrices

A suspension of *Bacillus* with OD of 0.446A was produced and an excitation emission matrix with 220nm to 320nm excitation and 280nm to 500nm emission was taken. The suspension was then irradiated with UV light until the bacteria was dead, and then the EEM was retaken. This procedure was repeated for a suspension of *E. coli* with OD 0.339A.

Principle Component Analysis

Ten fluorescence spectra with excitation wavelength of 285nm were taken of a sample of *E. coli*. The sample was then irradiated until the bacteria were killed, and the ten spectra were retaken. This procedure was repeated for two more samples of *E. coli* and two samples of *Bacillus*. PCA

was then performed on the spectra in order to determine if they can be reliably separated by their live/dead status.

CHAPTER III

RESULTS AND DISCUSSION

Bacterial Components

Emission and Synchronous Spectra

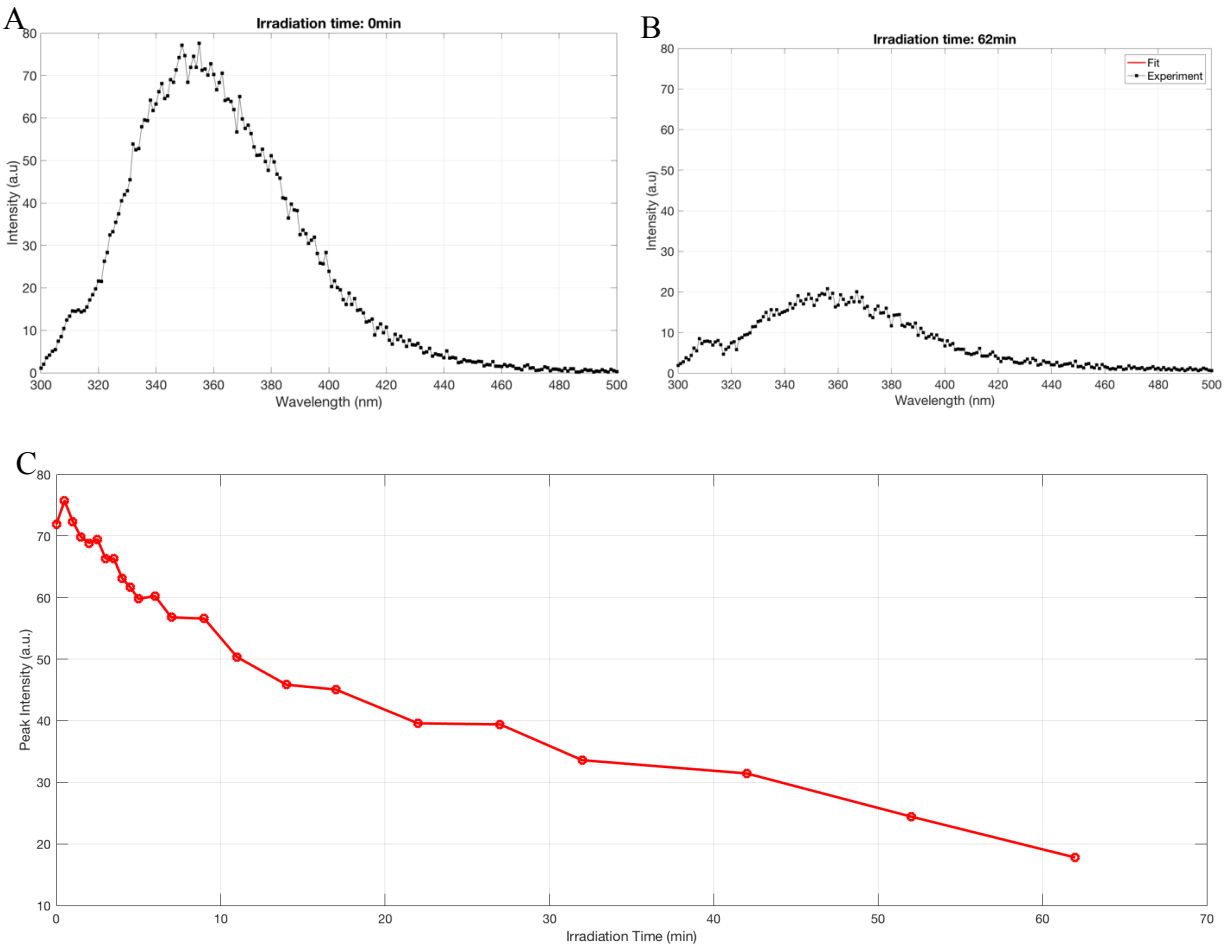

Fig. 1. Emission spectra of tryptophan. Figure 1A shows the emission spectrum before irradiation. Figure 1B shows the emission spectrum after 62 minutes of irradiation. Figure 1C shows the peak emission intensity as a function of irradiation time.

In figure 1, one can see the emission spectra, with 280nm excitation, from a sample of tryptophan with a concentration of $375\,\frac{ng}{ml}$. A Matlab program was used to plot the emission spectra after each period of irradiation and plot the peak intensities as a function of the irradiation time. Other than a small increase after 30 seconds of irradiation, the intensity of the spectra decreases as the time of irradiation increases. After 5 minutes of UV irradiation, the intensity had decreased by 15%; after 62 minutes, it had decreased by 74%. The peak intensity before and after irradiations occurs with an emission λ of around 353nm.

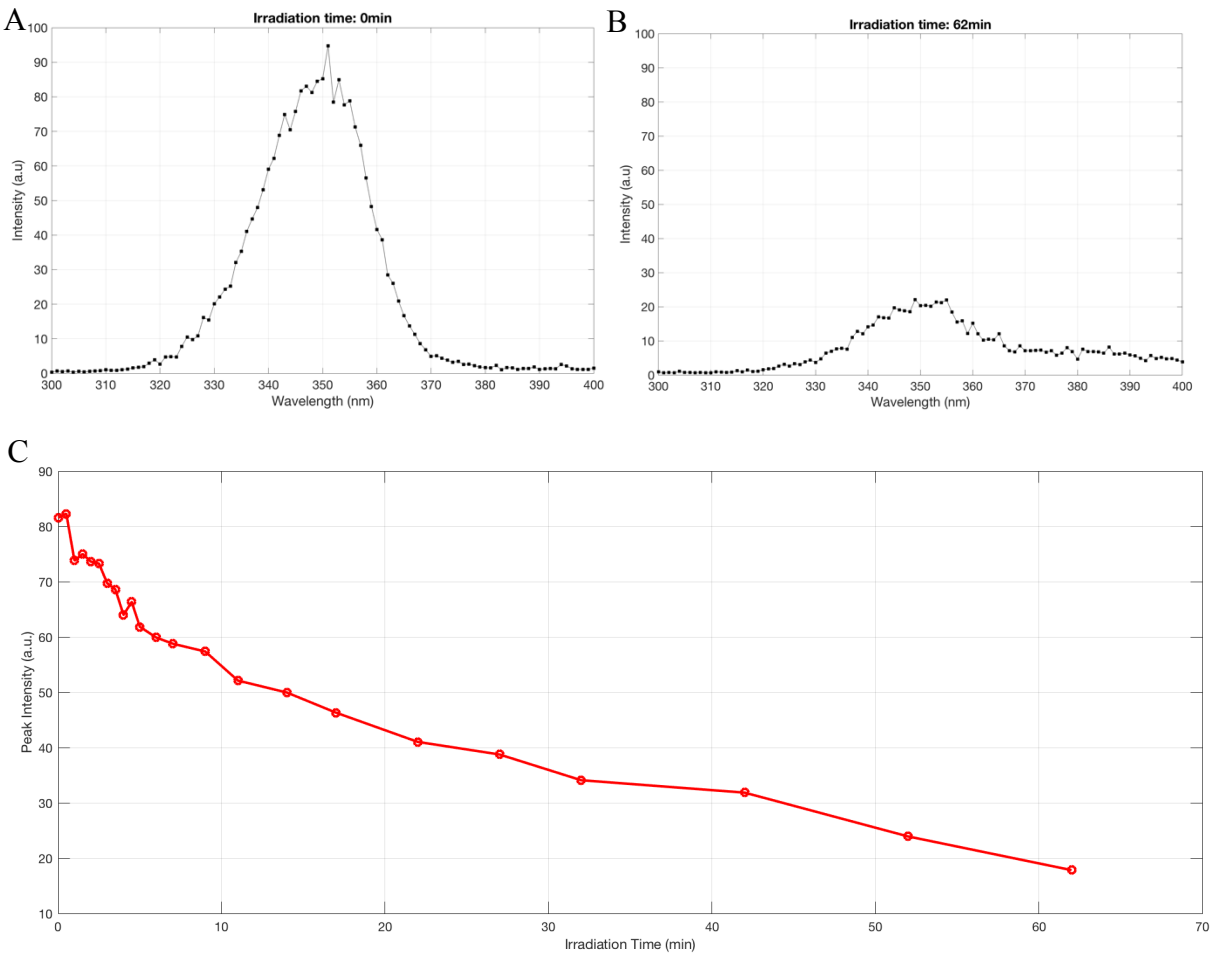

Fig. 2. Synchronous spectra of tryptophan with a delta lambda of 65nm. Figure 2A shows the synchronous spectrum of tryptophan before UV irradiation. Figure 2B shows the spectrum after 62 minutes. Figure 2C shows the peak intensity as a function of irradiation time.

Figure 2 shows the synchronous spectra of tryptophan. The peak decreases as the time the sample undergoes UV radiation increases. After 5 minutes of irradiation, the intensity had decreased by 24%; after 62 minutes, it had decreased by 78%. One can see that the general shape of the spectrum has changed slightly in figure 2B from its shape in figure 2A; there is increased emission at 385nm after irradiation. Both before and after irradiation, the peak intensity occurs with an emission λ of 350nm.

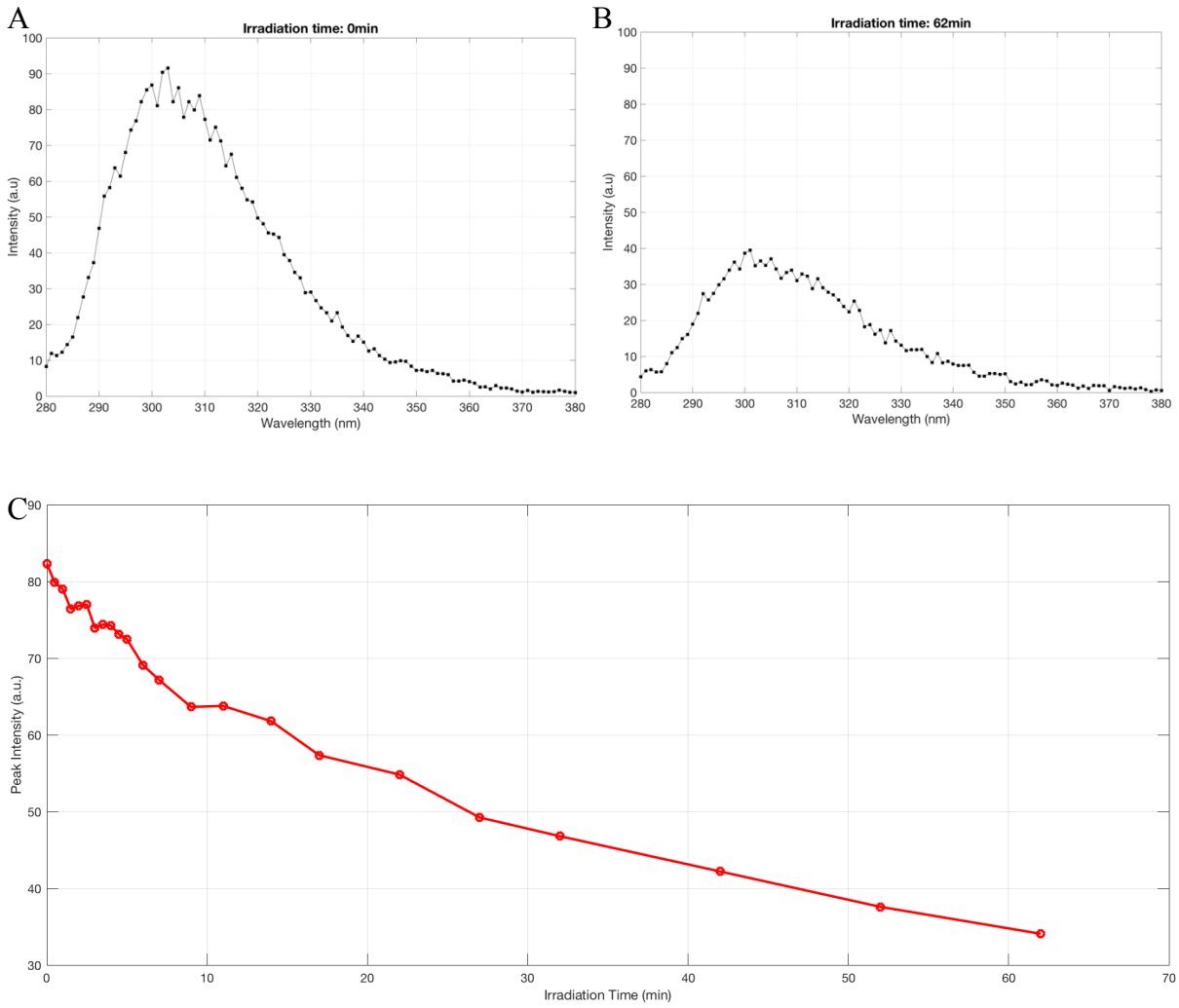

Fig. 3. Emission spectra of tyrosine. Figure 3A shows the emission spectrum of tyrosine before irradiation. 3B shows the spectrum after 62 minutes of irradiation. 3C shows the peak intensity as a function of time irradiated.

The emission spectra of tyrosine are shown in figure 3. The solution of tyrosine used had a concentration of $25\frac{ug}{ml}$. The emission spectra were measured at 280nm excitation and input to a Matlab program which plotted the emission spectra after each period of irradiation and the peak intensity of each spectra as a function of time irradiated. After 5 minutes of irradiation, the

intensity decreased 12%; after 62 minutes, it decreased 59%. Both before and after irradiation, the peak intensity occurred at an emission wavelength between 300nm and 302nm.

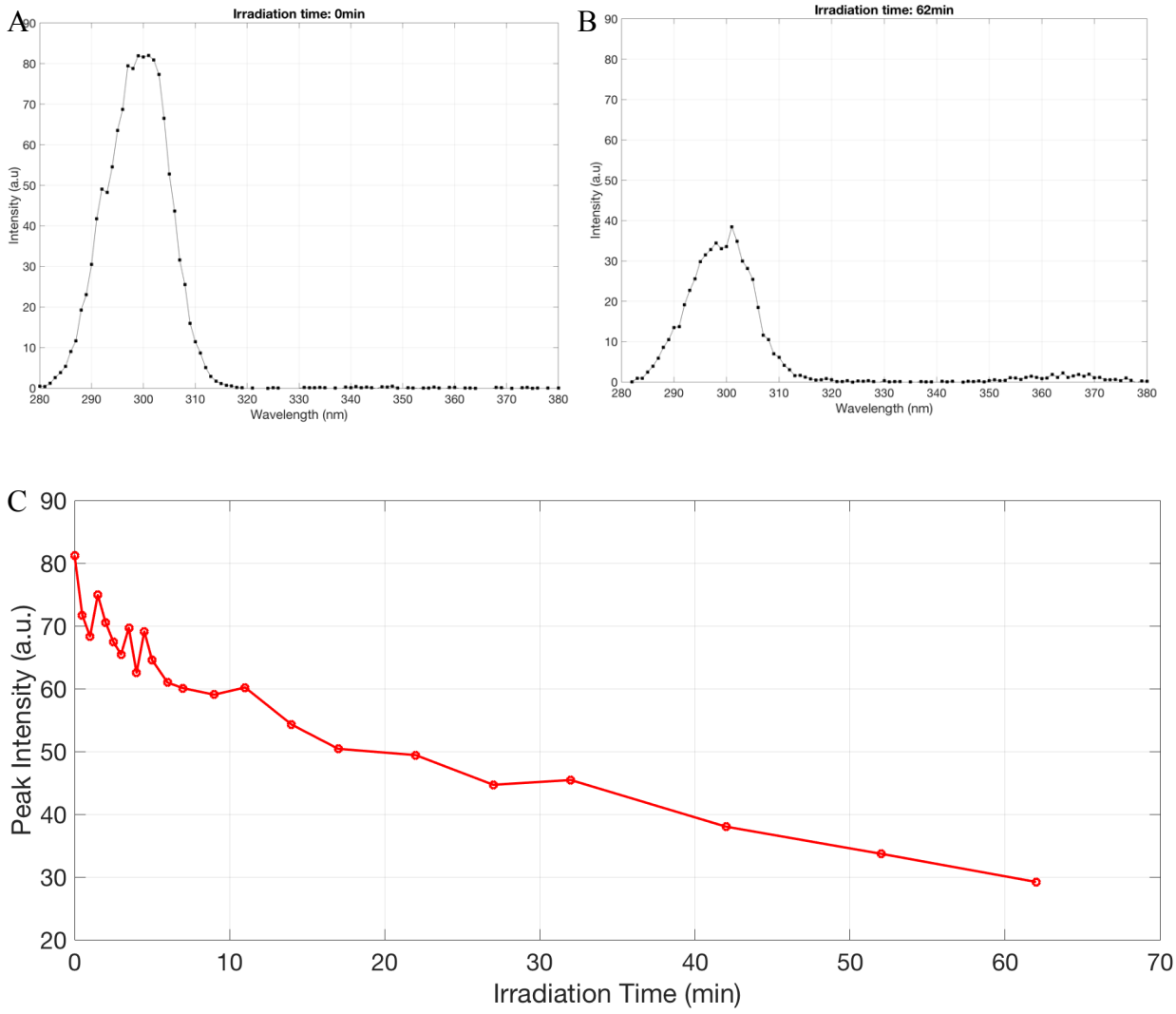

Fig 4. Synchronous spectra of tyrosine with a delta lambda of 20nm. Figure 4A shows the synchronous spectrum of tyrosine before UV irradiation. Figure 4B shows the spectrum after 62 minutes. Figure 4C shows the peak intensity as a function of irradiation time.

In figure 4, one can see that the peak intensity of tyrosine for a synchronous spectrum occurs at 300nm. Figure 4C shows that this peak is inversely proportional to the irradiation time of the

sample; the peak goes down as the irradiation time increases. After 5 minutes of irradiation, the intensity decreased by 21%; after 62 minutes of irradiation, the intensity decreased by 64%. Both before and after irradiation, the peak intensity occurred with an emission λ of 300nm.

While tryptophan, due to its higher quantum efficiency, has much higher intensity fluorescence for the same concentration, both amino acids experience a reduction in intensity as the irradiation time increases. The decrease in intensity that both experience is due to the UV light reacting with these molecules. For both emission and synchronous spectra, the intensity of tyrosine decreased at a slower rate than tryptophan.

Excitation Emission Matrices

Figure 5 shows the EEM of tryptophan. The peak occurs with an 280nm excitation and 350nm emission. One can see that the peak decreases in size but occurs at the same place and stays approximately the same shape as the solution is irradiated with UV. After irradiation, an addition peak forms with an excitation λ of 330nm and an emission λ of 400nm.

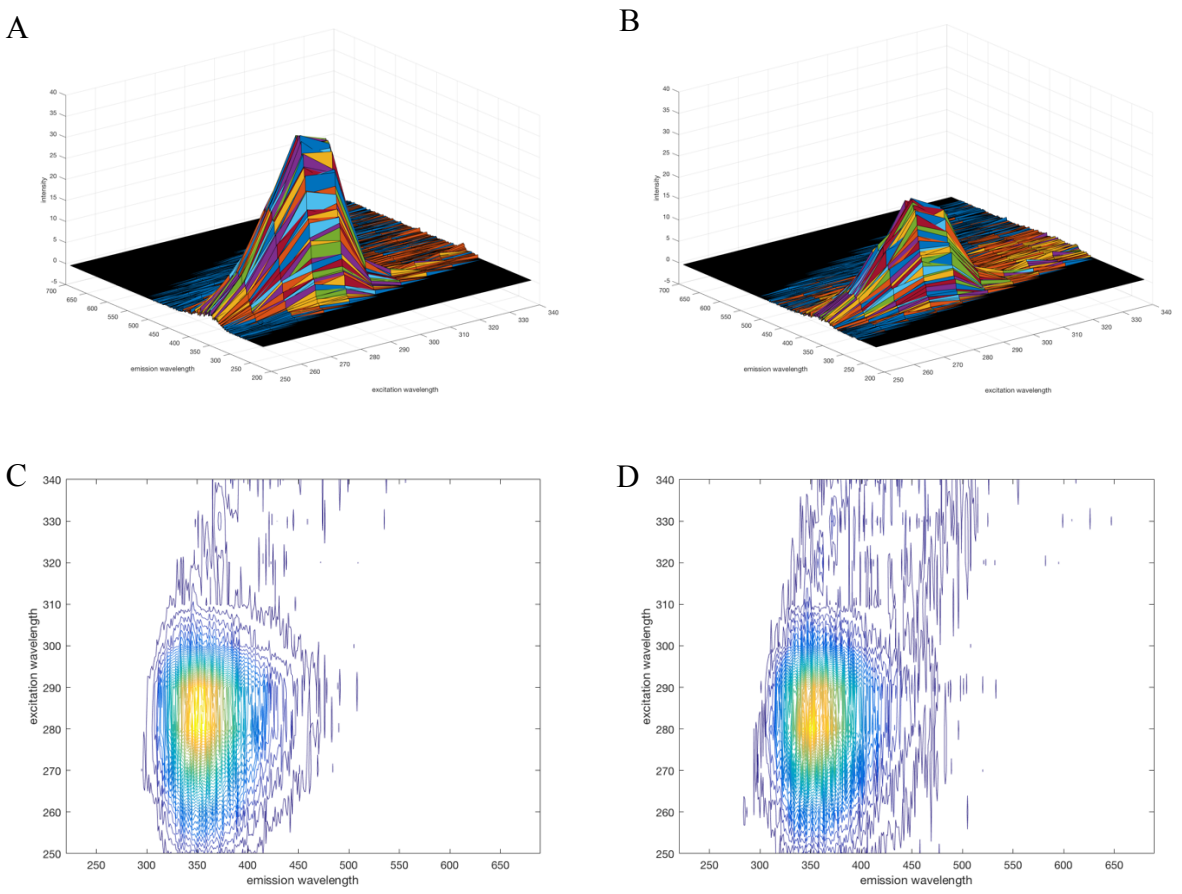

Fig. 5. EEM for tryptophan. Figure 5A shows the 3D plot of tryptophan before UV irradiation, and figure 5B shows the 3D plot for tryptophan after irradiation. Figures 5C and 5D are plots of the same data, but use contour plots instead of 3D plots.

In figure 6, one can see the same plots for tyrosine. The peak occurs with 280nm excitation and 300nm emission. After radiation, an additional peak forms with 400nm emission and 285nm excitation. The primary peak, at 280nm excitation and 300nm emission, does not change significantly in shape or position after irradiation; it only decreases in intensity.

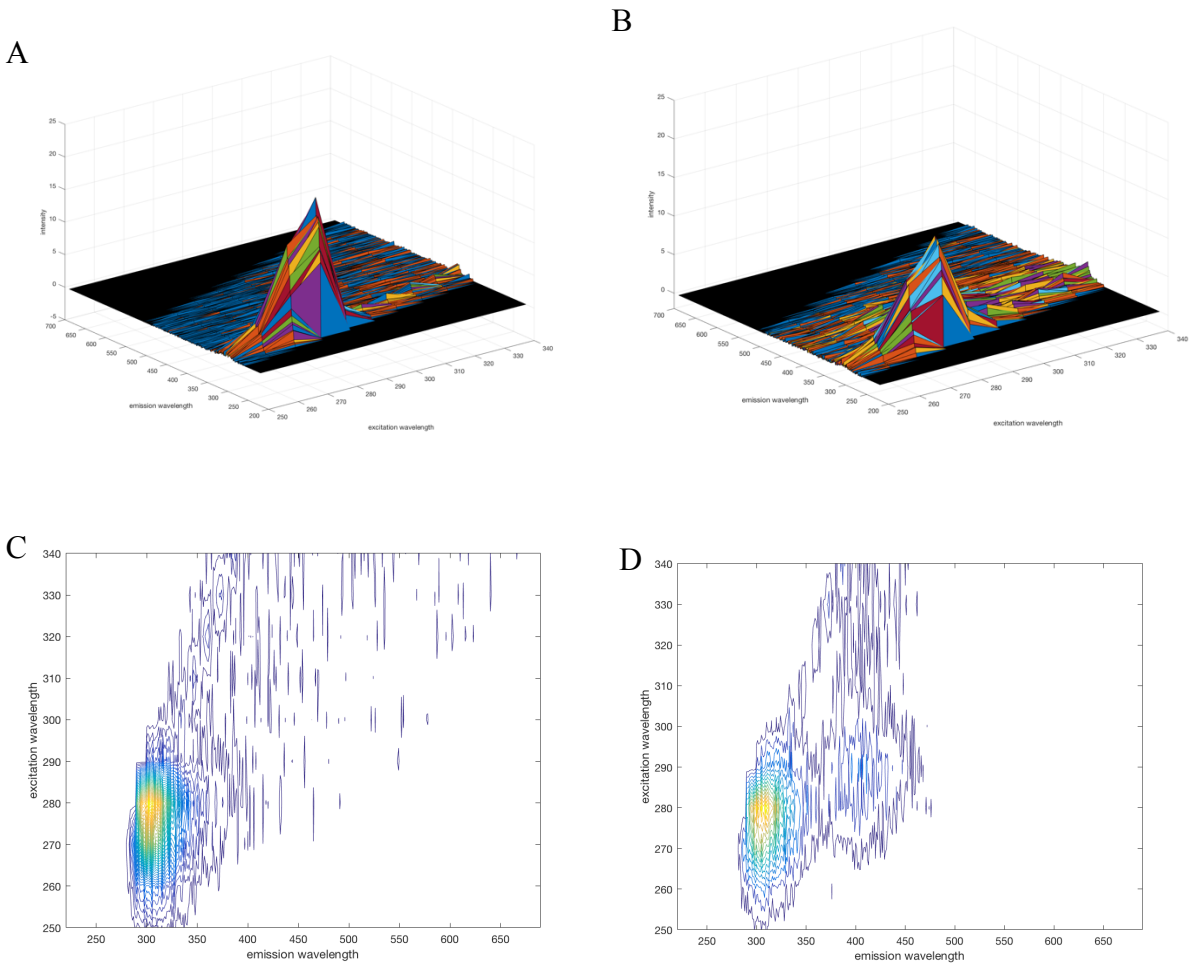

Fig. 6. EEM for tyrosine. Figure 6A shows the 3D plot of tyrosine before UV irradiation, and figure 6B shows the 3D plot for tyrosine after irradiation. Figures 6C and 6D are plots of the same data, but use contour plots instead of 3D plots.

Principle Component Analysis

The spectra from tryptophan were gathered into a single data table and input into a Matlab program which performed PCA on the data and plotted them by their first two principle components. This procedure was repeated for the tyrosine data. The results, shown in figure 7, indicate that the spectra from tryptophan and tyrosine both undergo changes detectable by fluorescence spectroscopy when they have been exposed to ultraviolet radiation.

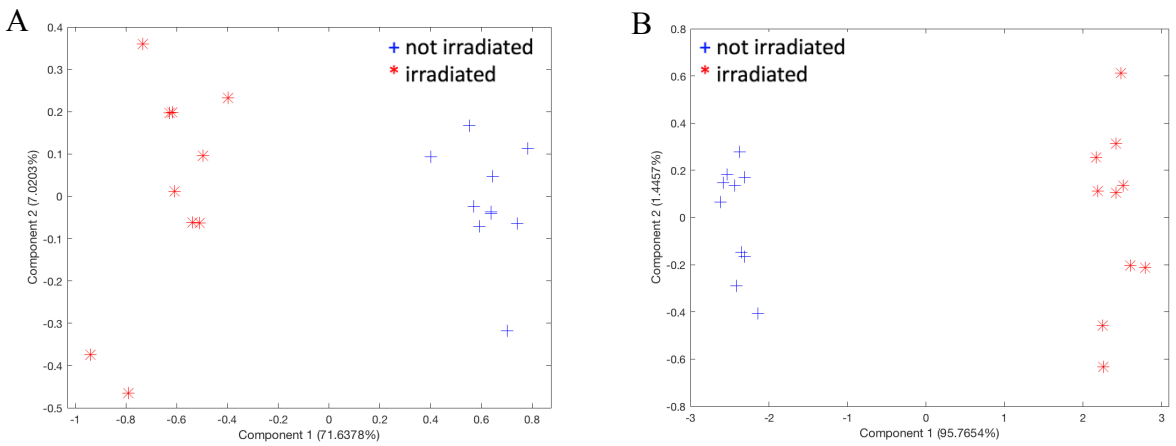

Fig. 7. PCA for samples of tryptophan and tyrosine. Figure 7A shows the score plot of the PCA performed on tryptophan, and figure 7B shows the score plot of the PCA performed on tyrosine.

Bacteria

Emission and Synchronous Spectra

Figure 8 shows the emission and synchronous spectra of *E. coli.* 20nm and 65nm were chosen for the $\Delta\lambda$ to isolate to tyrosine and tryptophan, respectively. The tyrosine within the bacteria decays at a slower rate than tryptophan.

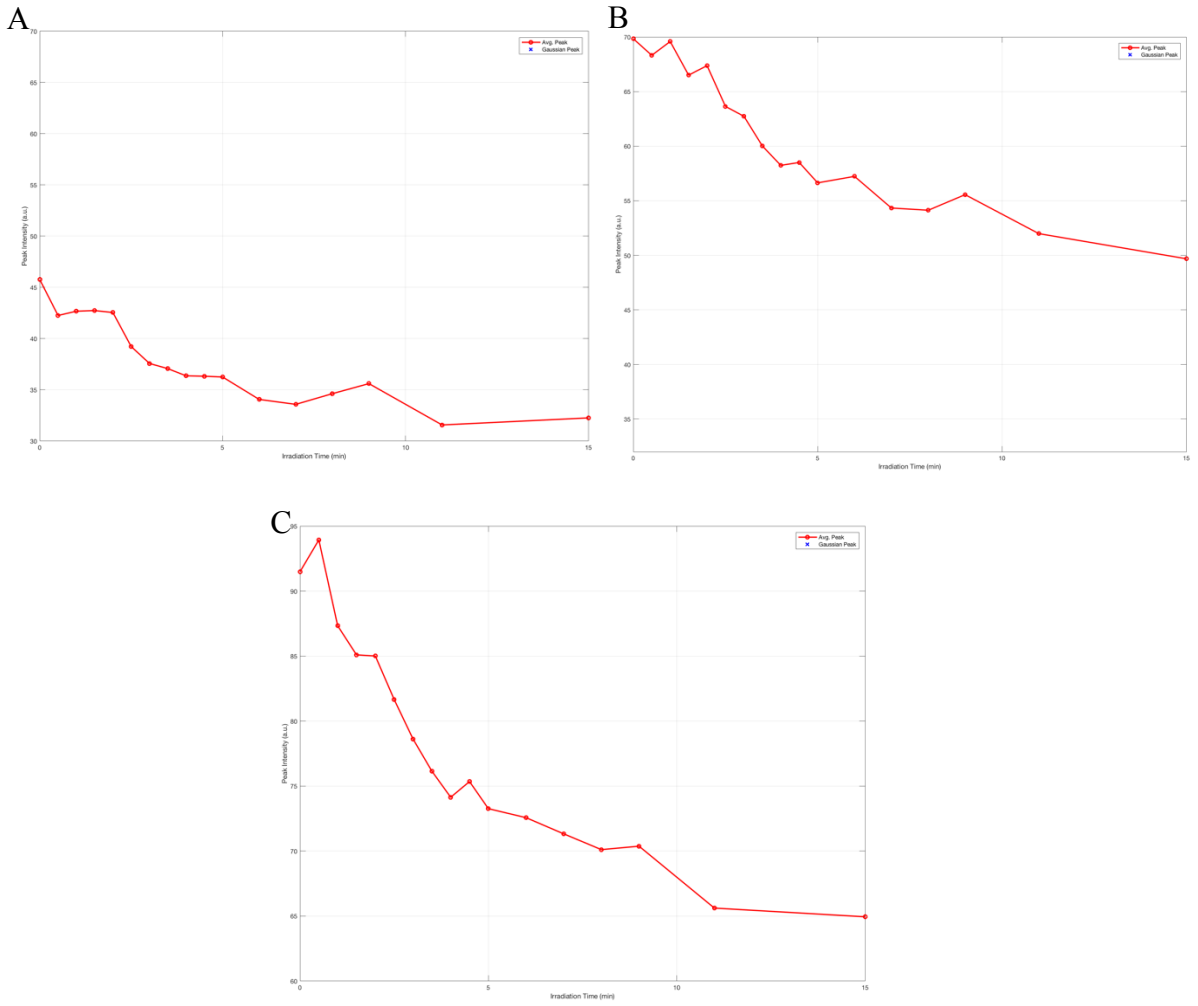

Fig 8. Spectra of *E. coli*. 8A shows the synchronous spectrum with a Δλ of 20nm. 8B shows the synchronous spectrum with Δλ of 65nm. 8C shows the emission spectrum excited at 285nm.

Figure 9 shows the emission and synchronous spectra of *Bacillus*. As with *E. coli,* 20nm and 65nm were chosen as the Δλ for the synchronous spectra to isolate tyrosine and tryptophan, in the bacteria, respectively. Tyrosine decays significantly slower than tryptophan, as was shown in *E. coli*.

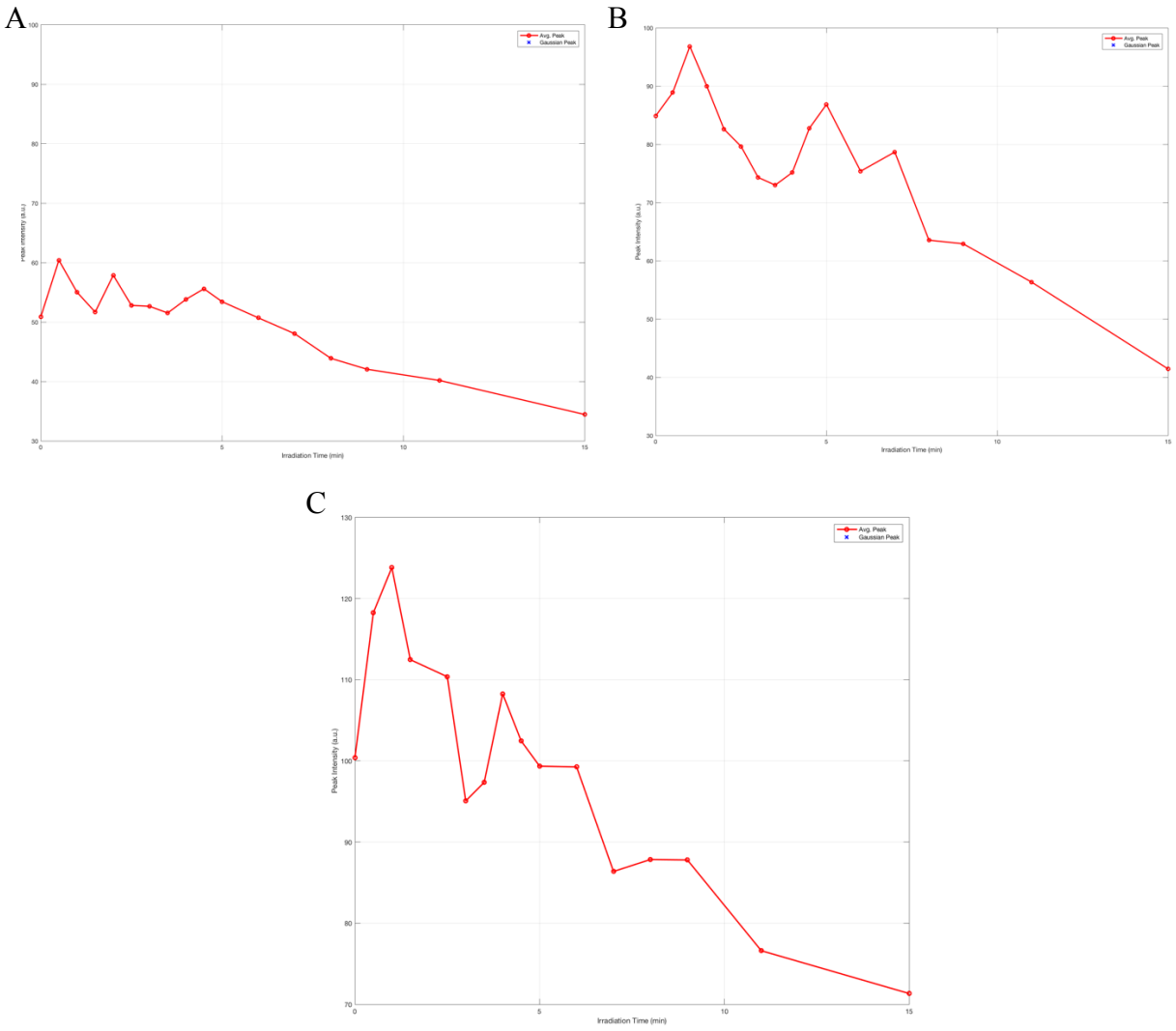

Fig 9. Spectra of *Bacillus*. 9A shows the synchronous spectrum with a Δλ of 20nm. 9B shows the synchronous spectrum with Δλ of 65nm. 9C shows the emission spectrum excited at 285nm.

As the spectrum of tyrosine decays slower than tryptophan, the ratio of the 20nm synchronous peak to the 65nm synchronous peak may be useful for determining the status of the bacteria. It is expected that the larger the ratio, the greater percentage of the bacteria in the sample that is dead.

Excitation Emission Matrices

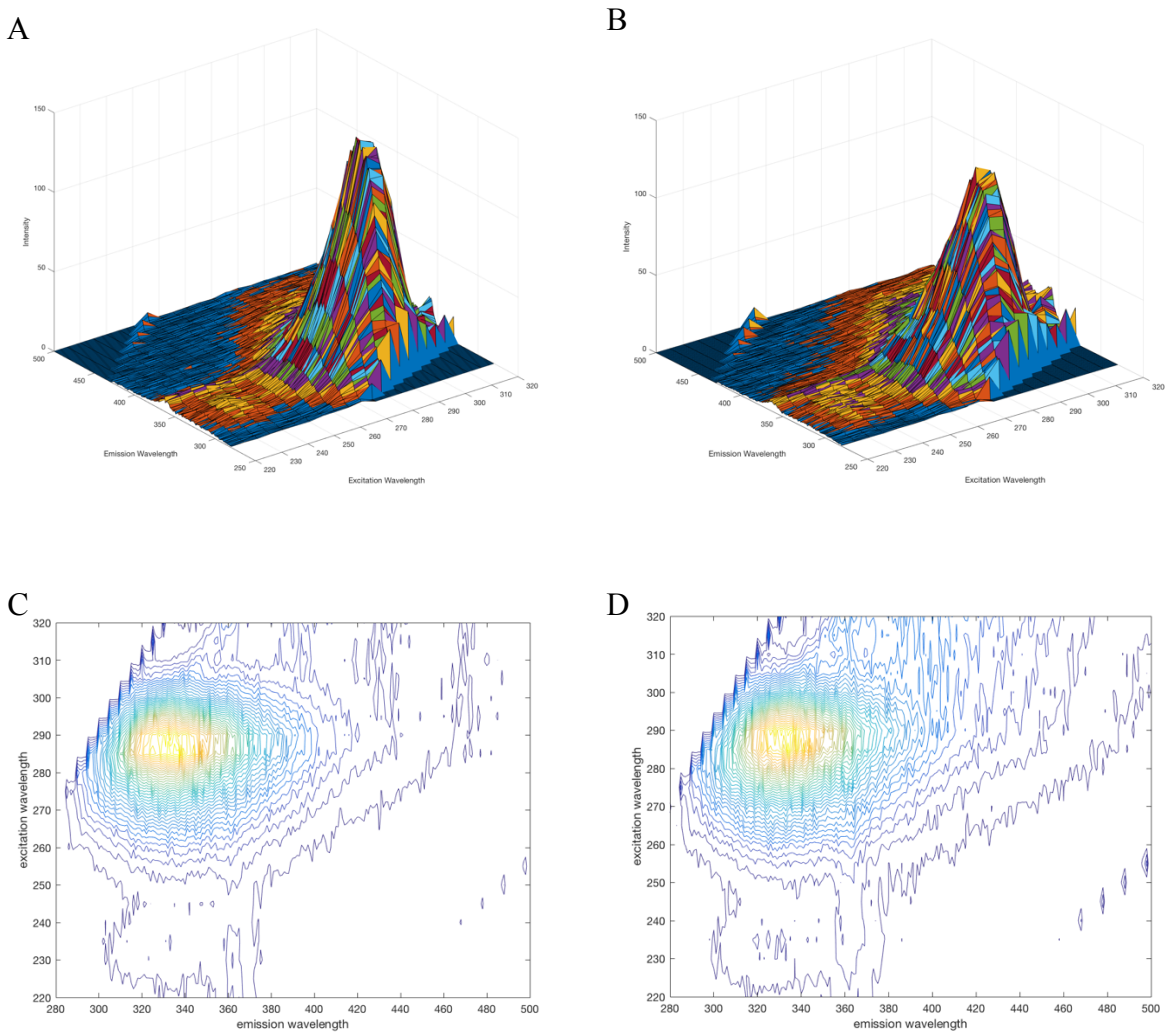

Fig. 10. EEM for *Bacillus*. Figure 10A shows the 3D spectrum of *Bacillus* before UV irradiation, and 10B shows the 3D spectrum of *Bacillus* after UV irradiation. 10C and 10D show the same information using a contour plot.

Figure 10 shows the EEM of *Bacillus* before and after ultraviolet irradiation. The peak emission occurs at 285nm when excited at 333nm. There are also additional peaks in the lower excitation wavelength range. The first occurs with 235nm excitation and 333nm emission, and the second occurs with 235nm excitation and 360nm emission. The ratio between the intensity of the peak at

ex:235nm and em:333nm to the intensity of the primary peak is 0.056 before irradiation, and 0.064 after. Additionally, the trough between the peak at ex:235nm and em:360nm filled in after irradiation, causing the two to be connected. After irradiation, there was significantly more fluorescence at the higher excitation wavelength range of the plot.

The same plots previously shown for *Bacillus* were taken for *E. coli* and are shown in figure 11. The peak emission occurs at 285nm excitation at 335nm emission. There is also an additional peak at 235nm excitation and 335nm emission. After irradiation, this peak moves to 235nm excitation and 335nm emission. The ratio between the intensity of the secondary peak to the intensity of the primary peak is 0.077 before UV irradiation and .0932 after.

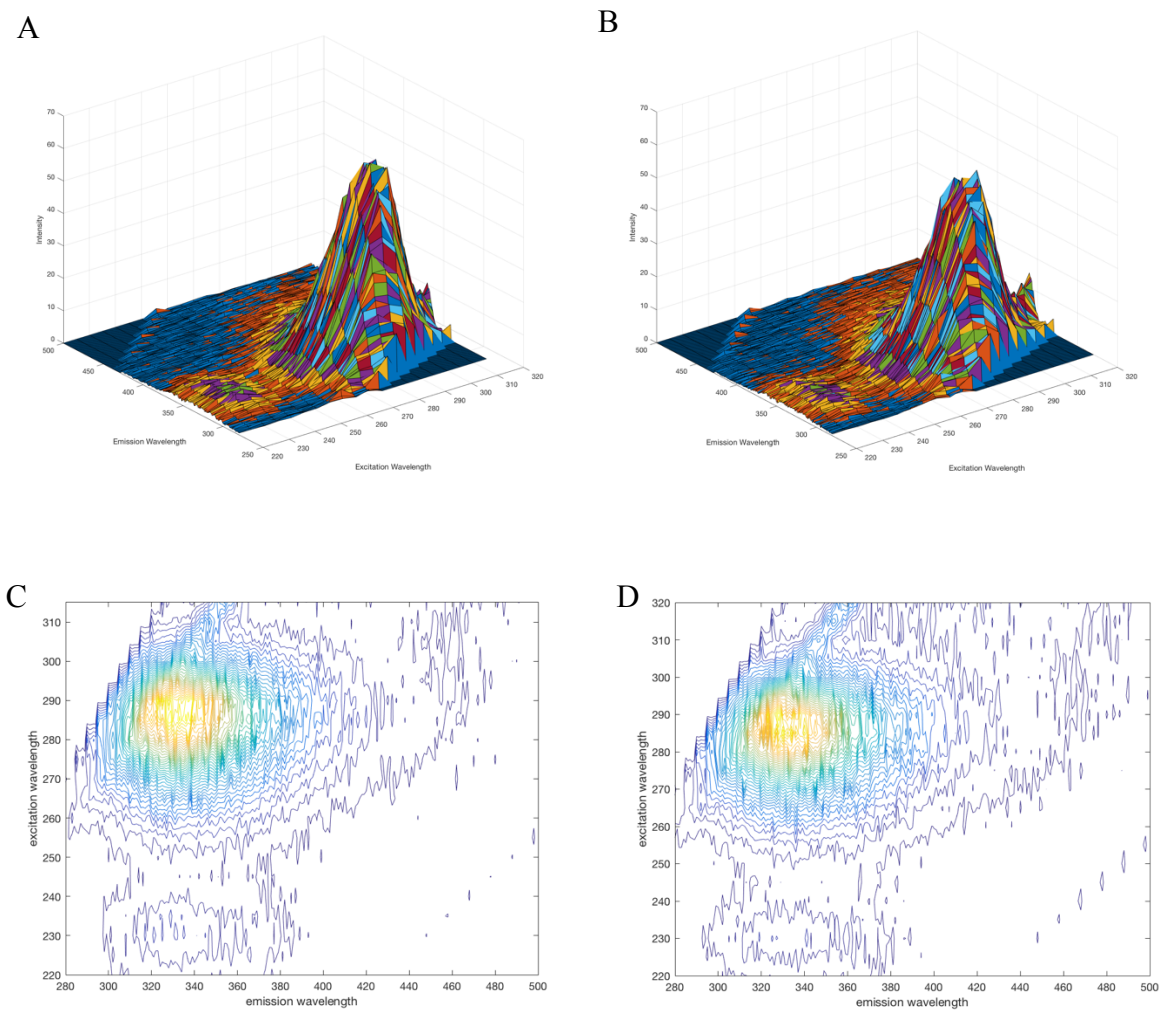

Fig. 11. EEM for *E. coli.* Figure 7A shows the 3D spectrum of *E. coli* before UV irradiation, and 7B shows the 3D spectrum of *E. coli* after UV irradiation.

Principle Component Analysis

Spectra from living and dead *Bacillus* and *E. coli* were gathered into data tables and input to a Matlab program that performed PCA. This was done with both *E. coli* and *Bacillus* individually as well as together. PCA was performed on the raw data as well as normalized data.

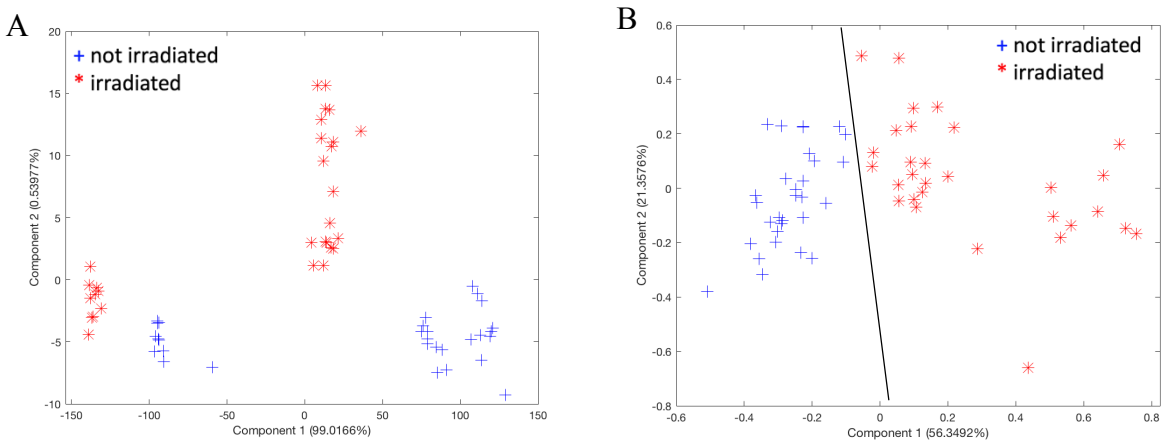

Fig. 12. PCA of *E. coli*. 12A shows the score plot of PCA performed on the set before normalization, and 12B shows the score plot of PCA performed after normalization. In 12B, a line is drawn to separate the points indicating live and dead bacteria.

From figure 12, we can see that *E. coli* undergoes fluorescence changes detectable by PCA upon death. Normalization of the data makes the separation cleaner and allows us to draw a line that separates living from dead.

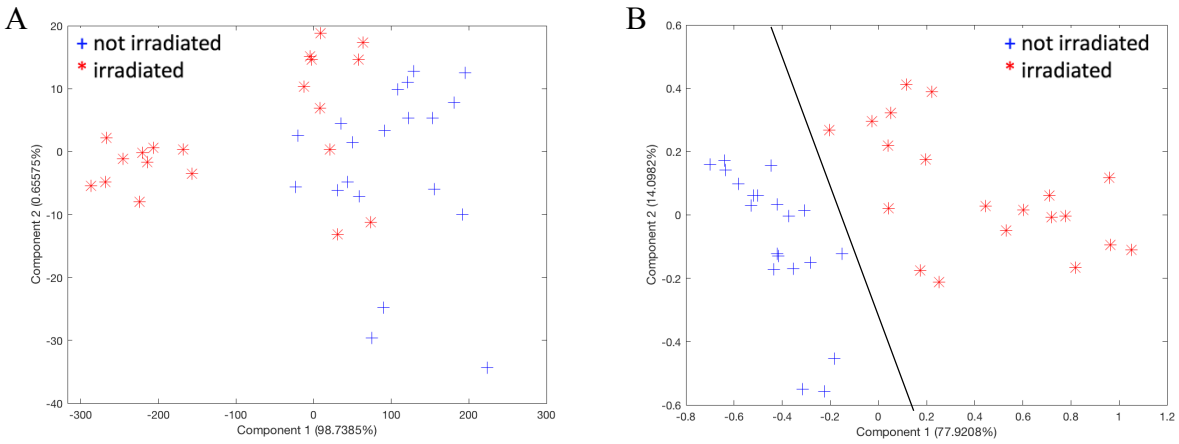

Fig. 13. PCA of *Bacillus*. 13A shows the score plot of PCA performed on the raw data set, and 13B shows the score plot of PCA performed on the normalized data set. In 13B, a line is drawn to separate the points indicating live and dead bacteria.

As for *E. coli*, living and dead *Bacillus* bacteria can be separated using PCA. Figure 13 shows the results of performing PCA on normalized and raw sets of *Bacillus* spectra. Similarly to *E. coli*, the separation of *Bacillus* is more accurate after the data is normalized.

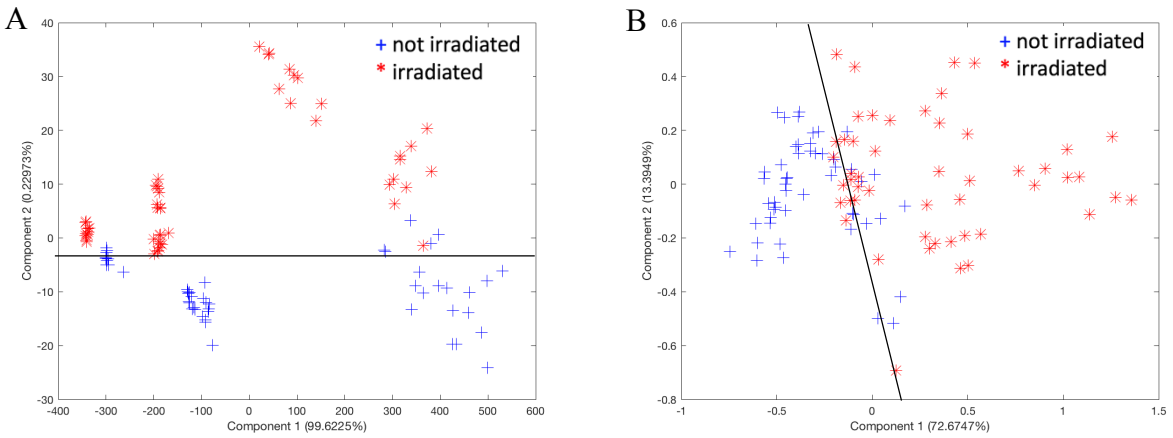

Fig. 14. Score plots of PCA performed on samples of live/dead *Bacillus* and *E. coli*. 14A shows the score plot of PCA performed on the raw data set, and 14B shows the score plot of PCA performed on the normalized data set. Both 14A and 14B have lines drawn to show the separation between the points indicating live and dead bacteria.

The score plot from the PCA performed on both *Bacillus* and *E. coli* bacteria is shown in figure 14. For the set that was not normalized, clear separation can be seen between the two bacteria as well as between the living and dead bacteria. The live/dead status of the bacterial sample seems to be indicated by its score for component 2 (y axis of the score plot) for both *Bacillus* and *E. coli.* This indicates that fluorescence spectroscopy, along with PCA, can differentiate between living and dead bacteria. Furthermore, since the differences between live and dead *E. coli* and *Bacillus* are plotted on the same axis, this implies that the spectra of *E. coli* and *Bacillus* undergo similar changes upon UV irradiation.

The normalized set also indicates that the bacteria change in similar ways, though the separation between the two types of bacteria is almost completely removed. There is some overlap between the spectra of living and dead bacteria in both principle components, but there is still good separation between them. This implies that both *Bacillus* and *E. coli* undergo similar changes to their fluorescence spectrum after irradiation. Further study is necessary to see if there is a way to increase this separation and lower the error rate.

Handheld Spectrometer Design

The above results suggest that fluorescence spectroscopy can be used to detect bacteria, by looking for the fluorescence of tryptophan and tyrosine, and identify whether they are dead or alive. This suggests the possibility of a handheld spectrometer that will be able to scan a surface, such as emergency room walls or operating tables, and perform the necessary spectroscopy and data processing to ascertain the presence of bacteria and their viability. Coupling this spectrometer with a powerful UV source, used for bacterial deactivation, would allow for in situ detection and destruction of bacteria and confirmation of the sterilization.

In figure 15, the design of the suggested handheld spectrometer is shown. All lenses, (L1 and L2) are convex lenses. The mirrors M1, M3, M4, and M6 are spherical mirrors; M2 and M5 are flat mirrors. The design utilizes a monochromator to select an excitation wavelength which is then transmitted through an optical fiber and shown on the surface or sample that is to be scanned. The fluorophores that are excited in the sample emit light that is collected by an optical fiber and a second grating is used to disperse the light across a CCD detector, which then sends the for processing and plotting of the spectrum. This system would be capable of synchronous spectroscopy, 3D fluorescence spectroscopy, and emission fluorescence spectroscopy. Any additional data processing, including PCA, may be handled by the onboard computer.

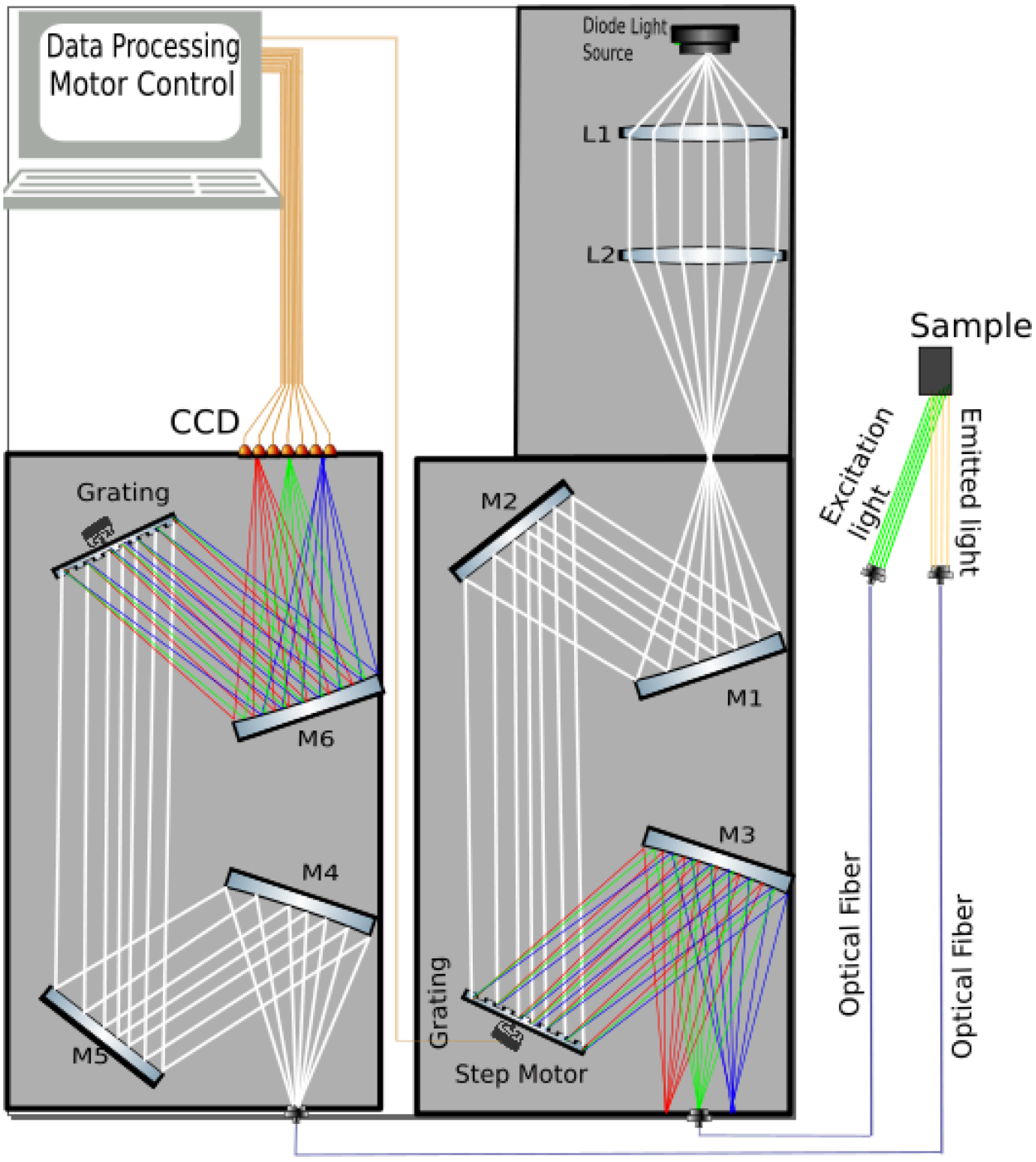

Fig. 15. Optical design of the handheld spectrometer.

CHAPTER IV

CONCLUSION

Fluorescence spectroscopy can be used to detect bacteria and determine their living status. This work has investigated the fluorescent properties of bacteria and has suggested methods for differentiating between live and dead bacteria. The results from this work show the potential for fluorescence spectroscopy, used in conjunction with PCA, for the detection of bacteria and the determination of whether the bacteria are alive or dead. To further that goal, this work has presented a design for a handheld spectrometer that will be capable of utilizing the techniques and methods discussed in the text: 3D fluorescence spectroscopy, synchronous spectroscopy, and PCA.

REFERENCES

[1] Center for Disease control and prevention, "Antibiotic Resistant Threats in the United States, 2013," 2013. [Online]. Available: http://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf. [Accessed: 30-Aug-2016].

[2] Center for Disease Control and Prevention, "HAI Data and Statistics | HAI | CDC," *CDC.gov*, 2014. [Online]. Available: http://www.cdc.gov/hai/surveillance/. [Accessed: 07-Sep-2016].

[3] I. F. R. Cockerill *et al.*, "Optimal Testing Parameters for Blood Cultures," *Clinical Infectious Diseases*, no. 12, p. 1724, 2004.

[4] Héctor Enrique Giana, Landulfo Silveira Jr., Renato Amaro Zângaro, and Marcos Tadeu T. Pacheco, "Rapid Identification of Bacterial Species by Fluorescence Spectroscopy and Classification Through Principal Components Analysis," *J. Fluoresc.*, vol. 13, no. 6, p. 489, Nov. 2003.

[5] B. Alizadeh Behbahani, F. Tabatabaei Yazdi, F. Shahidi, S. A. Mortazavi, and M. Mohebbi, "Principle component analysis (PCA) for investigation of relationship between population dynamics of microbial pathogenesis, chemical and sensory characteristics in beef slices containing Tarragon essential oil," *Microb. Pathog.*, vol. 105, pp. 37–50, Apr. 2017.

[6] P. Boguta, P. M. Pieczywek, and Z. Sokołowska, "A Comparative Study of the Application of Fluorescence Excitation-Emission Matrices Combined with Parallel Factor Analysis and Nonnegative Matrix Factorization in the Analysis of Zn Complexation by Humic Acids," *Sens. 14248220*, vol. 16, no. 10, pp. 1–20, Oct. 2016.

[7] X.-S. He and Q.-D. Fan, "Investigating the effect of landfill leachates on the characteristics of dissolved organic matter in groundwater using excitation–emission matrix fluorescence spectra coupled with fluorescence regional integration and self-organizing map," *Springer Verl.*, 2016.

[8] T. Vo-Dinh, "Multicomponent analysis by synchronous luminescence spectrometry," *Anal. Chem.*, vol. 50, no. 3, pp. 396–401, Mar. 1978.

[9] M. Sohn, D. S. Himmelsbach, F. E. Barton, and P. J. Fedorka-Cray, "Fluorescence Spectroscopy for Rapid Detection and Classification of Bacterial Pathogens," *Appl. Spectrosc.*, vol. 63, no. 11, pp. 1251–1255, Nov. 2009.